Traub, P., and Nomura, M. (1968a), *Proc. Nat. Acad. Sci. U. S. 59*, 777.
Traub, P., and Nomura, M. (1968b), *J. Mol. Biol. 40*, 391.
Van Duin, J., and Kurland, C. (1970), *Mol. Gen. Genet. 109*, 169.
Van Duin, J., van Knippenberg, P., Dieben, M., Kurland, C. (1972), *Mol. Gen. Genet. 116*, 181.
Wireman, J. W., and Sypherd, P. S. (1974), *Nature (London), New. Biol.* (in press).
Yuki, A. (1971), *J. Mol. Biol. 62*, 321.

Biosynthesis of Viomycin. I. Origin of α,β-Diaminopropionic Acid and Serine†

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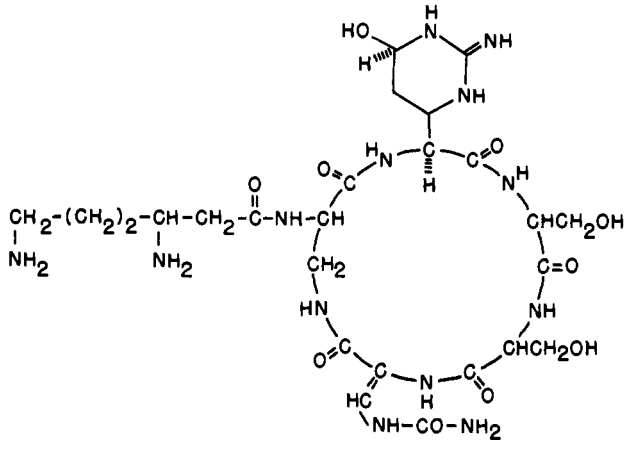
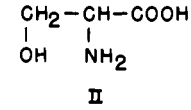
ABSTRACT: A study was made of the origin of the carbon skeletons of L-serine and L-α,β-diaminopropionic acid, two amino acids found in the polypeptide antibiotic viomycin. [^{14}C]Serine was incorporated into viomycin, and most of the radioactivity was present in those two amino acids. The labeling patterns of the serine and diaminopropionic acid from hydrolysates of viomycin produced using [1-^{14}C]- and [3-^{14}C]serine as precursors indicated that the carbon skeletons were both derived from serine without randomization. Radioactive glycine, formate, carbonate, and acetate were incorporated into viomycin, but the radioactivity was extensively randomized among all of the hydrolysis products. [U-^{14}C]Diaminopropionic acid was incorporated almost exclusively into the diaminopropionic acid residue of viomycin. The low amount of radioactivity in the serine residue indicated that the diaminopropionic acid was incorporated as such and did not involve prior conversion to serine.

Viomycin is a polypeptide antibiotic that has been used to a limited extent in the treatment of infections caused by *Mycobacterium tuberculosis* (Finlay *et al.*, 1951). The antibiotic is produced by several organisms including *Streptomyces puniceus* (Finlay *et al.*, 1951) and *S. floridae* (Bartz *et al.*, 1951). This latter organism is now referred to as *S. griseus* var. *purpureus* (J. Ehrlich, 1964, personal communication). Several structures have been proposed for viomycin (Bowie *et al.*, 1964; Dyer *et al.*, 1965; Lechowski, 1969; Bycroft *et al.*, 1968, 1971; Kitagawa *et al.*, 1968, 1972). Structure I was proposed by Bycroft *et al.* (1968).



I

The acid hydrolysis products of viomycin (Dyer *et al.*, 1964) are 2 equiv of serine (II) and 1 equiv each of L-α,β-diaminopropionic acid (III), L-3,6-diaminohexanoic acid (β-lysine)



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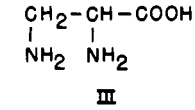

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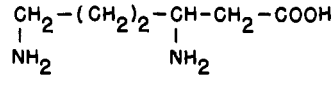

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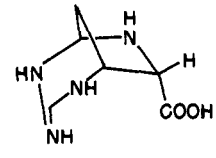

V

(IV), 2,4,6-triaza-3-iminobicyclo[3.2.1]octane-7-carboxylic acid (viomycidine) (V), ammonia, and carbon dioxide.[1] The structure of viomycidine was elucidated by X-ray crystallography (Floyd *et al.*, 1968; Bycroft *et al.*, 1968). This compound is not present as much in intact viomycin (Bycroft *et al.*, 1968) but rather represents a rearrangement product,

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[1] Urea has previously been observed to be a product of acid hydrolysis of viomycin (Bartz *et al.*, 1951), and we have observed that 1 equiv of urea was produced under these conditions (Dyer *et al.*, 1964). However, the commercial viomycin that we have used in recent years does *not* yield urea upon acid hydrolysis. Furthermore, the Streptomycete strain that we received from Charles Pfizer and Co. produced a viomycin that gave no urea upon acid hydrolysis. Also, from the 24 ^{14}C incorporation studies using this strain, no [^{14}C]urea was produced. These data suggest that the structure proposed for viomycin (Bycroft *et al.*, 1971; Kitagawa *et al.*, 1972), which include a ureido fragment, may be incorrect.

formed during acid hydrolysis, of one of the amino acids in viomycin.

L-α,β-Diaminopropionic acid is found in a number of plants. Several species of *Mimosa* (Gmelin *et al.*, 1959) and *Vicia baicalensis* (Bell and Tirimanna, 1965) contain the free amino acid, and the following derivatives have been isolated from other plants: L-α-amino-β-ureidopropionic acid (albizziine) from several *Mimosacae* (Gmelin *et al.*, 1959); β-*N*-oxalyl-diaminopropionic acid from *Lathyrus sativus* (Adiga *et al.*, 1963); β-uracil-3-yl-L-alanine (willardiine) from *Acacia willardiana* (Gmelin, 1959); β-(1,4-dehydro-3-hydroxy-4-oxo-pyrid-1-yl)-L-alanine (mimosine) from *Mimosa pudica* (Renz, 1936); and β-pyrazol-1-yl-L-alanine from *Citrillus vulgaris* (Noe and Fowden, 1960). In addition, it is present in the polypeptide antibiotic edeine (Roncari *et al.*, 1966), and its enantiomorph has been found in the digestive fluid of the fifth instar of the larvae of *Bombyx mori* (Wada and Toyota, 1965).

One report of the biosynthesis of this class of compounds concerned albizziine (Reinbothe, 1962). It was reported that [1-^{14}C]- and [3-^{14}C]serine and [1-^{14}C]- and [2-^{14}C]glycine were incorporated into albizziine. [U-^{14}C]Glyoxylic acid and [^{14}C]uric acid were incorporated slightly, but labeled carbon dioxide, formic acid, urea, [2-^{14}C]uracil, and [amidine-^{14}C]-arginine gave only negligible incorporation. It was suggested that serine was utilized by a direct pathway of biosynthesis of the diaminopropionic acid portion of albizziine, that glycine and formaldehyde were incorporated *via* serine, and that C-2 of uric acid in some unknown manner furnished the ureido carbon atom. The diaminopropionic acid derived from albizziine was not degraded, however, so the positions that were labeled are not known. This paper described studies of the origin of the serine and diaminopropionic acid residues of viomycin.

Experimental Section

Materials. D-[U-^{14}C]Glucose, DL-[1-^{14}C]serine, DL-[3-^{14}C]-serine, [1-^{14}C]glycine, sodium [^{14}C]formate, sodium [^{14}C]-bicarbonate, and sodium [2-^{14}C]acetate were obtained from the New England Nuclear Corp., and sodium [1-^{14}C]acetate was from Isotopes Specialities Co. [U-^{14}C]Diaminopropionic acid was obtained from viomycin isolated from cultures grown using [U-^{14}C]glucose as described in the text. Samples of viomycin sulfate were provided by Parke, Davis and Co. Sources of other materials are noted at appropriate points in the following text.

Culture Conditions. Freeze-dried cultures of actinomycete No. 16790, obtained from Charles Pfizer and Co., Inc., were maintained in serile soil and transferred to slants of Emerson's agar medium. After incubation for 4 days at 26°, spores were transferred from the slants to liquid media containing the following components (g/l.): glucose, 10; soybean meal, 10; sodium chloride, 5; and calcium carbonate, 1. The medium was adjusted to pH 7.0 prior to the addition of calcium carbonate. Radioactive compounds were dissolved in water and sterilized by filtration through ultrafine sintered glass funnels before addition to the medium. Cultures were grown in 250 ml of medium in 1-l. erlenmeyer flasks on a rotary shaker (150 rpm, 2.5-in. stroke). Under these growth conditions, the production of viomycin began between 48 and 72 hr after inoculation, and reached a maximum in 7–9 days. Labeled precursors were therefore added after 48 hr, and the viomycin was isolated after 9 days growth.

Isolation and Purification of Viomycin. Cultures from four flasks were combined and centrifuged to remove mycelium. The supernatant solution was adjusted to pH 7.8 with 1 N potassium hydroxide and applied to a column (18-mm i.d.) containing approximately 60 ml of Amberlite IRC-50 (50–100 mesh Na^+ phase) ion exchange resin (J. T. Baker Chemical Co.). The resin was washed with 250 ml of distilled water and viomycin was eluted using 0.5 N hydrochloric acid. Fractions that gave positive ninhydrin tests were then assayed spectrophotometrically (*cf.* below for spectrophotometric assay method). Fractions that contained viomycin were combined and the viomycin was converted to the sulfate salt by passage through a Dowex 2-X8 (50–100 mesh, J. T. Baker Chemical Co.) column in the sulfate phase. Ninhydrin-positive fractions were combined, and the volume was reduced to 6 ml using a rotary evaporator. This solution was cooled in an ice bath, and the inorganic salts that precipitated were removed by filtration.

The viomycin sulfate was purified by gel filtration through 400-ml Sephadex G-15 (Pharmacia Fine Chemicals, Inc.) columns (18-mm i.d.). The Sephadex G-15 was swollen in 0.01 N formic acid, and that solvent was used as the eluent. Ninhydrin-positive fractions were combined, and the viomycin content was determined. Both spectrophotometric and biological assays indicated that the viomycin was about 70–75% pure when compared with commercially available samples of viomycin sulfate. The volume of the combined viomycin-containing fractions was reduced to about 6 ml using a rotary evaporator, and the solution was subjected to gel filtration on a second Sephadex G-15 column identical with the one just described. The fractions containing viomycin were combined and freeze-dried. This viomycin sulfate, along with a sample of the commercial product for a standard, was further dried at room temperature, *in vacuo* over phosphorus pentoxide. The amounts of viomycin isolated by this procedure varied from 90 to 250 mg/l.; however, the yield was generally 120–200 mg/l. and represented about 60–75% recovery.

The procedure described above was used in nearly all of the studies with labeled precursors. In some later studies, however, the composition of the 9-day-old culture filtrates appeared to have changed so that the volume of IRC-50 resin required to absorb all of the viomycin from the brews was increased five- to tenfold. The isolation procedure was therefore modified by using the IRC-50 in the ammonium phase (150 ml of resin/l. of brew). The resin was eluted first with 500 ml of distilled water, then with 500 ml of 0.1 N formic acid, and finally, to elute the viomycin, with 0.25 N formic acid. The ammonium formate was removed from the dried viomycin by sublimation. Subsequent purification steps were carried out as described before.

Assay Methods. In order to establish the identity and purity of the products obtained by the growth of the streptomycete using labeled potential precursors, the isolated viomycin samples were subjected to three assay procedures. Biological activity was determined using spores of *Bacillus subtilis* (Difco Laboratories) as the test organism. The paper disk assay method was used, and commercial viomycin was always used as a standard.

Viomycin concentrations were also determined spectrophotometrically at each stage of purification. The viomycin used in these studies showed an absorption maximum in neutral or acidic solution at 269 nm (ϵ 24,600) that shifted to 290 nm (ϵ 16,100) in alkali. Aliquots were diluted to 3.0 ml with either 1 N hydrochloric acid or 1 N sodium hydroxide, and absorbance was determined at 269 and 290 nm using a Beckman DU spectrophotometer with a Gilford 2000 attachment. The concentration of viomycin in a given sample could thus be determined, and the probable reliability of the method could be estimated by comparing the 269 nm/290 nm absorbance, which was 1.528 for a pure sample.

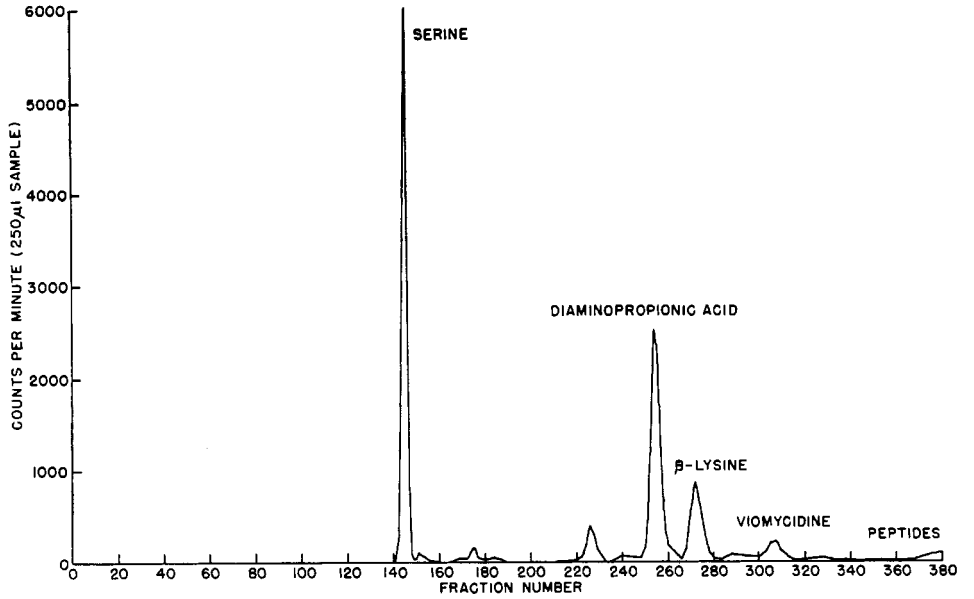

FIGURE 1: Ion exchange chromatography of the hydrolysis products of viomycin isolated from cultures grown in the presence of [U-^{14}C]-glucose.

Purified samples of viomycin were subjected to chromatography on cellulose-coated thin-layer sheets (Eastman Kodak Co.) with fluorescent indicator in a solvent containing *tert*-butyl alcohol–acetone–glacial acetic acid–5% ammonium hydroxide–water (5:5:5:3:1). Viomycin was detected on the chromatograms by exposure to an ultraviolet light source. Antibiotic activity was detected by placing the developed chromatograms on the surface of agar plates prepared in the same manner as that used to prepare the plates for quantitative biological assay.

Acid Hydrolysis of Viomycin. The [^{14}C]viomycin samples isolated from the cultures were dried to a constant weight in a vacuum desiccator at room temperature along with samples of commercial viomycin and then diluted to about 2.5 g with the dried carrier. The combined samples were then hydrolyzed in 6 N hydrochloric acid as described by Haskell *et al.* (1952). [^{14}C]Carbon dioxide was trapped by slowly bubbling nitrogen through the hydrolysis mixture and into a methanol solution of Hyamine 10-X (Rohme & Haas, Inc.).

After the hydrolysis was complete, the hydrolysate was concentrated to a volume of about 10 ml and stirred with Amberlite IR-45 (OH^- phase) resin until the pH had risen to about 5.

Purification of Viomycin Hydrolysis Products. The viomycin hydrolysis products were chromatographed on columns (2.4 cm × 450 cm) or resin (Dowex 50-W X-8, H^+ phase, 100–200 mesh, Baker Reagent 1930). The column was eluted using a hydrochloric acid gradient in which, at the start, the mixing chamber contained distilled water and the reservoir contained 300 ml of 3.6 N hydrochloric acid. Approximately 400 fractions (20 ml each) were collected from the column at a flow rate of about 1 ml/min. A typical elution pattern is shown in Figure 1. These particular data were obtained from an experiment in which the steptomycete had been grown in the presence of D-[U-^{14}C]glucose. The principal peaks were identified by thin-layer chromatography using silicic acid plates in a solvent system containing *tert*-butyl alcohol–acetic acid–water (2:1:1).

Degradation of Viomycin Hydrolysis Products. Serine, obtained by freeze-drying appropriately combined fractions from the ion exchange column, was dissolved in a small amount of water and neutralized with IR-45 (OH^- phase) resin. The serine was recrystallized from aqueous ethanol. Diaminopropionic acid from the columns was purified as the monohydrochloride salt by recrystallization of freeze-dried samples from aqueous ethanol.

The radioactivity present in C-3 of serine and diaminopropionic acid was determined by periodate oxidation of the amino acid and isolation of the formaldehyde derived from C-3 as the dimedone derivative. Aliquots of the amino acid, 100 μl, containing about 1 mg of the amino acid were diluted with 4.5 ml of 0.5 M sodium acetate buffer (pH 4.7). To this solution was added 2.4 ml of a freshly prepared solution of 0.5 M sodium metaperiodate, and the mixture was allowed to stand at room temperature for 2.5 hr. At this time, 160 mg of dimedone in 2 ml of ethanol was added, and the formaldehyde derivative was allowed to precipitate for 20 hr.

The radioactivity present in C-1 of serine was determined either by oxidation with *N*-bromosuccinimide or ninhydrin. Only the ninhydrin method was used to determine the radioactivity in C-1 of diaminopropionic acid. A modification of the method of Chappelle and Luck (1957) was used for the *N*-bromosuccinimide degradation. Aliquots of 50 μl, containing approximately 5 μmol of serine, were diluted with 3 ml of a 10% solution of succinimide in 1 M sodium acetate (pH 4.7). The mixture was allowed to equilibrate for 20 min, 0.5 ml of the *N*-bromosuccinimide reagent (2.5 g each of *N*-bromosuccinimide and succinimide in 25 ml of 1 M sodium acetate buffer (pH 4.7)) was added, and the mixture was agitated at room temperature for 3 hr. Carbon dioxide from C-1 was collected in Hyamine hydroxide.

For the ninhydrin degradations, 50-μl aliquots of a solution containing about 5 μmol of serine or diaminopropionic acid were diluted with 3 ml of 1 M citrate buffer (pH 2.5). Ninhydrin, 50 mg, was added and the mixtures were allowed to stand in a boiling water bath: 5 min for serine and 10 min for diaminopropionic acid. Carbon dioxide was collected in 0.5 ml of hydroxide of Hyamine.

Radioactivity Measurements. Radioactivity measurements were made using either a Packard Model 3003 or Model 3375 liquid scintillation spectrometer. Viomycin samples were counted in aqueous solution using scintillation solutions prepared by mixing 1540 ml of absolute ethanol and 2400 ml of toluene and dissolving 17.76 g of 2,5-diphenyloxazole and 59.2 mg of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene (both from Packard Instrument Co.) in the mixture. Toluene-soluble

TABLE I: Incorporation of ^{14}C-Labeled Compounds into Viomycin.

Precursor (dpm × 10^{-8})	Viomycin Produced			
	mg	dpm × 10^{-6}	Sp Act. (dpm/mmol × 10^{-7})	Incorpn (%)
D-[U-^{14}C]Glucose (8.08)	273	12.1	3.44	1.51
DL-[1-^{14}C]Serine (2.62)	125	8.21	5.07	3.13
DL-[3-^{14}C]Serine (9.53)	189	13.1	5.39	1.38
[1-^{14}C]Glycine (8.10)	36.6	0.40	0.85	0.05
Sodium [^{14}C]formate (10.8)	91	0.31	0.26	0.03
Sodium [^{14}C]bicarbonate (3.45)	166	0.11	0.05	0.03
Sodium [1-^{14}C]acetate (8.00)	160	0.70	0.34	0.09
Sodium [2-^{14}C]acetate (10.8)	154	1.87	0.94	0.17

samples, such as the hydroxide of Hyamine solutions of carbon dioxide, were counted in solutions of 5 g of 2,5-diphenyloxazole and 0.3 g of 1,4-bis[2-(5-phenyloxazolyl)]benzene per l. of redistilled toluene. The amino acids obtained from the acid hydrolyses and other water-soluble compounds were counted in solutions made up of 4 l. of redistilled toluene, 2000 g of Triton X-100 (Packard Instrument Co.), 13.872 g of 2,5-diphenyloxazole, and 0.347 g of 1,4-bis[2-(5-phenyloxazolyl)]-benzene.

Results

Incorporation of Radioactive Precursors into Viomycin. Table I shows the results of studies on the incorporation of ^{14}C-labeled compounds into viomycin by growing streptomycete cultures. Specific activities of the precursors are not shown because the concentrations of the unlabeled materials present in the cultures were not known. On a percentage basis, only glucose and serine were incorporated efficiently into viomycin. A similar result was obtained when the data were calculated in terms of specific activity. The specific activities were calculated before dilution with carrier viomycin and thus provide a better estimate of incorporation efficiency because they take into consideration the variation in viomycin production.

Incorporation of Radioactive Precursors into Viomycin Hydrolysis Products. D-[U-^{14}C]Glucose was used in the initial experiment to test the efficiency of that compound as a precursor of the amino acid components of viomycin in the presence of the unlabeled amino acids potentially available in the medium as soybean protein. Figure 1 shows the elution pattern from the Dowex 50 column of the viomycin hydrolysis products from viomycin isolated from the cultures grown in the presence of labeled glucose. The radioactivity in the carbon dioxide, produced during hydrolysis of the viomycin, and in the combined amino acid fractions is given in Table II. All four of the amino acids were labeled, although serine and diaminopropionic acid contained about 71% of the total radioactivity. Upon purification, the specific activities of serine and diaminopropionic acid were 6.75×10^5 and 8.65×10^5 dpm/mmol, respectively. The fractions labeled peptides are materials that were eluted from the column after viomycidine and appear to contain a mixture of peptides that contain viomycidine or closely related compounds.

Ion exchange chromatography of the hydrolyzed viomycin samples obtained from cultures grown using DL-[1-^{14}C]serine and DL-[3-^{14}C]serine gave the results shown in Table III. In the [1-^{14}C]serine experiment, the serine derived from viomycin accounted for about 81% of the total activity from the column; in the [3-^{14}C]serine experiment, it amounted to approximately 68%. The diaminopropionic acid contained about 16% of the total activity in both experiments.

Because glycine is known to be a precursor of serine in bacterial systems (Broquist and Trupin, 1966), and because it was incorporated into albizziine (Reinbothe, 1962), a growth experiment was conducted with that amino acid labeled with C-14 in the 1 position. Glycine was very poorly incorporated into viomycin (0.05%, Table I), probably because of extensive utilization of that amino acid in metabolic processes other than viomycin synthesis. Approximately 49% of the radioactivity in the viomycin and hydrolysis products was found in serine and about 14% in diaminopropionic acid. Most of the remaining radioactivity was found in the carbon dioxide and peptide fractions.

In addition to glucose and the amino acids, ^{14}C-labeled sodium formate, sodium bicarbonate, and sodium acetate were tested as potential precursors of viomycin. None of these compounds was incorporated well into viomycin (Table I),

TABLE II: Radioactivity in Principal Hydrolysis Products from D-[U-^{14}C]Glucose Experiment.

Fraction	Wt (mg)	Act. (dpm × 10^{-3})
Carbon dioxide		502
Serine	438	1570
Diaminopropionic acid·HCl	183	1210
β-Lysine·HCl	248	507
Viomycidine·HCl	160	244
Peptides	84	384

TABLE III: Radioactivity in Principal Hydrolysis Products from DL-[1-^{14}C]- and DL-[3-^{14}C]Serine Experiments.

Fraction	Wt (mg)		Act. (dpm × 10^3)	
	1-^{14}C	3-^{14}C	1-^{14}C	3-^{14}C
Carbon dioxide			580	106
Serine	623	575	2100	3410
Diaminopropionic acid·HCl	385	356	413	816
β-Lysine·HCl	332	456	42	430
Viomycidine·HCl	266	249	20	183
Peptides	146	133	18	171

TABLE IV: Labeling Patterns of Serine and Diaminopropionic Acid Derived from Labeled Viomycin.[a]

Precursor	Sp Act. (dpm/mg)	Act.[b] (dpm/mg of AA Oxidized)		
		C-1		
		N-Bromo-succinimide	Ninhydrin	C-3
[U-^{14}C]Glucose				
Serine	6480	2100	2130 (32.8)	2220 (34.3)
Diaminopropionic acid·HCl	6148		2110 (34.4)	2190 (35.6)
[1-^{14}C]Serine			(99.9)	(0.0)
Serine	4580	4560	4430 (96.8)	31 (0.1)
Diaminopropionic acid·HCl	1340		1290 (96.2)	13 (0.1)
[3-^{14}C]Serine			(7.8)	(91.8)
Serine	7020	517	573 (8.2)	6460 (91.9)
Diaminopropionic acid·HCl	2105		206 (9.8)	1890 (89.7)

[a] The numbers in parentheses are the percentages of radioactivity in the indicated carbon atom. For comparison, the distribution of radioactivity among the carbon atoms of the labeled serine precursors is also given. [b] The radioactivity in C-2 was determined by subtracting the radioactivity in C-1 plus C-3 from the total. With [U-^{14}C]glucose these values were for serine, 2130 dpm/mg (32.9%), and for diaminopropionic acid·HCl, 1848 dpm/mg (30.1%). With [1-^{14}C]- and [3-^{14}C]serine, radioactivity in C-2 of the serine and diaminopropionic acid·HCl was negligible (0–3%).

and the radioactivity was extensively randomized. Sodium formate and sodium bicarbonate were incorporated into serine to a greater extent than into the other amino acids. The radioactivity from acetate labeled at either C-1 or -2 was distributed among all the amino acids; the β-lysine had the greatest amount of radioactivity.

Incorporation of [^{14}C]Diaminopropionic Acid into Viomycin. The studies on the incorporation of labeled serine into viomycin indicated that this amino acid could serve as a precursor of both serine and diaminopropionic acid. Two alternatives were considered as possible origins of the diaminopropionic acid fragment: either it was synthesized as such and then incorporated into a polypeptide, or at some stage in the biosynthesis of viomycin, a serine residue, already bound in a peptide linkage, was converted to a diaminopropionic acid residue. In order to test these possibilities, streptomycete cultures were grown in the presence of [U-^{14}C]diaminopropionic acid. The labeled diaminopropionic acid was obtained from the hydrolysate of viomycin that had been isolated from cultures grown using D-[U-^{14}C]glucose. Growth of the organism using labeled glucose was accomplished in the manner described in the Experimental Section except that the viomycin samples from four 1-l. batches (5.4×10^9 dpm of glucose per l.) were combined for Sephadex G-15 chromatography and subsequent hydrolysis. The [U-^{14}C]diaminopropionic acid purified from the combined fractions from the ion exchange column contained 2.46×10^7 dpm (sp act. 3.34×10^7 dpm/mmol).

The viomycin obtained by growing the streptomycete using [U-^{14}C]diaminopropionic acid (2.09×10^7 dpm) contained 7.82×10^5 dpm (sp act. 3.57×10^6 dpm/mmol; 3.74% incorporation). Ion-exchange chromatography of the viomycin hydrolysis products indicated that only serine and diaminopropionic acid were radioactive. Of the total radioactivity recovered from acid hydrolysis 1.16×10^5 dpm (31%) was found in the carbon dioxide, 0.22×10^5 dpm (6%) in the serine, and 2.33×10^5 (63%) in the diaminopropionic acid.

The diaminopropionic acid was purified as described previously to give a product having a sp act. of 1.38×10^5 dpm/mmol.

Degradation of Serine and Diaminopropionic Acid. The purified samples of serine and diaminopropionic acid obtained from hydrolysis of viomycin isolated from the labeled glucose and serine experiments were degraded as described in the Experimental Section. The results of these degradations are given in Table IV. The radioactivity from [U-^{14}C]glucose was equally distributed among the three carbon atoms of both serine and diaminopropionic acid. Radioactivity from [1-^{14}C]-serine was incorporated almost exclusively into the C-1's of both serine and diaminopropionic acid, and that from [3-^{14}C]-serine into C-3 of the serine and diaminopropionic acid from viomycin. Furthermore, distribution of ^{14}C among the three carbons of the amino acids derived from viomycin was essentially the same as that of the starting material, serine. The diaminopropionic acid obtained from the acid hydrolysates of viomycin from the diaminopropionic acid growth experiment was also degraded by periodate and ninhydrin oxidations. C-1 contained 27.6% of the activity and C-3 contained 33.2%. C-2 contained, by difference, 39.2% of the activity.

Discussion

The data in Table I indicate that a variety of carbon compounds can serve as precursors of viomycin, but glucose and serine are incorporated approximately 10–20 times more efficiently than the next best compound, sodium [2-^{14}C]acetate. Relative efficiencies of incorporation are difficult to evaluate, however, because of the necessity for using a complex mixture of soybean proteins as a nitrogen source for the organism. An examination of the viomycin hydrolysis products from the [U-^{14}C]glucose experiment indicates that glucose is generally incorporated into the antibiotic (Table II). Recovered radioactivity indicates, however, that the serine and diaminopropionic acid residues are somewhat preferentially labeled. These variations probably reflect differences in utilization of glucose *vs.* the amino acid carbon skeletons of the soybean protein for the formation of the amino acids of viomycin. The differences in specific activity may also have been caused by the different viomycin components having been synthesized or incorporated into more complex viomycin precursors at different times during the growth of the cultures.

Recently Tam and Jordan (1972) reported negligible incorporation of serine into viomycin. In contrast we observed relatively good incorporation, particularly into the serine and diaminopropionic acid residues. [1-^{14}C]Serine, although incorporated to a greater extent (3.13%) into viomycin than [3-^{14}C]serine (1.38%), was no more effective in labeling the serine and diaminopropionic acid residues of the antibiotic (Table III). If the specific activities of the amino acids from viomycin are corrected for dilution with carrier, the values for serine and diaminopropionic acid from the [1-^{14}C]serine experiment become 1.1×10^4 and 0.52×10^4 dpm/mmol and from the [3-^{14}C]serine experiment, 1.2×10^4 and 0.45×10^4 dpm/mmol, respectively. Thus serine, labeled in either C-1 or C-3, is incorporated into these two amino acids in essentially the same manner, but the serine residues have about twice the specific activity of the diaminopropionic acid.

In addition to serine, Reinboth (1962) reported that [1-^{14}C]- and [2-^{14}C]glycine were incorporated into albizziine by *Acacia lophantha* plants. [1-^{14}C]Glycine was not well incorporated into viomycin, but serine and diaminopropionic acid were more heavily labeled than the other amino acids. There was insufficient radioactivity in these amino acids to determine labeling patterns; however, the results may indicate that a pathway of serine biosynthesis from glycine may be operative in the streptomycete as it is in other bacteria.

^{14}C-Labeled formate and bicarbonate were not incorporated appreciably into any of the amino acids from viomycin. These results agree with those of Reinbothe (1962) who reported poor incorporation of these compounds into albizziine. Acetate was also poorly incorporated into viomycin. The extensive randomization of activity among all the viomycin degradation fragments indicated that acetate was not on the direct pathway for their biosynthesis.

The labeling patterns of the individual carbon atoms of serine and diaminopropionic acid from viomycin are both virtually identical with the [1-^{14}C]- and [3-^{14}C]serine added to the growth media, and thus they confirm the hypothesis that serine is a precursor of diaminopropionic acid. Furthermore, they suggest that precursor serine is incorporated into both the serine and diaminopropionic acid residues directly and without randomization of the carbon skeleton.

The source of the two amino groups of diaminopropionic acid is not known. Reinbothe (1962) suggested that, in *A. lophantha*, diaminopropionic acid might be formed by oxidation of serine to aminomalonic acid semialdehyde and that a second amino group could be introduced into the molecule by transamination. Some preliminary results with ^{15}N- and ^{2}H-labeled serine (J. R. Dyer and P. D. Shaw, unpublished experiments) suggest that the amino group of serine might be incorporated into diaminopropionic acid; however, some doubly labeled diaminopropionic acid was observed in the mass spectra of diaminopropionic acid from an experiment with [3,3-$^{2}H_2$]serine. These results would suggest that the β-amino group is introduced into diaminopropionic acid without prior oxidation of C-3 of serine. These conclusions are only tentative, however, because of the low isotopic incorporation and because the structures of certain significant mass peaks could not be assigned with certainty. Attempts to detect enzyme systems that would oxidize serine, in the presence or absence of potential amino group donors, or that would form diaminopropionic acid by direct substitution into serine, acetylserine, or cysteine, using a variety of amino group donors have thus far been unsuccessful.

Our results show that radioactivity from diaminopropionic acid is incorporated almost exclusively into the diaminopropionic acid fragment of viomycin. The serine from this sample of viomycin contained only 10% as much radioactivity. The low activity of the serine suggests that either the serine–diaminopropionic acid interconversion is not readily reversible, or more likely that any serine derived from diaminopropionic acid is diluted rapidly by endogenous serine and further metabolized *via* nonviomycin related pathways.

The degradation of diaminopropionic acid from this experiment gave a radioactive labeling pattern among the three carbon atoms very similar to that of the diaminopropionic acid fragment of the viomycin from the [U-^{14}C]glucose experiment (Table IV). The nearly equal labeling of the three carbon atoms indicates that they were incorporated as a unit without fragmentation or dilution with endogenous one- or two-carbon precursors.

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References

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

Bartz, Q. R., Ehrlich, J., Mold, J. D., Penner, M. A., and Smith, R. M. (1951), *Amer. Rev. Tuberc. 63*, 4.

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

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

Broquist, H. P., and Trupin, J. S. (1966), *Annu. Rev. Biochem. 35*, 231.

Bycroft, B. W., Cameron, D., Croft, L. R., Hassanali-Walji, A., Johnson, A. W., and Webb, T. (1971), *Experientia 27*, 501.

Bycroft, B. W., Cameron, D., Croft, L. R., Johnson, A. W., Webb, T., and Coggon, P. (1968), *Tetrahedron Lett.*, 2925.

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

Du Bus, R., Dyer, J. R., Floyd, J. C., and Shaw, P. D. (1969), *Fed. Proc. Fed. Amer. Soc. Exp. Biol. 28*, 668.

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

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

Finlay, A. C., Hobby, G. L., Hockstein, F., Lees, T. M., Lenert, T. F., Means, J. A., P'An, S. Y., Regna, P. P., Routien, J. B., Sobin, B. A., Tate, K. B., and Kane, J. H. (1951), *Amer. Rev. Tuberc. 63*, 1.

Floyd, J. C., Bertrand, J. A., and Dyer, J. R. (1968), *Chem. Commun.*, 998.

Gmelin, R. (1959), *Hoppe-Seyler's Z. Physiol. Chem. 316*, 164.

Gmelin, R., Strauss, G., and Hasenmaier, G. (1959), *Hoppe-Seyler's Z. Physiol. Chem. 341*, 28.

Haskell, T. H., Fusari, S. A. Frohardt, R. P., and Bartz, Q. R. (1952), *J. Amer. Chem. Soc. 74*, 599.

Kitagawa, T., Miura, T., Fujiwara, K., and Taniyama, H. (1972), *Chem. Pharm. Bull. 20*, 2215.

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

Lechowski, L. (1969), *Tetrahedron Lett.*, 479.
Noe, F. F., and Fowden, L. (1960), *Biochem. J. 77*, 543.
Reinbothe, H. (1962), *Flora 152*, 545.
Renz, J. (1936), *Hoppe-Seyler's Z. Physiol. Chem. 244*, 153.
Roncari, G., Kurylo-Borowska, Z., and Craig, L. C. (1966), *Biochemistry 5*, 2153.
Tam, A. H.-K., and Jordan, D. C. (1972), *J. Antibiot. 25*, 524.
Wada, S., and Toyota, T. (1965), *Biochem. Biophys. Res. Commun. 19*, 482.

Biosynthesis of Viomycin. II. Origin of β-Lysine and Viomycidine†

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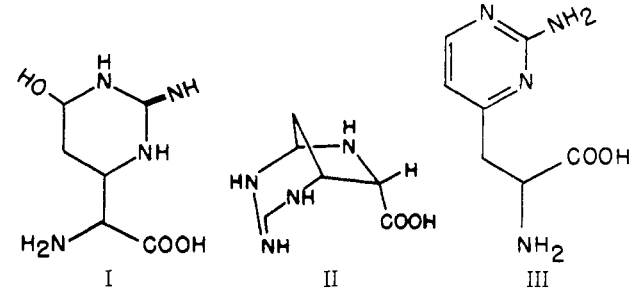
ABSTRACT: The biogenesis of L-3,6-diaminohexanoic acid (β-lysine) and 2,4,6-triaza-3-iminobicyclo[3.2.1]octane-7-carboxylic acid (viomycidine), two hydrolysis products of the polypeptide antibiotic viomycin, has been studied in growing cultures of a streptomycete. Labeling data indicate that the β-lysine carbon skeleton is derived from lysine without randomization of the lysine carbons. Radioactivity from aspartic acid, probably a precursor of lysine in this organism, was not incorporated well into viomycin, but most of the label was found in the β-lysine fragment, thus suggesting a possible biosynthetic pathway from aspartic acid to lysine to β-lysine. β-Lysine itself was incorporated well into viomycin, and only the β-lysine fragment was extensively labeled. This suggests that the displacement of the amino group in the α position of lysine to the β position of β-lysine occurs prior to the incorporation of the amino acid into the polypeptide. Viomycidine, which is a rearrangement product of one of the amino acids of the polypeptide, appears to be derived from arginine or a precursor of arginine. Labeled arginine was incorporated into viomycidine in what appears to be a specific manner. [1-^{14}C]-Ornithine was also incorporated almost exclusively into the amino acid residue which gives rise to viomycidine, but some randomization of activity was found in the resulting viomycidine. Radioactivity from specifically labeled glutamic acid was extensively randomized by the streptomycete, but it was incorporated somewhat preferentially into the viomycidine precursor. Labeled viomycidine was not incorporated specifically into any of the component amino acids of viomycin, thus confirming that viomycidine is not present as such in viomycin.

The structures of the polypeptide antibiotic viomycin proposed by Bycroft *et al.* (1971a) and Kitagawa *et al.* (1972) contain six amino acid residues. They are 2 equiv of L-serine and one each of L-α,β-diaminopropionic acid, L-3,6-diaminohexanoic acid (β-lysine), α-amino-β-ureidoacrylic acid, and a substituted hexahydropyrimidine derivative (I). These last two amino acids have never been isolated because of their instability to the acidic conditions used for the hydrolysis of the polypeptide. Rearrangement products of the hexahydropyrimidine fragment have been isolated, and the structure of one, 2,4,6-triaza-3-imino[3.2.1]octane-7-carboxylic acid (viomycidine, II), has been determined by X-ray crystallography (Floyd *et al.*, 1968; Bycroft *et al.*, 1968). A second guanidino compound, called viocidic acid, was formed in small amounts and has also been characterized (Bycroft *et al.*, 1969; J. R. Dyer and F. Suddath, unpublished results). A third guanidino compound has been isolated from viomycin hydrolysates by ion-exchange chromatography and has been referred to as "unknown guanidino-containing compound (UGCC)."[1] This compound has not been characterized, but has properties similar to viomycidine and is somewhat less basic (J. R. Dyer and K. C. Rice, unpublished results). A number of basic peptides are also produced during acid hydrolysis of viomycin. Several of these give rise to viomycidine upon further acid hydrolysis, so it is suspected that they are probably incomplete hydrolysis products of viomycin that contain viomycidine or related compounds bound to other amino acids (J. R. Dyer and J. C. Floyd, unpublished results).



I II III

β-Lysine, in addition to being a component of viomycin, has also been isolated from hydrolysis mixtures of other antibiotics including streptolin AB (Smissman *et al.*, 1953), streptothricin (Carter *et al.*, 1952), roseothricin (Nakanishi *et al.*,

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[1] Abbreviations used are: UGCC, unknown guanidino-containing compound.