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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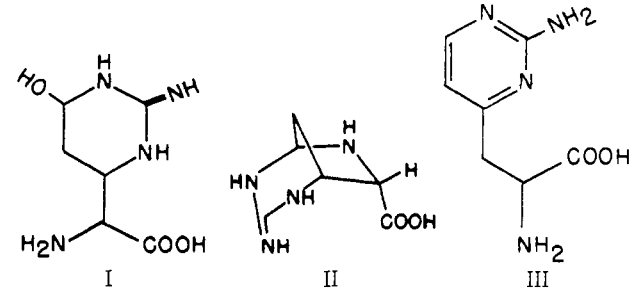
ABSTRACT: The biogenesis of L-3,6-diaminohexanoic acid (β-lysine) and 2,4,6-triaza-3-iminobicyclo[3.2.1]octane-7-carboxylic acid (viomycidine), two hydrolysis products of the polypeptide antibiotic viomycin, has been studied in growing cultures of a streptomycete. Labeling data indicate that the β-lysine 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).



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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.

1954), and geomycin (Brockmann and Musso, 1955). The biosynthesis of β-lysine has not been investigated in the organisms which produce these antibiotics; however, Costilow *et al.* (1966) have demonstrated that it is the first intermediate in the fermentation of L-lysine by *Clostridum*, SB4. The enzyme (lysine-2,3-aminomutase) that catalyzes the reaction has been isolated and purified by Chirpich *et al.* (1970).

In a previous paper (Carter *et al.*, 1974), the biosyntheses of the serine and diaminopropionic acid fragments of viomycin were reported. While ^{14}C-labeled serine was incorporated into β-lysine to some extent, neither serine nor any of the other labeled compounds tested were incorporated appreciably into viomycidine. This paper gives the results of experiments that were conducted to determine the origins of the carbon skeletons of these two amino acids.

Materials and Methods

DL-[1-^{14}C]Lysine, DL-[4-^{14}C]aspartic acid, DL-[5-^{14}C]arginine, DL-[amidine-^{14}C]arginine, DL-[1-^{14}C]ornithine, DL-[3,4-^{14}C]-glutamic acid, and DL-[5-^{14}C]glutamic acid were obtained from the New England Nuclear Corp. DL-[2-^{14}C]Lysine was obtained from Nuclear Research Chemicals, Inc., and DL-[1-^{14}C]arginine from Schwarz BioResearch, Inc. L-[^{14}C]β-Lysine and [^{14}C]viomycidine were isolated from the acid hydrolysate of viomycin from cultures grown in the presence of D-[1-^{14}C]glucose (Carter *et al.*, 1974). Viomycin was supplied by Parke, Davis and Co., and streptomycete cultures were the gifts of Parke, Davis and Co. and Charles Pfizer and Co.

Methods for the growth of the *Streptomyces* species, for the isolation, purification, and hydrolysis of viomycin, and for the purification of the hydrolysis fragments have been described previously (Carter *et al.*, 1974). Techniques for the degradation of serine and diaminopropionic acid and for the radioactivity measurements were described in that same publication.

Degradation of β-Lysine. A sample of β-lysine was converted to the *p*-hydroxyazobenzene-*p'*-sulfonate salt, which was purified by recrystallization from hot water. An aqueous solution of this purified salt (628 mg) was neutralized with IR-45 (OH^- phase) resin. After removal of the resin by filtration, the filtrates were freeze-dried to yield 172 mg of a brown glass. This glass was mixed with 250 mg of anhydrous sodium acetate and 625 mg of powdered phthalic anhydride. The mixture was ground and then heated in an oil bath at 170° for 30 min. To the cooled reaction mixture were added equal volumes of 1 N hydrochloric acid and ether. The ether layer was removed and evaporated to yield a colorless oil. The oil was extracted with boiling water to remove phthalic acid, the residue was dissolved in ether, and an ethereal solution of diazomethane was added. After 3 days at room temperature, white crystals (191 mg) of the methyl ester of di-(*N*-phthalyl)-β-lysine were deposited in the flask.

A portion of this methyl ester (100 mg) was diluted with 742 mg of unlabeled ester which had been prepared in the same manner, and the mixture was subjected to degradation by the Barbier–Wieland method. The benzophenone obtained from C-1 of the β-lysine derivative was chromatographed over an alumina column (1.0 cm × 14 cm) using benzene–ligroin (1:1) as the eluent. The yield was 14.40 mg (4.0%) based on the methyl ester).

The L-di(*N*-phthalyl)ornithine, present in the reaction mixture, was recrystallized from 95% ethanol and gave 70 mg (8.9% yield based on the original methyl ester) of pure material. A portion of this sample (68 mg) was mixed with 324 mg of unlabeled carrier prepared by the method of van Tamelen and Smissman (1953), and the phthalyl groups were removed by dissolving the sample in equal volumes (2.0 ml) of 1 M alcoholic hydrazine hydrate and 95% ethanol and allowing the solution to reflux for 1.5 hr. After crystallization from ethanol, the L-ornithine (isolated as the monohydrochloride, 80 mg; 4.7% yield) had an infrared spectrum identical with that of authentic material. A portion of this product was degraded using ninhydrin in the same manner as that described for serine and diaminopropionic acid (Carter *et al.*, 1974).

Degradation of Viomycidine. Viomycidine was degraded by a method involving acetylation, ozonolysis, and acid hydrolysis to yield aspartic acid, carbon dioxide, and guanidine (Dyer *et al.*, 1964). To freeze-dried samples of viomycidine were added 30 ml of dry pyridine and 30 ml of acetic anhydride. This solution was heated on a steam bath for 20 hr. Water (60 ml) was added, and after remaining at room temperature for 2 hr, the solution was evaporated to dryness. Another 40 ml of water was added, and the solution was again evaporated to give a syrup. This material was dissolved in 75 ml of 80% formic acid, and ozone was bubbled through the solution maintained at 0° for 1.5 hr. At this time, 5.0 ml of 30% hydrogen peroxide was added, and the solution was allowed to stand at room temperature overnight. Another 5.0 ml of 30% hydrogen peroxide was added, and the orange solution was allowed to stand for 1 hr. The solution was evaporated to give a syrup, and 75 ml of 6 N hydrochloric acid was added to the residue. This solution was heated on a steam bath for 22 hr and then decolorized using Darco G-60 (Atlas Chemical Industries, Inc.). The carbon was removed by filtration, and the filtrates were evaporated to a syrupy, crystalline mass.

The mixture, containing guanidine, aspartic acid, and other unidentified degradation products, was dissolved in 5.0 ml of water, and the components were separated by preparative paper chromatography on Whatman No. 17 paper. The chromatograms were developed by descending chromatography in a solvent containing 95% ethanol–3 N ammonium hydroxide (5:1). The components were detected on test strips by Weber's reagent and ninhydrin. The centers of the bands that contained guanidine and aspartic acid were removed, and the compounds were eluted with three 30-ml portions of hot water. The extracts were freeze-dried, the residues were redissolved in 3.00 ml of water, and 0.25-ml aliquots were removed for radioactivity measurement. By this method, C-1, -2, -3, and -4 of viomycidine could be isolated as C-1, -2, -3, and -4 of aspartic acid, C-5 as carbon dioxide, and C-6 as guanidine. Those samples of aspartic acid that contained appreciable radioactivity were further degraded using ninhydrin by the method described previously for the degradation of serine (Carter *et al.*, 1974).

In the experiment using [5-^{14}C]arginine as a precursor, attempts were made to collect the carbon dioxide at each stage of the degradation. All steps were carried out in a closed system, and the carbon dioxide was trapped in Hyamine hydroxide (Packard Instrument Co.). Excess ozone was removed by allowing the gas stream to pass through styrene prior to trapping the carbon dioxide. Oxygen was used to purge the system during the hydrogen peroxide step, and nitrogen was used to purge the system during the acid hydrolysis.

This method for viomycidine degradation, although the best available, suffers from several serious drawbacks. None of the reactions is quantitative, and multiple reactions occur.

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 (%)
DL-[1-^{14}C]Lysine (8.03)	389	41.7	8.32	5.18
DL-[2-^{14}C]Lysine (20.8)	111	39.8	27.8	1.91
DL-[4-^{14}C]Aspartic acid (7.95)	243	1.27	0.40	0.16
L-[^{14}C]-β-Lysine (0.143)	232	1.17	0.39	8.15
DL-[1-^{14}C]Arginine (7.06)	219	16.5	5.85	2.34
DL-[5-^{14}C]Arginine (10.8)	177	39.5	17.25	3.66
DL-[amidine-^{14}C]Arginine (4.94)	333	26.6	6.21	5.38
DL-[1-^{14}C]Ornithine (6.86)	254	15.8	4.83	2.30
DL-[3,4-^{14}C]Glutamic acid (8.07)	171	2.61	1.18	0.32
DL-[5-^{14}C]Glutamic acid (9.75)	169	1.63	0.75	0.17
[^{14}C]Viomycidine (0.069)	316	0.091	0.02	1.31

Furthermore, the low yields of guanidine and aspartic acid plus the soluble carbohydrates eluted from the paper during isolation made purification of these products and thus comparisons of specific activities impossible.

Results

Incorporation of Radioactive Precursors into Viomycin. Lysine, specifically labeled in C-1 or -2, was tested as a potential precursor of viomycin because of the reported role of that amino acid as a precursor of β-lysine in a *Clostridium* species (Costilow *et al.*, 1966). The results shown in Table I indicate that both labeled lysines were incorporated into viomycin. Aspartic acid is known to be a precursor of lysine in bacteria (Meister, 1965; Vogel, 1959) *via* the diaminopimelic acid pathway. Such a pathway has been demonstrated in the streptomycete used in these studies, and the typical pathway of higher organisms, which involves saccharopine as an intermediate, was absent (R. H. Du Bus and P. D. Shaw, unpublished results). Aspartic acid was, however, not so effective as lysine as a precursor of viomycin. β-[^{14}C]Lysine was incorporated very well into viomycin. In fact, of the many compounds examined, β-lysine gave the highest per cent incorporation.

In order to test the hypothesis that arginine might be involved in the biosynthesis of the portion of viomycin from which viomycidine is derived, cultures of the streptomycete were grown in the presence of arginine specifically labeled with ^{14}C in C-1 and -5 and in the amidine group. The radioactivity incorporated into viomycin from these three compounds is shown in Table I. In all three experiments, radioactivity was incorporated reasonably well into viomycin. Although arginine labeled in the amidine group gave the greatest per cent incorporation into viomycin, [5-^{14}C]arginine gave a product with the highest specific activity.

In bacteria, arginine is derived from glutamic acid, and ornithine is an intermediate in the pathway (Meister, 1965). The results in Table I show that the viomycin obtained from cultures grown in the presence of [1-^{14}C]ornithine was labeled to about the same extent as that from [1-^{14}C]arginine. The incorporation of both [3,4-^{14}C]- and [5-^{14}C]glutamic acid, however, was very poor. Small amounts of radioactivity were also incorporated into viomycin from [^{14}C]viomycidine.

TABLE II: Radioactivity in the Principal Hydrolysis Products from DL-[1-^{14}C]- and DL-[2-^{14}C]Lysine Experiments.

Fraction	Wt (mg): 1-^{14}C	2-^{14}C	Act. (dpm × 10^{-3}): 1-^{14}C	2-^{14}C
Carbon dioxide			403	23
Serine	462	795	1,782	3150
Diaminopropionic acid·HCl	295	355	329	744
β-Lysine·HCl	513	568	18,280	9780
Viomycidine·HCl	306	290	356	1760
Peptides	410	276	325	427

Determination of Radioactivity in Viomycin Hydrolysis Products. The distributions of radioactivity among the hydrolysis products of viomycin samples obtained from cultures grown in the presence of [1-^{14}C]- and [2-^{14}C]lysine are shown in Table II. In both experiments, most of the radioactivity was found in the β-lysine, although serine and, in the case of the [2-^{14}C]lysine, viomycidine contained appreciable radioactivity. Radioactivity from [4-^{14}C]aspartic acid, which was poorly incorporated into viomycin, was distributed among all the hydrolysis fragments (Table III); however, the β-lysine appeared to be the most heavily labeled. The β-lysine obtained from acid hydrolysis of viomycin grown in the presence of β-[^{14}C]lysine was the only component of the hydrolysate that contained radioactivity. Of the 1.17×10^6 dpm present in the viomycin, the β-lysine contained 1.03×10^6 dpm (88%), and the purified product had a specific activity of 6.17×10^5 dpm/mmol.

Table IV shows the distribution of radioactivity in the viomycin hydrolysis products using [1-^{14}C]-, [5-^{14}C]-, and [amidine-^{14}C]arginine and [1-^{14}C]ornithine as the precursor. With each of these compounds, relatively small amounts of activity were found in the carbon dioxide, serine, diaminopropionic acid, and β-lysine, whereas the viomycidine, UGCC, and the peptides were heavily labeled. If preliminary indications, that viomycidine and UGCC are derived from the same portion of the viomycin molecule, are correct and if the peptides is present largely as the viomycidine precursor, the nearly all (>90%) of the radioactivity in the isolated viomycin hydrolysis products can be accounted for in the viomycidine precursor.

Radioactivity from [3,4-^{14}C]- and [5-^{14}C]glutamic acid was poorly incorporated into viomycin (Table I), and it was

TABLE III: Radioactivity in the Principal Hydrolysis Products from DL-[^{14}C]Asparatic Acid Experiment.

Fraction	Wt (mg)	Act. (dpm × 10^{-3})
Carbon dioxide		75
Serine	709	43
Diaminopropionic acid·HCl	281	36
β-Lysine·HCl	460	152
UGCC·HCl	263	40
Peptides	128	34

TABLE IV: Radioactivity in the Principal Hydrolysis Products from DL-[1-^{14}C]-, DL-[5-^{14}C]-, and DL-[amidine-^{14}C]Arginine and DL-[1-^{14}C]Ornithine Experiments.

Fraction	Wt (mg) of Arginine [1-^{14}C]-	Wt (mg) of Arginine [5-^{14}C]-	Wt (mg) of Arginine [amidine-^{14}C]-	Wt (mg) of [1-^{14}C]-Ornithine	Act. (dpm × 10^{-3}) Arginine [1-^{14}C]-	Act. (dpm × 10^{-3}) Arginine [5-^{14}C]-	Act. (dpm × 10^{-3}) Arginine [amidine-^{14}C]-	Act. (dpm × 10^{-3}) [1-^{14}C]-Ornithine
Carbon dioxide					182	64	87	182
Serine	638	706	574	330	151	50	12	111
Diaminopropionic acid·HCl	325	359	223	162	66	241	122	366
β-Lysine·HCl	470	432	486	569	48	181	90	98
Viomycidine·HCl	234	176	207	264	2742	4650	2276	3740
UGCC·HCl	148	144	90	96	1275	3350	999	907
Peptides[a]	103	498	308	338	739	5248	2405	2254

[a] Combined weights and radioactivity of two peaks.

randomly distributed in the hydrolysis products. None of the amino acids contained appreciable activity. The viomycin from the [^{14}C]viomycidine-grown cultures was also hydrolyzed and the hydrolysate was chromatographed on an ion-exchange column. The low level of activity (7.6×10^4 dpm) present in the viomycin from [^{14}C]viomycidine grown cultures was distributed throughout the column fractions; therefore, radioactivity in the individual fractions was not determined.

Degradation of Viomycin Hydrolysis Products. The β-lysine, serine, and diaminopropionic acid samples obtained from the hydrolysis of viomycin isolated from cultures grown in the presence of labeled lysine were degraded to determine their labeling patterns. The β-lysine from the labeled aspartic acid cultures was not degraded because of the low activity, and the β-lysine from the labeled β-lysine cultures was not degraded because the labeling pattern of the starting material was not known. The results with the labeled lysine cultures are shown in Table V. Essentially all of the radioactivity in the β-lysine derived from [1-^{14}C]lysine was found in the benzophenone derived from C-1. The radioactivity in the benzophenone was negligible from the β-lysine derived from [2-^{14}C]lysine cultures. Within experimental error, all (104%) of the radioactivity was found in the ornithine derived from C-2 to C-6 of the β-lysine. Further degradation of the ornithine indicated that 84.3% of the radioactivity was present in C-1 of the ornithine and hence in C-2 of the β-lysine.

TABLE V: Labeling Patterns of Serine, Diaminopropionic Acid, and β-Lysine Derived from Labeled Viomycin.

	Act. (%) C-1	Act. (%) C-2	Act. (%) C-3
Precursor [1-^{14}C]Lysine			
β-Lysine	98.6	1.4	
Serine	100	0.0[a]	0.0
Diaminopropionic acid·HCl	100	0.0[a]	0.0
Precursor [2-^{14}C]Lysine			
β-Lysine	0.8	84.3	
Serine	2.4	62.9[a]	34.7
Diaminopropionic acid·HCl	2.1	61.0[a]	36.9

[a] Values determined by difference.

Because the ^{14}C of lysine was incorporated significantly into the serine and diaminopropionic acid fragments of viomycin, these two amino acids were also degraded to determine the positions of the labels. These results are given in Table V. It can be seen that the serine and diaminopropionic acid labeling patterns were nearly identical. [1-^{14}C]Lysine, however, was incorporated exclusively into C-1 of the serine and diaminopropionic acid, while the radioactivity from [2-^{14}C]-lysine was distributed in a ratio of about 2/1 between C-2 and -3, with only a trace of incorporation into C-1.

The samples of viomycidine obtained from viomycin isolated from cultures grown in the presence of labeled arginine and ornithine were degraded to determine their labeling patterns. The results of these degradations are shown in Table VI. The yields of the degradation fragments from viomycidine were low, so purification to homogeneity was not possible. The values for the radioactivity of the fragments are therefore in terms of absolute disintegrations per minute rather than in specific activities. The recoveries of radioactivity in all of the experiments were essentially the same (0.8–2.5%). It is therefore reasonable to assume that the yields of the products were similar, so a comparison of the activity in each of the fragments should give a reliable estimate of the labeling patterns.

If it is assumed that the carbon dioxide formed during ozonolysis contained little or no radioactivity, the results indicate a high degree of specificity of incorporation from [1-^{14}C]arginine and [amidine-^{14}C]arginine into viomycidine. Approximately 95% of the recovered radioactivity from [1-^{14}C]arginine was recovered in the aspartic acid fraction, and 91% was in C-1 and -4 of that amino acid. The viomycidine derived from [amidine-^{14}C]arginine appeared to be labeled almost exclusively in the guanidino carbon; 92% of the isolated activity was found in guanidine. Neither the aspartic acid nor the guanidine from the degradation of viomycidine from the [5-^{14}C]arginine experiment contained significant radioactivity. Essentially all of the isolated radioactivity was found in the carbon dioxide formed during ozonolysis.

From the known pathway for the conversion of ornithine to arginine in bacteria, one might have predicted that the incorporation of these amino acids, labeled in the carboxyl carbon, into viomycin would lead to similar labeling patterns in the degradation fragments. Such, however, was not the case. While both were incorporated primarily into the viomy-

TABLE VI: Labeling Patterns of Viomycidine Derived from Labeled Viomycin.

Precursor	Viomycidine			
	Sp Act. (dpm/mg)	Carbon	Act.[c] (dpm)	Total Act. per Atom (%)
DL-[1-^{14}C]Arginine	8,166	1,4	1575	91.4
		2,3	59[d]	3.4
		5	*a*	
		6	89	5.2
DL-[5-^{14}C]Arginine	20,220	1,2,3,4	170	<1.0
		5	2.04×10^5	100[b]
		6	13	<1.0
DL-[amidine-^{14}C]Arginine	8,237	1,2,3,4	140	7.9
		5	*a*	
		6	1820	92.1
DL-[1-^{14}C]Ornithine	11,608	1,4	1794	48.6
		2,3	1788[d]	48.4
		5	*a*	
		6	113	3.1

[a] Activity not determined and assumed to be zero. [b] This value is only approximate because of poor recovery of carbon dioxide. The absence of radioactivity in the other degradation fragments suggests that most if not all was present in C-5. [c] Activity is in terms of disintegrations per minute per 0.25-ml aliquot as described in the text. [d] Determined by difference in radioactivity of the aspartic acid and the carbon dioxide and by radioactivity in residue after ninhydrin degradation.

cidine fragment of viomycin, and the radioactivity in the viomycidine degradation products was almost exclusively in the aspartic acid, the aspartic acid from the [1-^{14}C]ornithine contained about one-half of the radioactivity and C-1 and -4 and the remainder in C-2 and -3. In order to establish that the ornithine used in this experiment was labeled exclusively in the carboxyl group, a sample was degraded with ninhydrin. The results of this degradation showed that 98% of the ornithine was labeled in C-1.

Discussion

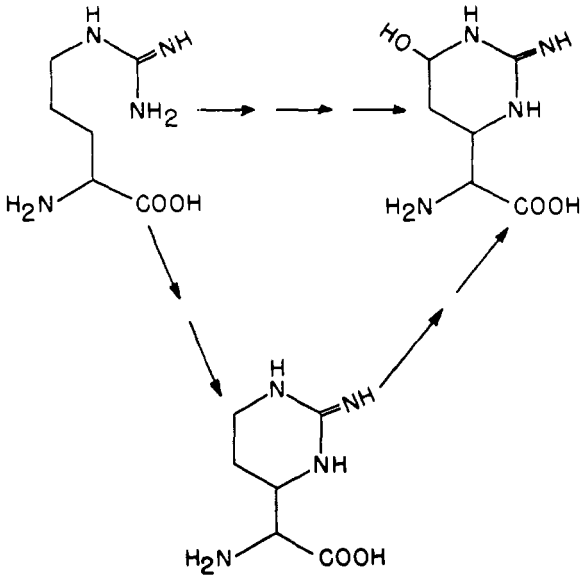
In contrast to the results reported by Tam and Jordan (1972), lysine was one of the best precursors of viomycin of the many compounds we tested, and radioactivity from labeled lysine was incorporated primarily into the β-lysine portion of the antibiotic. This indicates that lysine may be a direct precursor of β-lysine. Aspartic acid, although quite probably a precursor of lysine in this organism, was not incorporated well into viomycin, probably because of extensive metabolism by other, nonviomycin related pathways. In spite of this poor incorporation, the β-lysine was the most heavily labeled product (approximately 40% of the total). These results, therefore, suggest a pathway in which aspartic acid is converted into lysine and the lysine into β-lysine.

Degradation of the β-lysine indicated a high degree of specificity of incorporation of C-1 and -2 of lysine. Although nothing can be inferred from these results concerning the origin of the remaining four carbon atoms of the β-lysine, the data provide a good indication that the carbon skeleton of β-lysine may be derived from lysine directly and without randomization. This hypothesis is supported by results using [2,6-^{15}N]lysine (J. R. Dyer and K. C. Rice, unpublished results) that suggest that the amino groups of lysine may also be incorporated into β-lysine. The biosynthesis of β-lysine by the *Streptomyces* species used may therefore follow a similar pathway to that found in anaerobic bacteria (Chirpich *et al.*, 1970). A number of attempts have been made to detect an enzyme system similar to that reported by Chirpich *et al.* (1970), in extracts of this *Streptomyces* species. Under conditions intended to duplicate theirs, however, no conversion of lysine to β-lysine could be detected. A possible explanation for our failure to find this enzyme may be found in the instability of the enzyme and/or the low levels of activity in our preparations. Lysine represented the primary nitrogen source in the growth medium for the *Clostridium*, and lysine-2,3-aminomutase accounted for about 2% of the protein in the crude extracts. The enzyme was thus essential for the growth of the *Clostridium*, whereas viomycin, and presumably β-lysine, formation is not required for growth of the streptomycete organism, so the conversion of lysine to β-lysine may represent a very minor pathway.

The incorporation of β-[^{14}C]lysine exclusively into the β-lysine of viomycin indicates that, if lysine is assumed to be a precursor of β-lysine, the migration of the amino group occurs prior to the incorporation of β-lysine into a more complex viomycin precursor.

Arginine and ornithine were the only precursors to be incorporated almost exclusively into the fragment of viomycin that gives viomycidine upon acid hydrolysis. In spite of the fact that radioactivity in the viomycidine degradation fragments could not be related quantitatively to the specific activities of the starting materials, the distribution of activity indicates a high degree of specificity in the incorporation of arginine into the viomycidine precursor. If it is accepted that the radioactive carbon dioxide obtained from the ninhydrin degradation of aspartic acid obtained from the viomycidine of the [1-^{14}C]arginine experiment is derived from C-1 of the aspartic acid, the labeling patterns of the three viomycidine samples from the labeled arginine experiments suggest that all of the carbon skeleton including the carbon of the amidine group of arginine is incorporated as a unit into the viomycidine precursor. These results would tend to rule out a proposed pathway (Paśś and Raczynska-Bojańowska, 1969; Raczyńska-Bojanowska *et al.*, 1969) which involved the transfer of the arginine amidine group to some unspecified acceptor. The

SCHEME I



mechanism by which arginine is utilized for viomycin biosyntheses is not known. Our results suggest that the other unusual amino acids found in viomycin, α,β-diaminopropionic acid and β-lysine, are utilized performed for viomycin biosynthesis, so by analogy one might predict that the structure of arginine may also be altered prior to incorporation into the polypeptide. The lack of specificity of viomycidine incorporation into viomycin suggests that viomycidine is not a precursor of viomycin and confirms previous results that it is not present as such in viomycin but is an artifact of the acid hydrolysis.

The poor incorporation of glutamic acid into viomycin and the low specificity of its incorporation into the hydrolysis products probably reflect the multiple pathways by which that amino acid can be utilized by the streptomycete. The loss of the amino group by transamination or oxidation would give α-ketoglutarate, the carbon skeleton of which could be degraded and reutilized for the biosynthesis of a wide variety of compounds. It is also possible that this organism synthesizes arginine by a pathway that does not involve glutamic acid.

Although radioactivity from [1-^{14}C]ornithine was incorporated into viomycidine to about the same extent as [1-^{14}C]-arginine, the labeling patterns of the resulting viomycidine samples were different. Three alternatives were considered that might account for the observed distributions of radioactivity. (1) The ornithine could have contained radioactivity in positions other than the carboxyl group. (2) Either radioactive material may have cochromatographed with the aspartic acid, and this material did not produce radioactive carbon dioxide upon degradation with ninhydrin or the aspartic acid was incompletely degraded. (3) The radioactivity in the ornithine carboxyl group was somehow randomized between one or more of the remaining four carbon atoms. The first of these possibilities was proven incorrect by ninhydrin degradation of the ornithine, which showed that essentially all of the radioactivity was in the carboxyl group. The second possibility cannot be ruled out, but is would appear unlikely in view of the fact that all of the viomycidine degradations and the isolations of aspartic acid were carried out in the same manner and that in none, including that from [1-^{14}C]-arginine, was there any apparent randomization of activity. Furthermore, concurrent degradations (in duplicate) of aspartic acid from [1-^{14}C]arginine and [1-^{14}C]ornithine always gave the results shown in Table VI. The third alternative would thus seem the most likely; however, we are unaware of any pathway of ornithine metabolism in which C-1 becomes randomized with one or more of the other four carbons.

A proposed pathway for the biosynthesis of the viomycidine precursor is shown in Scheme I. The transformation of arginine would require a cyclization of the guanidino group to C-3 and an oxidation at C-5. It is interesting to speculate that arginine might also be involved in the biosynthesis of the closely related compound, capreomycidine (Deoxy-I), an amino acid found in a closely related antibiotic, capreomycin (Bycroft *et al.*, 1971b). A similar pathway has been proposed for the biosynthesis of lathyrine (III), by *Lathyrus tingitanus* (Bell, 1961; Bell and Foster, 1962). This compound is an unsaturated homolog of capreomycidine, and it is found in the seeds of several species of the genus *Lathyrus* along with other unusual amino acids such as homoarginine, γ-hydroxyhomoarginine, and both α- and β-*N*-oxalyl-α,β-diaminopropionic acids (Bell, 1962, 1963; Rao *et al.*, 1963; Bell and O'Donovan, 1966; Adiga *et al.*, 1963). A pathway was proposed in which homoarginine is oxidized to γ-hydroxyhomoarginine, and this compound is cyclized to lathyrine.

The similar labeling patterns of the serine and diaminopropionic acid obtained from viomycin grown in the presence of specifically labeled lysine provide a confirmation for the proposal that serine is a direct precursor of diaminopropionic acid (Carter *et al.*, 1974). Furthermore, the extent of incorporation would suggest that the conversion of lysine to these two amino acids may represent a fairly major pathway of lysine metabolism. The labeling patterns of the serine and diaminopropionic acid, however, do not provide any obvious explanation for the nature of such a pathway. It would appear that this represents an alternate mechanism for lysine metabolism and not subsequent steps in the β-lysine pathway, because only traces of radioactivity from that amino acid were incorporated into either serine or diaminopropionic acid.

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References

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

Bell, E. A. (1961), *Biochim. Biophys. Acta 47*, 602.

Bell, E. A. (1962), *Biochem. J. 85*, 91.

Bell, E. A. (1963), *Nature (London) 199*, 70.

Bell, E. A., and Foster, R. G. (1962), *Nature (London) 194*, 91.

Bell, E. A., and O'Donovan, J. P. (1966), *Phytochemistry 5*, 1211.

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

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

Bycroft, B. W., Cameron, D., Croft, L. R., Hassanli-Walji, A., Johnson, A. W., and Webb, T. (1971b), *Nature (London) 231*, 301.

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

Bycroft, B. W., Croft, L. R., Johnson, A. W., and Webb, T. (1969), *J. Antibiot. 22*, 133.

Carter, H. E., Hearn, W. R., Lansford, E. M., Jr., Page, A. C., Jr., Salzman, N. P., Shapiro, D., and Taylor, W. R. (1952), *J. Amer. Chem. Soc. 74*, 3704.
Carter, J. H., Du Bus, R. H., Dyer, J. R., Floyd, J. C., Rice, K. C., and Shaw, P. D. (1974), *Biochemistry 13*, 1221.
Chirpich, T. P., Zappia, V., Costilow, R. N., and Barker, H. A. (1970), *J. Biol. Chem. 245*, 1778.
Costilow, R. N., Rochovansky, O. M., and Barker, H. A. (1966), *J. Biol. Chem. 241*, 1573.
Dyer, J. R. Hayes, H. B., Miller, E. G., Jr., and Nassar, R. F. (1964), *J. Amer. Chem. Soc. 86*, 5363.
Floyd, J. C., Bertrand, J. A., and Dyer, J. R. (1968), *Chem. Commun.*, 998.
Kitagawa, T., Miura, T., Fujiwara, K., and Taniyama, H. (1972), *Chem. Pharm. Bull. 20*, 2215.
Meister, A. (1965), Biochemistry of the Amino Acids, New York, N. Y., Academic Press.
Nakanishi, K., Ito, T., and Hirata, Y. (1954), *J. Amer. Chem. Soc. 76*, 2845.
Paśś, L., and Raczynska-Bojańowska, K. (1969), *Acta Biochim. Pol. 16*, 297.
Raczyńska-Bojanowska, K., Tye, M., Paśś, L., and Kotula, Z. (1969), *Bull. Acad. Pol., Sci. Ser. Sci. Biol., 17*, 203.
Rao, S. L. N., Ramachandran, L. K., and Adiga, P. R. (1963), *Biochemistry 2*, 298.
Smissman, E. E., Sharpe, R. W., Aycock, B. F., van Temelen, E. E., and Peterson, W. H. (1953), *J. Amer. Chem. Soc. 75*, 2029.
Tam, A. H.-K., and Jordan, D. C. (1972), *J. Antibiot. 25*, 524.
van Tamelen, E. E., and Smissman, E. E. (1953), *J. Amer. Chem. Soc. 75*, 2031.
Vogel, H. J. (1959), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 18*, 345.

Polysaccharide Production by Cultured B-16 Mouse Melanoma Cells†

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ABSTRACT: Polysaccharide and glycoprotein production by two clones of the B-16 mouse melanoma and a primary explant culture of syngeneic normal iris melanocytes was studied by exposure of cultures to [^{3}H]glucosamine and $^{35}SO_4^{2-}$. Products were fractionated by differential salt extraction and porous glass bead chromatography. Neither the melanotic nor the amelanotic clones produce significant quantities of hyaluronic acid, a product which accounts for about half of the total labeled fraction for the iris melanocytes. A high molecular weight chondroitin of varying sulfate content, predominantly 4-sulfate, is a major product of the cloned lines; this polysaccharide was not detected in the iris cultures. Heparitin sulfate was present in all cells but appeared to be somewhat higher in content for the tumorigenic clones.

Cell surface properties are often markedly altered as a result of neoplastic transformation. Properties such as agglutination by plant lectins, the expression of tumor associated antigens, loss of histocompatibility antigens and variations in glycolipid and glycoprotein components have been described. We have recently reported on the alteration of mucopolysaccharide synthesis associated with virus-induced cellular transformations (Satoh *et al.*, 1973a). The present study characterizes and compares the complex polysaccharides produced by B-16 mouse melanoma cell cultures (both melanotic and amelanotic clones) and a control population of normal melanotic melanocytes obtained from syngeneic irises. A preliminary report has been presented (Satoh *et al.*, 1973c).

Materials and Methods

The origins of the B-16 melanotic melanoma cell line used in these experiments have been previously described (Kreider *et al.*, 1973). The amelanotic clone was isolated from the stock B-16 tumor maintained by the Jackson Laboratory, Bar Harbor, Maine. Clonal isolates were obtained by the glass chip method (Martin, 1973); the resulting line produces only very small amounts of melanin. All cells were routinely propagated in 16-oz prescription bottles and fed with minimum essential medium with Earle's salts, 10% heat-inactivated fetal calf serum supplemented with nonessential amino acids, sodium pyruvate, and twice the usual concentration of vitamins.

Cell pellets were periodically checked for bacterial contamination in tryptose phosphate and thioglycollate broths and for yeast contamination in Sabouraud broth. *Mycoplasma* testing was performed by both culture (House and Waddell, 1967) and autoradiography of tritiated thymidine uptake (Studzinski *et al.*, 1973).

Mouse iris melanocytes were obtained by a modification of a previously described method (Ephrussi and Temin, 1960). C57BL/6J mice, syngeneic to the B16 tumor cells, were killed by cervical dislocation following ether anesthesia. The irises were aseptically excised, and cultured as primary explants for 41 hr with 20 μCi/ml of [^{3}H]glucosamine and 20 μCi/ml of $^{35}SO_4^{2-}$.

Prescription bottles containing 10–15 million cells were incubated for 24–48 hr with the same isotopically labeled precursors at identical concentrations. After the incubation period, the media was decanted and saved and the cell monolayers were released from the glass with 0.25% EDTA in

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