Biogenetic Origin of the D-Isoleucine and N-Methyl-L-Alloisoleucine Residues in the Actinomycins

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Studies with ¹⁴C-labeled isoleucine stereoisomers have established that L-alloisoleucine, D-alloisoleucine, and D-isoleucine may function as precursors for the biogenesis of D-isoleucine and N-methyl-L-alloisoleucine residues in actinomycin. L-[¹⁴C]isoleucine appears to be employed chiefly for D-alloisoleucine (and N-methylisoleucine [?] formation); however, its role in the biosynthesis of D-isoleucine and N-methylalloisoleucine remains unclear. The potential pathway of biosynthesis of D-isoleucine and N-methyl-L-isoleucine is discussed.

Previous investigations have demonstrated that synthesis of novel actinomycins as well as enhanced formation of minor components occurs during antibiotic production in the presence of one of the four stereoisomers of isoleucine (16, 22, 33, 34). D-Isoleucine and N-methyl-L-alloisoleucine were identified as amino acid constituents of the isoleucine-induced antibiotics elaborated by various *Streptomyces* species (20, 38, 39). The observation that D-isoleucine is a constituent of certain monamycin congeners constituted the first report concerning an exception to the rule of α -epimerization (4, 5, 7). Radioisotope experiments clearly revealed that L-isoleucine, L-alloisoleucine, and D-alloisoleucine can serve as precursors of the D-isoleucine moiety (11). To clarify the biogenetic origin of the D-isoleucine and N-methyl-L-alloisoleucine residues in the actinomycins, experiments were carried out employing the ¹⁴C-labeled stereoisomers of isoleucine. The results of these investigations are presented in this communication.

MATERIALS AND METHODS

Organisms and conditions of cultivation. *Streptomyces antibioticus* (ATCC 14888), *Streptomyces chrysomallus* (ATCC 11523), and *Streptomyces parvulus* (ATCC 12434) were cultivated on slants of glucose-yeast extract-malt extract agar medium (15). For production of actinomycin, an organism was grown first in NZ amine medium for 48 h; synthesis of actinomycin mixtures was effected subsequently in glutamic acid-galactose-glucose or fructose-glutamic acid-histidine medium as described previously (19, 21, 23, 39).

Chemicals and reagents. D-Isoleucine and DL-isoleucine (mixture of four stereoisomers) were purchased from Sigma Chemical Co. (St. Louis, Mo.). L-Alloisoleucine was purified from commercial L-alloisoleucine (Schwarz BioResearch, Orangeburg, N.Y.) or from a mixture of stereoisomeric racemates by the method of Greenstein and Winitz (13). D-Alloisoleucine was kindly supplied by Ted Otani (National Cancer Institute, Bethesda, Md.). L-Isoleucine was purchased from Calbiochem (San Diego, Calif.). N-methyl-DL-isoleucine was provided by Jun'ichi Shoji (Shionogi Research Laboratories, Osaka, Japan), and N-methyl-D-alloisoleucine was a gift from Tapan Audhya (Dalhousie University, Halifax, Nova Scotia, Canada). N-methyl-DL-valine was prepared by the method of Cook and Cox (10). All other amino acids were from commercial sources.

D- and L-amino acid oxidases were purchased from Worthington Biochemicals Corp. (Freehold, N.J.) and the Ross Allen Reptile Institute (Silver Springs, Fla.), respectively. Acylase I (hog kidney) and silicic acid (sil-B-200, 60 to 200 mesh) were from Sigma Chemical Co. (St. Louis, Mo.). Silicic acid (100 mesh) was procured from Mallinckrodt Chemical Works (St. Louis, Mo.).

Radioisotopes. Uniformly labeled L-[¹⁴C]isoleucine (312 mCi/mmol) was purchased from Amer-sham-Searle (Arlington Heights, Ill.) and New England Nuclear Corp. (Boston, Mass.), and DL-[3,3',4,5-¹⁴C]alloisoleucine (165 mCi/mmol) was a gift from Schwarz/Mann BioResearch Inc. (Orangeburg, N.Y.). D- and L-[¹⁴C]alloisoleucine were purified from the DL-racemate by the following method. A preliminary experiment (high-voltage electrophoresis, autoradiography) revealed that the ¹⁴C-labeled racemate was contaminated with several radioactive substances. To purify the radiolabeled amino acid (50 μ Ci), preparative high-voltage electrophoresis was carried out. After autoradiography, the area of the paper corresponding to alloisoleucine was cut out, and the amino acid was eluted with water by descending chromatography. The eluate was acidified with 6 N HCl to pH 1.0 and applied to

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a Dowex 50W $\times$ 8 column (H^+ form, 0.4 by 5 cm). The column was washed with 0.1 N HCl and then water until neutral. The amino acid was eluted with 1 N NH_4OH . After evaporation to dryness, the residue was mixed with 5 mg of unlabeled L-alloisoleucine and acetylated with 0.05 ml of acetic anhydride and 1 ml of glacial acetic acid. Acetic acid was removed in vacuo after the reaction; the residue was treated with 0.5 mg of acylase I and 2.5 mg of cobaltous acetate in a final volume of 2.5 ml (pH 7.5 with 1 N NH_4OH) for 48 h at 38 C. After acidification with glacial acetic acid to pH 5.0, protein was removed by centrifugation. The volume of the supernatant was reduced to approximately 1 ml in vacuo, and the amino acid solution was applied as a streak to Whatman 3MM paper (46 by 46 cm) and developed with the solvent mixture (1-butanol-acetic acid-water, 12:3:5) for 16 h. The air-dried paper was subjected to autoradiography; two bands were visible on the X-ray film. Free L-alloisoleucine was eluted from lower band (R_f , 0.66) and N-acetyl-D-alloisoleucine was eluted from the higher band (R_f , 0.92) with water. The L- $[^{14}C]$ alloisoleucine solution was purified further through a Dowex 50W $\times$ 8 column; the NH_4OH eluate was evaporated to dryness. The yield of L-alloisoleucine was 10.5 μCi (1.96 mg). The solution containing acetyl-D-alloisoleucine was evaporated to dryness, and the residue was refluxed for 2 h in 1.0 ml of 2 N HCl. The hydrolysate was evaporated to dryness and purified by Dowex 50W $\times$ 8 (H^+ form) chromatography. The yield of D- $[^{14}C]$ -alloisoleucine was 14.5 μCi .

D- $[^{14}C]$ isoleucine was prepared from DL- $[^{14}C]$ alloisoleucine in the following manner. A mixture of DL- $[^{14}C]$ alloisoleucine (4.24 μCi , 6.8×10^6 counts/min) and unlabeled L-alloisoleucine (0.4 μmol) was added to each of two flasks of a 48-h-old culture of *S. antibioticus* actively synthesizing actinomycin, and the incubation was resumed for 4 h. The combined culture filtrates (190 ml) were extracted two times with 200 ml of ethyl acetate. The organic phase (containing $[^{14}C]$ actinomycin) was washed five times with 100 ml of water. After removal of the ethyl acetate by evaporation in vacuo, the actinomycin mixture (5.06 μmol , 3.38×10^6 counts/min per μmol) was hydrolyzed in 6 N HCl, and the amino acids in the hydrolysate were separated by high-voltage paper electrophoresis. $[^{14}C]$ isoleucine on the electropherogram was located by autoradiography. After elution with water, the ^{14}C -labeled amino acid was further purified by Dowex 50W $\times$ 8 ion-exchange chromatography. The D configuration of the amino acid was established with the use of D- and L-amino acid oxidases (23, 25, 30).

Incorporation of ^{14}C -labeled amino acids into the actinomycins. Experiments were carried out with a Dubnoff metabolic shaking incubator (Precision Scientific Co., Chicago, Ill.) or with a reciprocating shaking incubator (New Brunswick Scientific Co., New Brunswick, N.J.) at 30 C (23).

Distribution of radioactivity in the constituent amino acids of actinomycin. After incubation, the culture filtrate was separated from the mycelium by filtration. The mycelium was washed with deionized water, and the washings were combined

with the culture filtrate. Actinomycin mixtures were extracted from the aqueous solution with ethyl acetate (1:2). The organic phase was washed four times with an equal volume of water and then evaporated to dryness under vacuum. The actinomycins were purified by silicic acid chromatography (23, 35). The antibiotic synthesized by *S. antibioticus* or *S. parvulus* was hydrolyzed in 6 N HCl as detailed elsewhere (23). In most experiments, *S. chrysomallus* mixtures were first separated into individual components by descending paper chromatography and then purified by the procedure of Yajima et al. (39) prior to acid hydrolysis.

Separation of amino acids in hydrolysates was achieved by high-voltage electrophoresis (23, 38, 39). Amino acid standards were also applied to locate the amino acids. For samples of relatively high specific radioactivity, sections (1 cm long by 2.5 cm wide) from a single run were cut from the unstained paper and transferred to counting vials for radioactivity measurements in Omnifluor. For samples of low specific radioactivity, aliquots of a hydrolysate were applied to Whatman 3MM paper at 0.25-inch (approximately 0.63 cm) intervals (approximately 0.1 μmol /sample) and subjected to high-voltage electrophoresis. After separation, the amino acids were located with ninhydrin (0.02% in acetone) and then eluted from the paper with 75% acetone in water. Eluates were transferred to counting vials and evaporated to dryness, and radioactivity was measured in 10 ml of Omnifluor.

For the preparative isolation of amino acids by electrophoresis (23, 38), samples (containing 0.1 μmol of each amino acid) were applied to Whatman 3MM paper at 0.25-inch intervals. Amino acid standards were placed at both sides and the center of the paper. After electrophoresis, the standards were located with ninhydrin. The individual amino acids were eluted with water or 0.01 N HCl by descending chromatography. One- and two-dimensional paper chromatography techniques followed by autoradiography were then employed to identify the ^{14}C -labeled amino acids.

Radioactive measurements. Radioactive measurements were effected in a refrigerated Nuclear-Chicago Mark I liquid scintillation spectrometer. ^{14}C -labeled preparations of actinomycin and aqueous samples of amino acids were counted in 10 ml of Bray's solution (8). Strips cut from electropherograms were measured in Omnifluor. Quenching was corrected by counting samples in the presence and absence of a ^{14}C -labeled standard.

Autoradiography was accomplished using DuPont Cronex 4 medical X-ray films. ^{14}C -labeled amino acids also were located on paper chromatograms with an autoradiogram scanner (Packard model 7201).

Analytical procedures. The concentration of actinomycin was determined spectrophotometrically (23). Determination of amino acids was accomplished with the Beckman Spinco automatic amino acid analyzer (model 120C) (38, 39) or by a modification of the paper chromatographic procedure described by Naftalin (23, 29). Optical configuration of amino acids was established with dialyzed prepa-

rations of L- or D-amino acid oxidase (23, 25, 30). Radioactive assays were conducted by the method of Katz and Weissbach (23).

Chromatographic procedures and high-voltage electrophoresis. Silicic acid column chromatography was employed to purify ^{14}C -labeled actinomycin preparations (23, 25). Actinomycin in benzene (4 ml) was applied to the column (1 by 10 cm) which was washed successively with 200 ml of benzene and then 300 ml of a benzene-methanol mixture (99:1) to remove impurities. Actinomycin was eluted with 200 ml of benzene-methanol (95:5). The actinomycins produced by *S. chrysomallus* were separated by descending paper chromatography with the solvent system 10% sodium *o*-cresotinate-isopropyl ether-chloroform (3:2:1) and purified as described in an earlier publication (39). Each component was rechromatographed with the same solvent system. Homogeneity of the components was established by circular paper chromatography (19) with the solvent systems: (i) 10% sodium *o*-cresotinate-*n*-butyl acetate-dibutyl ether (3:1) and (ii) 10% sodium *o*-cresotinate-amyl acetate.

Amino acids in hydrolysates were separated by high-voltage electrophoresis (Gilson Manufacturing Co., Middleton, Wis.; 4% formate buffer, pH 1.9, 3 h, 3,500 to 4,000 V, 200 mA) or by a combination of high-voltage electrophoresis in one dimension and ascending paper chromatography in the second dimension, using 1-butanol-acetic acid-water (4:1:5, organic phase) (23, 38, 39). In some experiments, amino acids were eluted from electropherograms and further separated by ascending paper chromatography with the butanol-acetic acid-water system. Unless stated otherwise, amino acids were visualized with 0.2% ninhydrin in acetone. Homogeneity of amino acids were routinely checked with the Beckman Spinco automatic amino acid analyzer.

Purification of L-alloisoleucine from a mixture of the stereoisomeric racemates. Commercial L-alloisoleucine (200 mg) (alloisoleucine-isoleucine [77.5:22.5]) was dissolved in 10 ml of 0.2 M ammonium acetate buffer containing 40% ethanol (pH 3.80) and applied to a column (3 by 90 cm) of Dowex 50W $\times$ 8 (NH_4^+ form) which had been equilibrated previously with the same buffer. The column was developed with the same buffer at 30 ml/h, and 10-ml fractions were collected. L-Alloisoleucine fractions (580 to 680 ml) were combined and evaporated to dryness in vacuo. Residual ammonium acetate was removed by sublimation at 50 to 60 C for 2 to 3 h under reduced pressure. After removal of ammonium acetate, the residue was dissolved in 2 ml of hot water, and activated Norit A charcoal was added. The charcoal was removed by filtration while the solution was still hot, and L-alloisoleucine was crystallized from hot water-ethanol. The yield was 98 mg.

Purification of L-alloisoleucine was achieved also by the method of Greenstein and Winitz (13). DL-Isoleucine (98 g) (a mixture of isoleucines and alloisoleucines) was mixed with 1.2 liters of glacial acetic acid and 180 ml of acetic anhydride, and the mixture was maintained at boiling temperature for

2 min. *N*-acetyl-DL-alloisoleucine was recovered and crystallized from hot 50% acetic acid. Recrystallization from hot 50% acetic acid was repeated four times. The yield of crystalline *N*-acetyl-DL-alloisoleucine was 38.9 g (60.1% yield, containing 3% of isoleucine).

Free L-alloisoleucine was obtained by treatment of *N*-acetyl-DL-alloisoleucine with an L-amino acid-specific acylase. *N*-acetyl-DL-alloisoleucine (0.2 mol, 34.6 g) and 0.5 g of cobaltous acetate were suspended in 1.5 liters of water, and the pH was adjusted to 7.6 with 3 N NH_4OH . Acylase I (10 mg) and water were added to make a final volume of 2 liters. The mixture was incubated at 38 C for 18 h. The reaction was monitored by means of an analytical amino acid analyzer until the amount of alloisoleucine became constant. L-Alloisoleucine was recovered from the mixture and recrystallized from ethanol-water. The yield was 8.6 g (65.6%).

Purity of L-alloisoleucine from both preparations was checked by high-voltage electrophoresis, paper chromatography, and analytical amino acid analysis. Optical purity was determined by optical rotatory dispersion and L- and D-amino acid oxidase treatment.

RESULTS

Incorporation of ^{14}C -labeled amino acids into actinomycins. The incorporation of ^{14}C -labeled isoleucines into the actinomycins elaborated by *S. antibioticus* is shown in Fig. 1 and 2 and Table 1. Short-term experiments revealed that the most effective precursor was L-alloisoleucine followed by D-alloisoleucine and D-isoleucine. The kinetics of incorporation of L-isoleucine were atypical, for after a period of rapid labeling there was a slow, continuous loss of radioactivity (Fig. 1). Identical results were obtained in two separate short-term experiments with different preparations of L- ^{14}C isoleucine. At first, the loss was attributed to a rapid turnover of a labile form of radiolabeled actinomycin. However, a quantitative recovery of amino acids from actinomycin was obtained, whereas in two experiments 80 to 85% of the ^{14}C label derived from L-isoleucine was lost after acid hydrolysis and subsequent Norit A charcoal treatment of the hydrolysate. Separation of the ^{14}C -labeled contaminant (80 to 85% of the radioactivity, maximum absorption at 315 to 320 nm) was ultimately achieved by means of silicic acid chromatography using benzene-methanol (99:1); actinomycin (15 to 20% of the radioactivity) was eluted from the column with 5% methanol in benzene. The nature of the radiolabeled material has not been investigated further.

The results obtained with *S. antibioticus*, *S. chrysomallus* and *S. parvulus* with ^{14}C isoleucines as substrates are strikingly similar (Ta-

bles 1 to 5). Thus, L-[^{14}C]alloisoleucine was employed by all three organisms, principally as precursor for synthesis of D-isoleucine and N-methyl-L-alloisoleucine. With *S. chrysomallus* and *S. parvulus*, a limited interconversion of the amino acid to D-alloisoleucine was observed also. Due to the small amount available, D-[^{14}C]isoleucine was employed in only a single experiment with *S. antibioticus*, and the data were essentially the same as those obtained with L-[^{14}C]alloisoleucine (Table 3).

FIG. 1. Incorporation of ^{14}C -labeled amino acids into actinomycins synthesized by *S. antibioticus* during short-term incubations with L-alloisoleucine ($\circ$), D-alloisoleucine ($\triangle$), and L-isoleucine ($\bullet$). The amino acid (40 nmol, 2×10^4 counts/min) was pipetted into 50-ml Erlenmeyer flasks. Five milliliters of a 48-h-old culture of the organism previously cultivated in production medium was introduced. Incubation was carried out in a Dubnoff metabolic shaking incubator at 30 C. Radioactivity incorporated was determined by the method of Katz and Weissbach (23).

Previous investigations had demonstrated that L-isoleucine could be utilized as precursor for synthesis of the D-alloisoleucine residues in actinomycins C_2 and C_3 (1). Unfortunately, the

FIG. 2. Incorporation of ^{14}C -labeled amino acids into actinomycins synthesized by *S. antibioticus* during long-term incubations. Symbols: $\bullet$, L-isoleucine (two flasks, 765 μmol , 6.5×10^6 counts/min); $\circ$, L-alloisoleucine (two flasks, 153 μmol , 3.14×10^6 counts/min); $\triangle$, D-alloisoleucine (two flasks, 76.5 μmol , 4.7×10^6 counts/min). *S. antibioticus* was incubated in production medium (100 ml per flask) for 24 h. Then, the ^{14}C -labeled amino acid was added, and incubations were continued for an additional 120 to 192 h. Radioactive assays were carried out by the method of Katz and Weissbach (23).

TABLE 1. Incorporation of [^{14}C]isoleucine stereoisomers into actinomycins by *S. antibioticus*

Organism	Radioactive precursor	Amount supplied		Incubation volume	Duration of incubation	Amino acid incorporated		
		nmol	counts/min			nmol	counts/min	%
<i>S. antibioticus</i> ^a	L-[^{14}C]isoleucine	1,600	1.28×10^7	400	30	87.0	7.0×10^5	5.4
	L-[^{14}C]isoleucine	2,000	1.58×10^7	500	240	56.0	4.51×10^5	2.8
	D-[^{14}C]alloisoleucine	400	1.6×10^6	100	240	26.0	1.05×10^5	6.5
	L-[^{14}C]alloisoleucine	400	1.57×10^6	100	120	34.4	1.35×10^5	8.6
	D-[^{14}C]isoleucine		5.4×10^4	5	360		4.8×10^3	8.8
<i>S. antibioticus</i> ^b		μmol	counts/min	ml	h	μmol	counts/min	%
	L-[^{14}C]isoleucine	1,900	1.27×10^7	500	96	34.0	2.3×10^5	1.8
	L-[^{14}C]isoleucine	765	6.5×10^6	200	120	12.2	1.03×10^5	1.6
	D-[^{14}C]alloisoleucine	76.3	4.7×10^6	200	192	6.7	4.13×10^5	8.7
	L-[^{14}C]alloisoleucine	153	3.14×10^6	200	192	9.9	2.04×10^5	6.5

^a Short-term experiments. The organism was cultivated for 48 h in production medium, at which time the ^{14}C -labeled radioisotope was added to the culture. Incubation was resumed for the period designated.

^b Long-term experiments. The organism was cultivated for 24 h in production medium, the ^{14}C -labeled amino acid was supplied, and the incubation was resumed as shown.

TABLE 2. Incorporation of [^{14}C]isoleucine stereoisomers into actinomycins by *S. chrysomallus* and *S. parvulus*

Organism	Radioactive precursor	Amount supplied		Incubation volume	Duration of incubation	Amino acid incorporated		
		nmol	counts/min	ml	min	nmol	counts/min	%
<i>S. chrysomallus</i>	L-[^{14}C]isoleucine ^a	460	2.9×10^7	1,000	90	47	2.8×10^6	9.7
	D-[^{14}C]alloisoleucine ^a	5,600	5.6×10^6	300	240	360	3.58×10^5	6.4
	L-[^{14}C]alloisoleucine ^a	4,500	4.28×10^6	300	360	240	2.25×10^5	5.3
		μmol	counts/min	ml	h	μmol	counts/min	%
	L-[^{14}C]isoleucine ^b	950	1.62×10^7	500	72	17.1	2.92×10^5	1.8
<i>S. parvulus</i>		nmol	counts/min	ml	min	nmol	counts/min	%
	L-[^{14}C]isoleucine ^c	2,000	1.49×10^7	500	60	28.7	2.14×10^5	1.43
	L-[^{14}C]alloisoleucine ^c	4,500	4.3×10^6	300	240	1,590	1.51×10^6	35.1
		μmol	counts/min	ml	h	μmol	counts/min	%
	L-[^{14}C]isoleucine ^d	1,140	7.6×10^6	300	96	43.9	2.9×10^5	3.8

^a Incubation in production medium was for 72 h prior to addition of ^{14}C -labeled amino acid.^b Incubation in production medium was for 48 h prior to addition of ^{14}C -labeled amino acid.^c Incubation was for 48 h prior to addition.^d Incubation was for 24 h prior to addition.TABLE 3. Distribution of ^{14}C -radiolabeled precursor in amino acids derived from actinomycins synthesized by *S. antibioticus*

Radioisotope precursor	Amino acid	Short-term expt (%)		Long-term expt (%)	
		1	2	1	2
L-[^{14}C]isoleucine	Unknown	1	3	3	6
	Sarcosine	1	4	6	10
	Valine	1	4	6	7
	Isoleucine	7	6	4	8
	Alloisoleucine	69	48	14	7
	Threonine	1	10	17	14
	Proline	2	9	33	39
	N-methylvaline	1	4	8	7
	N-methylisoleucine	16	11	7	1
	N-methylalloisoleucine	1	0	0	0
D-[^{14}C]alloisoleucine	Unknown		5		0
	Isoleucine		63		66
	Alloisoleucine		0		9
	N-methylalloisoleucine		30		25
L-[^{14}C]alloisoleucine	Unknown		5		6
	Isoleucine		56		56
	N-methylalloisoleucine		36		34
D-[^{14}C]isoleucine	Isoleucine		49		
	Alloisoleucine		7		
	N-methylalloisoleucine		43		

role of D-alloisoleucine as a substrate was not established by Albertini et al. (1). Our results have revealed that both *S. antibioticus* and *S. chrysomallus* are able to convert D-alloisoleucine to peptide-bound D-isoleucine and N-methylalloisoleucine. In addition, a significant portion of the ^{14}C -labeled amino acid was incorporated as D-alloisoleucine into the actinomycins (C_1 , C_2 , and C_3) synthesized by *S. chrys-*

omallus. When L-[^{14}C]isoleucine was supplied to this organism, virtually all of the radiolabel was present in the D-alloisoleucine residues of the C components. The specific radioactivity of the peptide-bound amino acid was equal to that of actinomycin C_2 and it was one-half that of actinomycin C_3 (data not shown), confirming the results of Albertini et al. (1). By contrast, L-isoleucine was extensively metabolized by

TABLE 4. Distribution of ^{14}C -radiolabeled precursor in amino acids derived from actinomycins synthesized by *S. chrysomallus*

Radioisotope precursor	Amino acid	Short-term expt (%)				Long-term expt (C mixture) (%)
		C ₁	C ₂	C ₃	E ₁ -E ₂	
L- ^{14}C]isoleucine	Isoleucine	2	3	2		11
	Alloisoleucine	98	97	98		84
	Proline	0	0	0		5
D- ^{14}C]alloisoleucine	Sarcosine	3	0	1	8	
	Isoleucine	25	44	12	21	
	Alloisoleucine	28	38	46	20	
	<i>N</i> -methylalloisoleucine	44	18	41	51	
L- ^{14}C]alloisoleucine	Sarcosine	2	0	0	2	
	Isoleucine	34	48	32	26	
	Alloisoleucine	14	17	16	12	
	<i>N</i> -methylalloisoleucine	50	34	52	60	

TABLE 5. Distribution of ^{14}C -radiolabeled precursor in amino acids derived from actinomycins synthesized by *S. parvulus*

Radioisotope precursor	Amino acid	Short-term expt (%)	Long-term expt (%)
L-[¹⁴ C]isoleucine	Unknown	2	3
	Sarcosine	1	2
	Valine	1	6
	Isoleucine	15	7
	Alloisoleucine	58	21
	Threonine	4	12
	Proline	4	37
	N-methylvaline	1	6
	N-methylisoleucine	7	2
	N-methylalloisoleucine	6	2
L-[¹⁴ C]alloisoleucine	Isoleucine	50	
	Alloisoleucine	6	
	N-methylalloisoleucine	43	

S. antibioticus and *S. parvulus* with considerable randomization of the ^{14}C radiolabel noted particularly in the long-term experiments. The short-term studies did reveal that both organisms utilized the L-enantiomorph for D-alloisoleucine synthesis. However, the formation of D-isoleucine and *N*-methylalloisoleucine from L-isoleucine was minimal in contrast to our previous investigations (20, 38). In fact, the short-term experiments with L-isoleucine suggested that the amino acid may undergo methylation to yield peptide-bound *N*-methylisoleucine.

Identification of radiolabeled amino acids. In each experiment the relevant ^{14}C -labeled amino acids formed (isoleucine, alloisoleucine, and *N*-methylalloisoleucine) were isolated and subjected to autoradiography and radiochromatographic scanning techniques. It was estab-

lished that the ^{14}C -labeled amino acid co-electrophoresed and co-chromatographed with standards of isoleucine, alloisoleucine, and *N*-methylalloisoleucine, respectively. Treatment of the [^{14}C]isoleucine or [^{14}C]alloisoleucine derived from hydrolysates with L- and D-amino acid oxidase established that the ^{14}C -labeled compounds possessed the D configuration.

DISCUSSION

D-Amino acids have been found as constituents of microbial cell walls (32), peptidolipids (2), capsules (37), toxins (14), and antibiotics (6, 9). Racemases for various amino acids have been observed in microorganisms (28); however, the mechanism of biosynthesis of the D-amino acid residues in most of these peptide metabolites remains unclear. In some cases,

both the L- and D-enantiomorphs of an amino acid can be utilized for synthesis of the peptide-bound D-amino acid (18). In other instances there is evidence to suggest that only the L isomer is used for the biogenesis of D-amino acids in the peptide structures (12, 23). For example, Troy (37) recently reported that a particulate fraction from an encapsulated strain of *Bacillus licheniformis* catalyzed the polymerization of L-glutamic acid (but not the D isomer) to form a high-molecular-weight polymer of γ -D-glutamic acid. It has also been observed that the D-amino acid may actually inhibit peptide synthesis (16, 22, 36); such inhibition is reversed by the L-enantiomorph.

Several hypotheses have been advanced to explain the biogenetic origin of D-amino acids in microbial peptides. Mauger (27) has postulated that D-amino acids in antibiotics are formed from L-amino acids after incorporation of the latter into stereochemically labile intermediates such as cyclic dipeptides (e.g., diketopiperazines). Bycroft (9) has proposed that a combined form of a dehydroamino acid derived from the corresponding L-amino acid might be reduced stereospecifically in vivo to the D isomer during antibiotic formation. These hypotheses have not been confirmed experimentally. Studies concerned with the cell-free synthesis of tyrocidine and gramicidin S, however, have revealed that an adenosine 5'-triphosphate-dependent racemization of phenylalanine occurs while the amino acid is enzyme bound in thioester linkage (24).

The formation of D-valine, D-alloisoleucine, and D-isoleucine has been investigated during actinomycin synthesis in vivo. Radioisotopic (23) and ^{15}N experiments (31) suggest that L-valine (but not D-valine) is the precursor of the D-valine residues in an actinomycin molecule and that the inversion of the L-amino acid to its D-enantiomer does not involve a loss of the alpha nitrogen. Further, D-valine inhibits actinomycin synthesis, and this inhibition is reversed by L-valine (16, 22). Albertini et al. (1) similarly showed that L-isoleucine can serve as precursor for D-alloisoleucine synthesis, and our present results confirm these observations. The role of D-alloisoleucine as a precursor was not explored prior to the present investigation. Precursor pressure studies have indicated that the four stereoisomers of isoleucine may be employed for the production of actinomycins containing D-isoleucine (and D-alloisoleucine) and N-methyl-L-alloisoleucine (20, 38, 39). The interconversion of the enantiomorphous pairs of the isoleucines was postulated to involve α -keto acid intermediates (38, 39).

The radioisotopic data reported here have demonstrated that of the isoleucine isomers examined D-alloisoleucine (*S. antibioticus* and *S. chrysomallus*), L-alloisoleucine (*S. antibioticus*, *S. chrysomallus*, and *S. parvulus*) and D-isoleucine (*S. antibioticus*) were readily employed for the biogenesis of the D-isoleucine and N-methylalloisoleucine moieties in the actinomycin peptides. On the other hand, the utilization of these amino acids for D-alloisoleucine synthesis by *S. antibioticus* and *S. parvulus* was considerably less efficient. All three organisms can synthesize D-alloisoleucine from L-isoleucine. In this respect, our findings with *S. antibioticus* and *S. parvulus* were somewhat unexpected, as these organisms normally elaborate actinomycins containing D-valine (17). Thus, the potential to utilize L-isoleucine for D-alloisoleucine formation during actinomycin production is present in these microorganisms, but it is generally not expressed. Conceivably, this is due to the absence of sufficient concentrations of the amino acid in the cell pool as a consequence of stringent regulation of its biosynthesis or to its extensive metabolism.

Studies conducted in our laboratory with L-isoleucine over an extended period of time (16, 20, 22, 38, 39) have proved to be inconsistent and puzzling. Consequently, we have been unable to confirm fully, by means of the ^{14}C -labeled experiments, the results obtained previously employing unlabeled L-isoleucine (16, 20, 22, 38). In fact, even the latter studies with L-isoleucine from different sources have yielded somewhat conflicting data. For example, in experiments conducted with L-isoleucine purified in the laboratory of J. Greenstein (National Institutes of Health [NIH]) (20, 38) or purchased from a commercial source (Calbiochem) (16, 22), we have observed that new actinomycins were produced (*S. antibioticus*, and *S. chrysomallus*). Moreover, quantitative examination of acid hydrolysates of the actinomycins revealed that D-isoleucine and N-methyl-L-alloisoleucine were present at significantly high levels, replacing D-valine (or D-alloisoleucine) and N-methylvaline, respectively (20, 38). Subsequently, these results could not be reproduced using a different isoleucine preparation from the same commercial source (unpublished data). Recently, in a similar study with the same L-isoleucine preparation, small amounts of peptide-bound D-isoleucine (*S. parvulus*) and N-methylalloisoleucine (*S. chrysomallus*) were produced during antibiotic formation (39). Confirmation of these results has been provided by the ^{14}C tracer studies described here. In all of the previous

investigations in which L-isoleucine was utilized for the synthesis of novel biosynthetic actinomycins, N-methylalloisoleucine (but not N-methylisoleucine) and/or D-isoleucine were identified as constituent amino acids in hydrolysates (20, 38, 39). Preliminary evidence (presented in Tables 3 and 5) indicate that N-methylisoleucine may also be formed, to a limited extent, from the [^{14}C]isoleucine precursor. Confirmation of this finding is now under investigation. In this connection it is of interest to note that Audhya and Russell observed that N-methyl-L-isoleucine and N-methyl-L-alloisoleucine were present in hydrolysates of an enniatin A preparation produced by the fungus *Fusarium sambucinum* (3).

Both the NIH preparation and the commercial preparation used in recent experiments were carefully checked for purity by means of the analytical amino acid analyzer, high-voltage electrophoresis, paper chromatography, and the use of L- and D-amino acid oxidase. All of the tests showed satisfactory homogeneity and optical purity. The isoleucine from commercial sources was examined also by optical rotatory dispersion and by diastereoisomeric dipeptide synthesis (26). By contrast, the isoleucine from NIH was provided by the laboratory of J. Greenstein, where the most rigorous standards of purity for amino acids were developed. The authenticity of this compound was attested to by former associates of J. Greenstein as well as by our own analyses. Thus, both L-isoleucine preparations have a high degree of chemical and optical purity. The inconsistent results obtained with ^{14}C -labeled and unlabeled L-isoleucine may be due to strain variation which could have occurred during the years of maintenance of the organism. Cloning may provide a more homogeneous, physiologically constant strain for future experiments.

^{14}C radioisotope experiments have established that L-isoleucine, L-alloisoleucine and, to a lesser extent, D-alloisoleucine can serve as precursor of the D-isoleucine residues in the monamycins (11). Davies et al. (11) proposed that the α -keto acids most likely function as intermediates which through interconversion by inversion at C-3 could serve as a source of D-isoleucine. They suggested that D-isoleucine units are incorporated directly into the monamycins.

Although α -keto acid intermediates undoubtedly play a role in the interconversion of the isoleucine stereoisomers during actinomycin synthesis, there is little evidence to support the view that the D-amino acids (D-alloisoleucine and D-isoleucine) are used di-

rectly for synthesis of the peptide-bound form of the respective amino acids. Previous investigations had revealed that the D-amino acids are inhibitory to actinomycin formation (16, 18, 22). In the current study, D-[^{14}C]alloisoleucine in fact generally proved to be a more effective precursor for peptide-bound N-methylalloisoleucine and/or D-isoleucine than for D-alloisoleucine (*S. antibioticus* and *S. chrysomallus*). Further, with D-[^{14}C]isoleucine (*S. antibioticus*) we noted an approximately equal labeling of both D-isoleucine and N-methylalloisoleucine residues, suggesting that L-alloisoleucine is a common precursor for both amino acids. However, the possibility that a D-amino acid is used directly for the peptide-bound form of the amino acid cannot be excluded by the data presented here.

Despite a number of unanswered questions concerning the metabolic relationships of the branched-chain amino acids in *Streptomyces*, we wish to suggest the following tentative scheme to explain the isoleucine interconversions:

The recent studies concerning the adenosine 5'-triphosphate-dependent racemization of phenylalanine during gramicidin S and tyrocidine formation (24) suggest that perhaps a single enzyme with rather broad specificities for the L form of the branched-chain amino acids catalyzes an energy-dependent biosynthesis of D-amino acids. The mechanism of inversion remains to be established.

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