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Effect of D-Leucine on the Biosynthesis of Polymixin D*

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Although much has already been learned about the detailed mechanism of protein synthesis, very little is now known about the synthesis of polypeptides. The study of this problem is made difficult by the circumstance that in most cases of polypeptide synthesis the small amount of polypeptide synthesized is obscured by a much greater synthesis of protein containing, in general, the same amino acids. For this reason, certain antibiotic peptides that contain amino acids not present in proteins afford a particularly favorable material for study. Moreover, these polypeptides are synthesized in considerable quantity under appropriate conditions and may be detected by sensitive bioassay methods.

We have chosen for such studies polymyxin D, an antibiotic peptide produced by strains of *Bacillus polymyxa* (1). No structural formula has been assigned as yet to polymyxin D, but from evidence available for other polymyxins (2), it appears to be a cyclic octapeptide composed of 4 molecules of L-α,γ-diaminobutyric acid, 2 of L-threonine, 1 of D-leucine, and 1 of D-serine, joined to a side chain composed of 1 L-threonine, 1 L-α,γ-diaminobutyric acid, and 1 isopelargonic acid residue.

The present paper reports a study of the effect of D-leucine on the biosynthesis of the antibiotic polymyxin D and the mode of entry of D-leucine into the molecule of the antibiotic.

EXPERIMENTAL PROCEDURE

Bacillus polymyxa (A.T.C.C. 10401) was maintained on agar slants of medium of the following composition: tryptone, 10 g; yeast extract, 5 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; $FeSO_4 \cdot 7H_2O$, 10 mg; glucose, 10 g; Na_2HPO_4, 4 g; KH_2PO_4, 0.6 g; agar, 15 g; and H_2O, 1 liter.

Inocula were prepared by transferring bacteria from such slants to liquid medium of the same composition lacking agar, and incubating at 28° for 14 hours on a rotary shaker (200 rotations per minute). Cells were centrifuged, washed, and suspended in sterile 0.9% NaCl solution and used to inoculate the growth media. Two different liquid media were used. Medium A was composed of (grams per liter) KH_2PO_4, 2; $MgSO_4 \cdot 7H_2O$, 0.5; $FeSO_4 \cdot 7H_2O$, 0.01; NaCl, 0.05; yeast extract, 5; $(NH_4)_2SO_4$, 20; and glucose, 10. Medium B was the same as Medium A, except that yeast extract was omitted and 0.02 g of thiamine and 0.1 mg of biotin were added per liter of medium.

Both media afforded fair growth and antibiotic production (Table I, Experiment 1). Unless otherwise stated, in all the incorporation experiments, the amino acids were sterilized by filtration and added to the medium 24 hours after inoculation, *i.e.* at the onset of antibiotic production (see Fig. 2). Radioactive D-leucine was always supplied at a concentration that had negligible inhibitory effect on antibiotic synthesis (2 μg per ml). All incubations were carried out for 72 hours at 28° on a rotary shaker (200 rotations per minute).

Extraction and Purification of Polymyxin—The antibiotic[1] was recovered from the incubation liquids by two extractions with 0.5 volume of isopropanol (3). The alcoholic phases were separated from the aqueous phases by addition of ammonium sulfate to saturation. The alcoholic phases from the two extractions were combined and evaporated to dryness on a rotary evaporator at a temperature not higher than 40°. The dry residue was taken up in water, brought to pH 3 with 1 N HCl, and applied to a glass column, 30 × 2 cm, packed with Amberlite IRC-50 (analytical grade). The exchange resin had been previously converted to the Na^+ form by treatment with 3% NaOH and washing with glass-distilled water until the effluent was neutral. After adsorption, the column was washed with 500 ml of water, then with 300 ml of 0.05 N HCl, and the antibiotic was eluted by an HCl gradient obtained by using 500 ml of 0.4 N HCl in the reservoir and 50 ml of 0.05 N HCl in the mixing flask. A typical diagram of elution is given in Fig. 1.

Antibiotic and ninhydrin assay, and when feasible, radioactivity assay, helped to locate the tubes into which polymyxin was eluted. The contents of such tubes were pooled and dried by lyophilization.

The antibiotic so purified was often contaminated with substantial amounts of NaCl concomitantly eluted from the column. Desalting was accomplished by passing a solution of the lyophilized antibiotic through a column, 30 × 2 cm, packed with Sephadex G-25 (Pharmacia, Uppsala, Sweden) that had been previously equilibrated with glass-distilled water. Elution was performed with glass-distilled water, and the antibiotic so purified was dried *in vacuo* over P_2O_5 and NaOH.

Degradation of Polymyxin—The purified antibiotic was dissolved in 6 N HCl and hydrolyzed in a sealed tube at 110° for 18 hours. The hydrolysate was dried, taken up in water, and extracted three times with 0.5 volume of ethyl ether. The ether fractions, containing the isopelargonic acid, were combined, evaporated, and counted as such. The aqueous phase, containing the amino acids, was applied to a column, 70 × 0.9 cm,

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[1] The strain, as determined by the amino acid composition of the purified antibiotic, produces only, or mainly, polymyxin D. Such results have been confirmed by Dr. P. G. Stansly (personal communication).

packed with Dowex 50-W4, 100 to 200 mesh (H^+ form), and washed with 200 ml of 0.01 N HCl. The amino acids were eluted with 300 ml of 1.5 N HCl, followed by 100 ml of 2.5 N HCl. Amino acids in the eluate were located by the ninhydrin test. Elution of serine and threonine was accomplished after approximately 60 ml of 1.5 N HCl, and of α,γ-diaminobutyric acid after a further 150 ml. Leucine was eluted after addition of approximately 30 ml of 2.5 N HCl. The identity of each amino acid was established by comparison with known standards in a descending paper chromatography system using Whatman No. 1 filter paper and a mixture of 1-butanol-acetic acid-water (52:14:34). Radioactivity was determined on aliquots of the combined fractions of each peak.

Preparation of Cell-free Extracts—Cells grown on Medium A were harvested by centrifugation, washed twice with 0.001 M EDTA, pH 7.2, and suspended in 0.066 M phosphate buffer and 0.002 M GSH, pH 6.8. Disruption of the cells was achieved by treatment in an M.S.E. (Measuring and Scientific Equipment Company) ultrasonic disintegrator for 10 minutes. During ultrasonic treatment, heating was prevented by cooling the cell container in an ice-ethanol bath. After centrifugation in the cold at 10,000 × *g* for 20 minutes, the cell-free supernatant fluid was dialyzed in the cold for 24 hours against 2 liters of 0.001 M EDTA, pH 7.2.

TABLE I

Effect of some amino acids on growth and polymyxin production

All amino acids were added 24 hours after inoculation of the medium. Except in the last experiment of the fourth group, in which 100 μg per ml of L-leucine were used, in all experiments the amino acids were used at a concentration of 10 μg per ml. After addition of the amino acids, the incubations were continued for another 48 hours, and the culture supernatant was assayed for antibiotic production. Each result is the average of at least two flasks.

Medium	Addition	Growth	Polymyxin	
		O.D. at 540 mμ	*μg/ml*	%
Experiment I				
Medium A	None	2.40	80	100
Medium B	None	0.910	44	55
Experiment II				
Synthetic	None	0.850	53	100
	L-α,γ-Diamino-butyric acid	0.790	72	136
	L-Threonine	0.870	54	102
	L-Leucine	0.840	54	102
	D-Threonine	0.830	56	106
	D-Serine	0.890	57	108
	D-Leucine	0.860	8	15
	D-Phenylalanine	0.800	29	55
Experiment III				
Synthetic	None		40	100
	D-Leucine		12	30
	D-Phenylalanine		18	45
	D-Leucine + D-phenylalanine		9	23
Experiment IV				
Synthetic	None	0.900	44	100
	L-Leucine	0.880	40	91
	D-Leucine	0.840	4	9
	D-Leucine + L-leucine	0.910	38	86

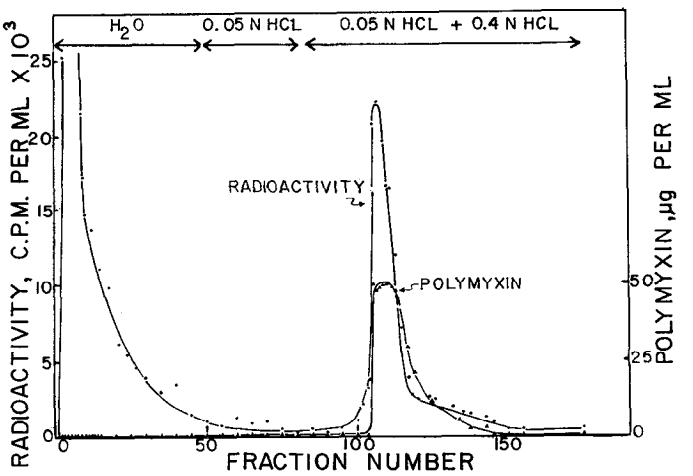

FIG. 1. Elution of radioactive polymyxin from IRC-50 column. Crude radioactive polymyxin (approximately 250,000 c.p.m.), 2.1 mg, was applied to the column, which was treated with 500 ml of water, 300 ml of 0.05 N HCl, and a gradient between 500 ml of 0.4 N HCl and 50 ml of 0.05 N HCl. The flow rate was approximately 50 ml per hour. Fractions of 10 ml were collected. ●, radioactivity; ▲, polymyxin.

Preparation of Soluble RNA—The cell-free extract was centrifuged at 105,000 × *g* for 240 minutes. The supernatant fluid was extracted for 1 hour in the cold with 1 volume of 90% phenol. The phenol phase was then extracted with 1 volume of water. The aqueous layers of the two extractions were combined. Then 0.1 volume of 30% potassium acetate was added and the preparation was alcohol-precipitated by the addition of 3 volumes of cold 98% ethanol. After centrifugation, the precipitate was dissolved and dialyzed overnight against 1 liter of 0.01 M Tris buffer, pH 7.2.

Analytical Methods—Amino acid-dependent exchange of $P^{32}P_i$ with ATP was assayed essentially by the procedure of DeMoss and Novelli (4) with the slight modifications already reported (5). Transfer of radioactive amino acids to sRNA[2] (aminoacid RNA ligases, 6.1.1) was measured by the procedure of Hoagland, Keller, and Zamecnik (6). Polymyxin production was assayed by the agar diffusion method of Stansly and Schlosser (7) with the use of polymyxin B sulfate (Calbiochem, C grade) as a standard.

Growth was determined turbidimetrically at 540 mμ against a blank of uninoculated medium. Radioactivity was determined by pipetting aliquots of the solution to be measured onto metal planchets, drying, and counting in a Tracerlab windowless flow counter or in an S.E.L.O. (Societa Elettronica Lombarda) thin mica (2 mg per cm^2) window counter. All samples were counted under conditions under which self-absorption was negligible and were corrected for background activity and counting efficiency.

Protein was determined by the method of Lowry *et al.* (8) with the use of crystalline egg albumin (Sigma Chemical Company) as standard, and inorganic phosphate, by the method of Fiske and SubbaRow (9).

Materials

$Na_4{}^{32}P_2O_7$ (specific activity, 4.49 to 8.75 mc per mmole), DL-leucine-1-^{14}C (specific activity, 7.2 mc per mmole), L-leucine-^{14}C (uniformly labeled) (specific activity, 7.06 to 24.8 mc per mmole),

[2] The abbreviation used is: sRNA, soluble ribonucleic acid.

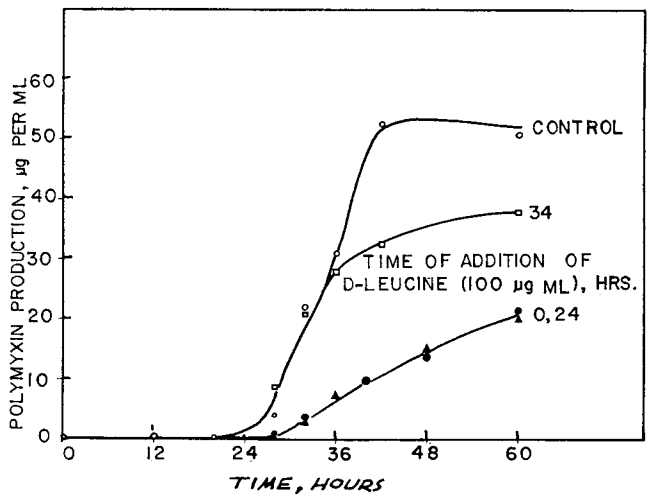

FIG. 2. Influence of D-leucine on production of polymyxin. *B. polymyxa* was grown on Medium B; D-leucine, 100 μg per ml, was added at the hours indicated: ○, control (no D-leucine); ●, 0 hours; ▲, 24 hours; □, 34 hours.

and L-phenylalanine-^{14}C (uniformly labeled) (specific activity, 7.85 mc per mmole) were obtained from the Radiochemical Centre, Amersham, England. L-α,γ-Diaminobutyric acid was obtained from Sigma; all other L-amino acids were obtained from Schwarz BioResearch, Inc. (O.S. grade). D-Leucine was obtained from Roche Laboratories, Inc., Calbiochem (C grade), and Nutritional Biochemicals Corporation; D-alanine (A grade), from Calbiochem; and D-phenylalanine and D-valine from Roche.

Resolution of D-leucine-1-^{14}C from the racemic mixture was achieved by subjecting a solution of the mixture to the action of L-amino acid oxidase from lyophilyzed venom of *Ancistrodon piscivorus piscivorus*.[3] To the central compartment of a Warburg flask were added 50 μmoles of Tris buffer, pH 7.2, and 10 μmoles of DL-leucine-1-^{14}C 0.2 ml of 20% KOH was added to the central well. Venom, 4 mg, dialyzed overnight against cold 0.1 M KCl was added to the side arm. After temperature equilibration at 37°, the reaction was initiated by tipping in the content of the side arm.

The reaction reached completion (uptake of 1 mole of O_2 per mole of L-leucine) after 30 to 45 minutes of incubation. The incubation was continued for another 15 minutes, and then 10 μmoles of L-leucine were added from the side arm. After oxidation of this aliquot, a second aliquot of L-leucine, together with 1 mg of venom, was added to insure complete oxidation of all radioactive L-leucine. The contents of the Warburg flasks were recovered, and protein was precipitated by adding trichloroacetic acid to a final concentration of 5%. After it had stood overnight in the cold, the solution was centrifuged and HCl was added to the supernatant to a final concentration 0.05 N. Trichloroacetic acid and α-ketoisocaproic acid were removed by extracting this supernatant five times with 0.5 volume of ethyl ether. The aqueous phase containing the radioactive D-leucine was neutralized with NaOH and used without further treatment.

The purity of the nonradioactive D-amino acids used in the tests for ATP-$P^{32}P_i$ exchange was assayed by the same procedure. Oxygen uptake with all three commercial preparations of D-leucine indicated a contamination by an L-amino acid ranging from 1 to 3%. Paper chromatography did not reveal any ninhydrin-positive spots other than that of leucine, and it was therefore surmised that the contaminant was probably L-leucine. The tested samples of D-alanine, D-phenylalanine, and D-valine appeared to be free of detectable contaminations of L-amino acids. Accordingly, the latter were used without further treatment, whereas D-leucine was purified by the same procedure reported for the radioactive samples.

In experiments in which D-leucine was not purified, activation of this amino acid was detected by determining the ATP-$P^{32}P_i$ exchange, but with the purified preparation of D-leucine, the results were negative (see Table III).

[3] Obtained from Ross Allen's Reptile Institute, Silver Springs, Florida.

TABLE II

Effect of D- and L-leucine on production of polymyxin

D-Leucine or D-leucine + L-leucine was added 24 hours after inoculation of Medium B. The incubations were continued for another 48 hours, after which the culture supernatants were assayed for antibiotic production. Each result is the average of at least two flasks.

D-Leucine	Inhibition					
	0*	1:10	1:5	1:1	5:1	10:1
μg/ml	%					
10	42			47		17
50	62		64		29	
100	75	69	68	44	34	

* Figures in the subboxheads give the ratio of L-leucine to D-leucine.

RESULTS

Effect of Amino Acids on Biosynthesis of Polymyxin—The effect of the addition to the synthetic medium of amino acids known to be present in polymyxin D or other polymyxins is reported in Table I, Experiment 2. All amino acids tested are without effect on the growth of *B. polymyxa*. D-Leucine and D-phenylalanine have a striking inhibitory effect on antibiotic production. As D-phenylalanine has not been found in polymyxin D, the principal polymyxin synthesized by our strain, its effect was not investigated in further detail.

Addition of L-α,γ-diaminobutyric acid was found to stimulate antibiotic production, but it should be noted that this compound was found to be contaminated with another ninhydrin-positive substance, which could be responsible for the stimulation.[4] D-Serine, the other D-amino acid present in the molecule of polymyxin D, has no detectable inhibitory effect.

Addition of 10 μg, or more, per ml of D-leucine to the growth medium (Table I, Experiments 3 and 4) resulted in inhibition of antibiotic production, and this inhibitory effect was also found when the amino acid was supplied after antibiotic production had begun (Fig. 2). The degree of inhibition of antibiotic synthesis was found to be roughly proportional to the D-leucine concentration; thus, at concentrations of 10, 50, and 100 μg per ml, D-leucine was found to inhibit antibiotic production by 42, 62, and 75%, respectively (Table II).

[4] Paper chromatography of L-α,γ-diaminobutyric acid showed the presence of a ninhydrin-positive contaminant (R_F, 0.29 in 1-butanol-acetic acid-water, 54:14:34; 0.56 in pyridine-methanol-10 N HCl-water, 10:80:2.5:17.5, v/v; R_F for the L-α,γ-diaminobutyric acid, 0.14 and 0.41, respectively).

The addition of L-leucine together with D-leucine was found to overcome almost completely the inhibitory effect of D-leucine (Table I, Experiment 4). The ratio of L-leucine to D-leucine that gave 50% removal of inhibition was roughly 5:1. At high concentration of D-leucine, however, L-leucine was relatively more effective at reversing the inhibition; *i.e.* the extent of removal of inhibition appeared to depend not only on the ratio of D- to L-leucine, but also on their absolute concentration.

The present results parallel earlier findings of other workers regarding inhibition of antibiotic production upon addition to the growth medium of a D amino acid present in the molecule of an antibiotic (10–12).

Activation of Amino Acids (Aminoacid-RNA Ligases: 6.1.1) in Cell-free Extracts—Before attempting experiments on the incorporation of amino acids into polymyxin, it was thought of some interest to investigate the presence in cell-free extracts of *B. polymyxa* of enzymes capable of activating amino acids by determining whether an ATP-pyrophosphate exchange reaction was catalyzed. The results in Table III indicate activation of L-amino acids, but not of the D-amino acids tested. We have reported similar results with a strain of *B. polymyxa* that produces polymyxin B, a strain of *Bacillus circulans* producing circulin, and other antibiotic-producing microorganisms (5). Similar results have been found in the case of an actinomycin-producing streptomycete (13). On the other hand, activation of D-amino acids by cell-free extracts from gramicidin-producing strains of *Bacillus brevis* (14, 15) has been reported; in these experiments, however, there was no indication of the purity of the D-amino acids used. As shown in Table III an apparent activation of D-leucine was obtained when the amino acid of commercial origin had not been freed of a contaminating L-amino acid. The apparent absence

TABLE III

Amino acid activation in cell-free extracts of B. polymyxa

The assay system contained, in a final volume of 0.5 ml: 20 μmoles of Tris buffer (pH 7.9), 5 μmoles of potassium-ATP (pH 6.5), 2.5 μmoles of $MgCl_2$, 3.2 μmoles of $Na_4{}^{32}P_2O_7$ (corresponding to approximately 340,000 c.p.m. on an S.E.L.O. thin mica window counter), 10 μmoles of amino acid, and dialyzed cell-free extract corresponding to from 0.23 to 0.47 mg of protein. The reaction was carried out at 37° for 15 minutes and was terminated by addition of 1 volume of 10% trichloroacetic acid. After removal of precipitated proteins by centrifugation, aliquots of the supernatant were treated with 100 mg of acid-washed Norit A suspended in 0.1 M sodium acetate. Norit A was recovered by centrifugation and was washed three times with 0.05 M sodium acetate (pH 4.5) and once with glass-distilled water. It was then suspended in 2 ml of 1 N HCl and hydrolyzed in a boiling water bath for 15 minutes. Following filtration, aliquots of the hydrolysate were used to determine radioactivity or inorganic phosphate. The value obtained in the control, in which no amino acid was added, was subtracted from all experiments. A zero (0) indicates that experimental values were not more than 10% higher than controls.

Amino acid	$P^{32}P_i$ exchanged*		
	μmoles/15 min/mg protein		
L-Alanine	0	0	0
L-Arginine	0.013	0.031	
L-Aspartic acid	0.080	0.031	
L-Cysteine	0.243	0.016	
L-α,γ-Diaminobutyric acid	0	0	0.017†
L-Glutamic acid	0		
Glycine	0	0	
L-Histidine	0	0	
L-Isoleucine	2.00	2.03	
L-Leucine	2.07	1.79	0.666
L-Lysine	0.094	0.045	
L-Methionine	0.278	0.274	
L-Phenylalanine	0.074	0.014	0.002
L-Proline	0.144	0.139	
L-Serine	0	0	0
L-Threonine	0.210	0.167	0.010
L-Tryptophan	0.083	0.068	
L-Valine	0.106	2.10	
D-Alanine	0		
D-Leucine (crude)	1.84	1.54	0.605
D-Leucine (purified)	0	0	0
D-Phenylalanine	0	0	0
D-Serine	0	0	0
D-Threonine	0		0
None (control)	0.133	0.130	0.030

* Some of the data of this column have been already reported (5).

† See the text, Footnote 4.

TABLE IV

Incorporation of D- and L-leucine ^{14}C into polymyxin D

D-Leucine-1-^{14}C, 29.6 μc (16.5 μmoles), alone or with 80 μmoles of L-leucine and 29 μc (4.1 μmoles) of L-leucine-^{14}C (uniformly labeled), were added to each 1000 ml of Medium B 24 hours after inoculation of the medium. After a further 48 hours of incubation, the polymyxin was recovered and purified as reported in the text.

Amino acid	Specific activity of external amino acid	Specific activity of polymyxin
	c.p.m./μmole	
L-Leucine-^{14}C	314×10^4	22.2×10^4
D-Leucine-^{14}C	792×10^3	5.6×10^3
D-leucine-^{14}C + L-leucine	792×10^3	3.4×10^3

TABLE V

Distribution of radioactivity in cells grown in presence of D- and and L-leucine-^{14}C

Cells were recovered from the experiments reported in Table IV, and the cell-free extracts were prepared as described under "Experimental Procedure," except that dialysis was omitted. The extract was divided into 3 aliquots. One was counted as such (*Cell-free extract*). Another was precipitated with 5 volumes of 5% trichloroacetic acid, and the precipitate was extracted several times with the same solvent, with 10% NaCl, and with ethanol-ether (3:1), and was dried and counted (*Proteins*). The third aliquot was centrifuged at 105,000 × *g* for 2 hours, and the precipitate from the ultracentrifugation was counted as such (*Ribosomes*); the supernatant was extracted with 1 volume of 90% phenol, then with 1 volume of water, and the two aqueous layers were combined, dried, and counted (*sRNA*).

Cells grown on Medium B	Percentage of total radioactivity			
	Cell-free extract	Proteins	Ribosomes	sRNA
L-Leucine-^{14}C	8.6	4.17	4.02	0.028
D-Leucine-^{14}C	0.42	0.002	0.063	<0.001
D-leucine-^{14}C + L-leucine	0.38	0.001	0.035	<0.001

of D-aminoacid-RNA ligases has been confirmed by showing that D-leucine is not transferred to sRNA, whereas the L isomer as well as L-phenylalanine are transferred to an appreciable extent.

Incorporation of D- and L-Leucine-^{14}C into Polymyxin—Growing cells of *B. polymyxa* incorporate the two stereoisomers of leucine with different efficiencies, when one or the other radioactive isomer is supplied in the medium (Table IV). L-Leucine was consistently incorporated to a greater extent than D-leucine.

Furthermore, the addition, together with radioactive D-leucine, of a 5-fold excess of L-leucine further decreased the incorporation of D-leucine into polymyxin. In all cases (Table IV), upon degradation of the labeled polymyxins the radioactivity was recovered only, or mainly, in the D-leucine portion of the antibiotic. In a typical experiment in which 500 μg of polymyxin corresponding to 10,500 c.p.m. were degraded, 150 c.p.m. (1.4%) were recovered in the isopelargonic acid fraction, 26 c.p.m. (2.4%) in the α,γ-diaminobutyric acid, 8,350 c.p.m. (79.5%) in the leucine fraction, and no radioactivity in the serine + threonine fraction. Such results conform to the general pattern that the L-amino acid can be the precursor of the D-amino acid of antibiotic peptides, as demonstrated for the valine in the case of penicillin (16), actinomycins (17), and valinomycin (18). A study of the distribution of radioactivity in the different fractions of cells grown in the presence of either D- or L-leucine appears to substantiate the results of the incorporation of these amino acids into polymyxin (Table V). Slight but measurable radioactivity was present in cell-free extracts of cells grown in the presence of D-leucine-^{14}C; only traces of this radioactivity, however, were detected in the ribosomes, the proteins, or sRNA. If, on the contrary, cells were grown in the presence of L-leucine-^{14}C, all fractions were labeled. Since exogenous L-leucine is a precursor of cellular proteins, this distribution of radioactivity gives no information regarding the route of in-incorporation of L-leucine into the antibiotic.

DISCUSSION

According to previous results (5) and those presented in this paper regarding the synthesis of antibiotic peptides, activation of amino acids by formation and transfer of the aminoacyl moieties of aminoacyladenylates to sRNA is not involved in the formation of peptide bonds between D- and L-amino acids.

Other workers (14, 15) have, however, reported this type of activation of D-phenylalanine by gramicidin-producing organisms. Conceivably, peptide synthesis may indeed proceed by different paths in different organisms. Other explanations for this apparent discrepancy, however, should be considered. Namely, the D-amino acid-activating enzyme may have thus far escaped detection because of its lability. Alternatively, the apparent activation of D-phenylalanine in the cited study might be an artifact due to (*a*) contamination of the D-amino acid preparation by a small amount of the L form, or (*b*) presence of an amino acid racemase.

Our finding of depression of polymyxin D production by D-leucine, the D-amino acid component of this antibiotic molecule, parallels results reported for the antibiotics actinomycin (11) and penicillin (10). With regard to the possible mechanism of this phenomenon, it should be emphasized that our finding that addition of D-leucine does not cause an immediate inhibition of antibiotic production does not rule out inhibition by D-leucine of an enzyme, or enzymes, involved in antibiotic synthesis, as experiments were not done to determine either the time required for entry of D-leucine into the cell or the time required for excretion of antibiotic synthesized subsequent to addition of D-leucine.

Externally supplied D-leucine seems to be a less favorable precursor of polymyxin D than either externally supplied or internally synthesized L-leucine. Similarly, in the cases of penicillin (16), actinomycin (17), and valinomycin (18), L-valine is favored over D-valine as a precursor of antibiotic.

The preferential incorporation of the L forms into the D forms of peptides may be related to the finding that addition of the L-amino acid overcomes the inhibition of synthesis of antibiotic caused by the D-amino acid.

From our present results, we cannot rigorously conclude that internal L-leucine is a better precursor of antibiotic peptide than internal D-leucine, since we are ignorant of both the permeability of the cell to exogenous D-leucine and of the relative sizes of internal D- and L-leucine pools. The inhibition of antibiotic production by D-leucine, however, lends support to the hypothesis of preferential utilization of internal L-leucine for antibiotic synthesis, as this inhibition is simply interpreted as due to competitive inhibition by D-leucine of an early step in incorporation of L-leucine into antibiotic. Such an interpretation also accounts in the simplest way for the observed reversal of D-leucine inhibition of polypeptide production by addition of L-leucine to the growth medium. It must, however, be pointed out that inhibition of polymyxin production by D-leucine could be due to inhibition of excretion rather than synthesis, or to some rather unspecific effect on the general metabolism of the cell.

At what point in the biogenesis of these peptides the L-amino acid is converted to the D form is largely a matter of speculation. Cell-free extracts of *B. polymyxa* were tested unsuccessfully for the presence of a racemase converting L-leucine to D-leucine or a transaminase system capable of converting α-ketoisocaproic acid into D-leucine. Moreover, no significant incorporation into protein was observed when D-leucine was added to the medium.

SUMMARY

The production of polymyxin D by a strain of *Bacillus polymyxa* is inhibited by the addition to the growth medium of D-leucine. Such inhibition is reversed, at least in part, by the addition of L-leucine.

Cell-free extracts of the microorganism are unable to activate D-leucine as judged by adenosine triphosphate-pyrophosphate exchange and to transfer the amino acid to soluble ribonucleic acid, whereas these reactions occur with L-leucine.

Experiments with the radioactive enantiomorphs of leucine lend support to the hypothesis that L-leucine, rather than D-leucine, is the precursor of the D-leucine of the antibiotic.

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