ROLE OF VALINE AND ISOLEUCINE AS REGULATORS OF ACTINOMYCIN PEPTIDE FORMATION BY *STREPTOMYCES CHRYSOMALLUS*

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Received for publication April 24, 1961

ABSTRACT

KATZ, EDWARD (Rutgers, The State University, New Brunswick, N. J.), CLARENCE R. WALDRON, AND MARY LOU MELONI. Role of valine and isoleucine as regulators of actinomycin peptide formation by *Streptomyces chrysomallus*. J. Bacteriol. **82**:600–608. 1961—D-Valine is an effective inhibitor of actinomycin synthesis by *Streptomyces chrysomallus*; L-valine stimulates actinomycin production and reverses the inhibition due to the D-enantiomorph. The incorporation of L-valine into the medium results, particularly, in a marked increase in actinomycin IV formation. In studies with various isoleucine isomers it was shown that L-isoleucine enhances actinomycin VII production; the principal effect of D-alloisoleucine and, especially, D-isoleucine, is to bring about synthesis of two new actinomycins which contain *N*-methylisoleucine. Both L- and D-threonine were observed to have an effect similar to that obtained with L-isoleucine. An interpretation of these findings has been discussed.

Streptomyces chrysomallus synthesizes a mixture of chromopeptide antibiotics, the actinomycins, which differ solely at the 'D-valine-D-alloisoleucine' positions of the peptides (Fig. 1) (Brockmann, 1960). Actinomycin IV represents approximately 8 to 10% of the mixture formed in chemically defined media containing glycine or nitrate as the sole nitrogen source; however, it may increase to as much as 83% of the actinomycins produced if DL-valine is added to the medium (Schmidt-Kastner, 1956*a*). Whether D-, L-, or both isomers of valine are important for the augmented synthesis of actinomycin IV is not evident from the data presented. It has further been shown that supplementation of a nitrate-glycerol medium with DL-isoleucine results in the formation of two new actinomycins (Schmidt-Kastner, 1956*a*, *b*). These antibiotics, designated actinomycins E_1 and E_2, contain 1 and 2 moles of *N*-methylisoleucine, respectively, instead of a corresponding number of *N*-methylvaline residues.

In contrast, the actinomycins produced by *Streptomyces antibioticus* each contain 2 moles of D-valine per mole of antibiotic (Roussos and Vining, 1956; Johnson and Mauger, 1959). It was demonstrated that D-valine, D-isoleucine, D-alloisoleucine, and α-methyl-DL-valine inhibit actinomycin synthesis by the organism (Katz, 1960). L-Valine, on the other hand, reversed the inhibition due to D-valine and α-methyl-DL-valine, and stimulated actinomycin synthesis when employed in the absence of the D-isomer. It was suggested that L- rather than D-valine is probably the precursor of the D-valine present in an actinomycin molecule. Recently, Kawamata, Kimura, and Fujita (1960) confirmed the fact that D-isoleucine is an inhibitor of actinomycin formation; moreover, they reported that L-isoleucine and D- and L-threonine reversed the inhibition.

In light of these results it was of interest to investigate the influence of the various isomeric forms of valine and isoleucine upon actinomycin formation by *S. chrysomallus*. The findings of these studies are presented in this communication.

MATERIALS AND METHODS

S. chrysomallus strain 3657 was used throughout the investigation. The method of cultivation, composition of the glutamic acid-galactose-mineral salts medium (basal medium) for production of actinomycin, procedure for collecting the mycelium formed during growth, and microbiological assay technique for determining the antibiotic titer of culture filtrates have been

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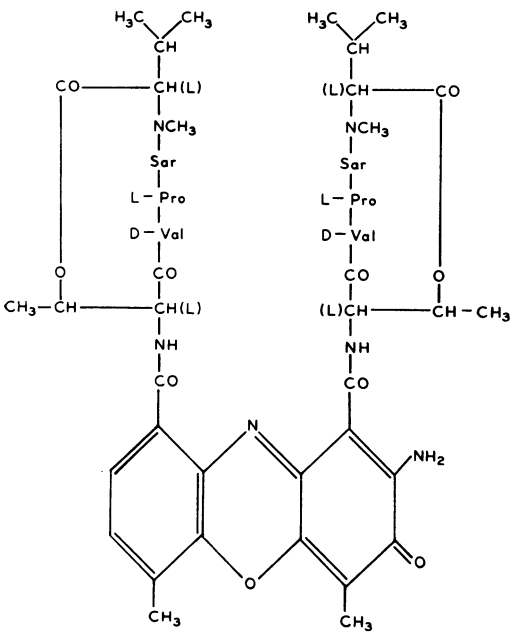

FIG. 1. *Structure of actinomycin IV* (C_1). *Sequence of amino acids:* L-*threonine,* D-*valine,* L-*proline, sarcosine, N-methyl-*L *valine. Actinomycin VI* (C_2)*—1 mole of* D-*valine is replaced by 1 mole of* D-*alloisoleucine; actinomycin VII* (C_3) *2 moles of* D-*valine are replaced by 2 moles of* D-*alloisoleucine.*

described previously (Goss and Katz, 1957; Katz, Pienta, and Sivak, 1958; Katz and Goss, 1959). Actinomycins IV, VI, and VII possess similar antimicrobial activity; therefore, a crystalline preparation of actinomycin IV was employed as assay standard.

The D-, L-, and DL-valine, the D- and L-threonine, and the D-alloisoleucine were obtained from the California Corporation for Biochemical Research. The D-, L-, and DL-isoleucine were purchased from the Nutritional Biochemicals Corporation. The D-alloisoleucine was described as purity grade B.

Unless stated, the amino acids were added to the medium at the time antibiotic production began, i.e., 12 to 24 hr after inoculation of the organism. Duplicate flasks were used for each test series.

The actinomycin mixture synthesized by *S. chrysomallus* was extracted from culture fluids with *n*-butanol and concentrated to dryness in a Rinco rotary flash evaporator. The actinomycin mixtures were separated by circular paper chromatography with the solvent system: 10% aqueous sodium-*o*-cresotinate, *n*-dibutyl ether, and symmetrical tetrachloroethane (4:3:1) (Katz et al., 1958; Katz and Goss, 1959). Determination of the percentage of a component in an actinomycin mixture was carried out by a spectrophotometric method (Goss and Katz, 1957).

RESULTS

Influence of D-, L-, *and* DL-*valine on actinomycin formation and growth of S. chrysomallus.* 1) D-Valine:—As observed previously with *S. antibioticus* (Katz, 1960), D-valine markedly inhibits actinomycin synthesis by *S. chrysomallus*; the extent to which antibiotic formation is blocked depends upon the concentration of D-valine used and the length of incubation of the organism (Fig. 2). It can further be seen that the inhibition was particularly striking early in the fermentation.

D-Valine probably interferes directly with some stage in the synthesis of the actinomycin molecule rather than with the growth of the organism,

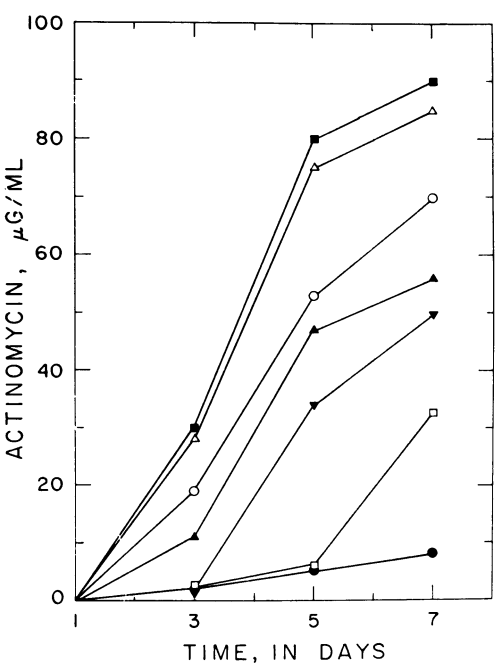

FIG. 2. *Influence of* D- *and* L-*valine on actinomycin production by Streptomyces chrysomallus in basal medium: no valine,* ○——○; D-*valine: 10 μg per ml,* ▲——▲; *250 μg per ml,* ▼——▼; *500 μg per ml,* □——□; *1,000 μg per ml,* ●——●; L-*valine: 250 μg per ml,* △——△; *1,000 μg per ml,* ■——■.

TABLE 1. *Influence of* D-, L-, *and* DL-*valine on actinomycin synthesis and growth of Streptomyces chrysomallus**

Valine isomer	Concn	Inhibition or stimulation of actinomycin synthesis	Mycelium dry wt	Actinomycin component in mixture		
				IV	VI	VII
	μg/ml	%	*mg/100 ml*	%	%	%
None	0	0	177	19.0	41.8	39.2
L-	10	+11	174	14.4	42.4	43.2
D-	10	−42	172	19.6	41.3	39.1
DL-	20	−32	175	13.6	44.2	42.2
L-	250	+47	199	24.2	36.7	39.1
D-	250	−89	183	15.6	43.3	41.2
DL-	500	−79	198	42.3	31.7	26.0
L-	500	+47	216	33.9	30.2	35.9
D-	500	−89	184	18.3	33.9	47.8
DL-	1,000	−79	193	53.2	20.9	25.9
L-	1,000	+58	225	58.8	28.1	13.1
D-	1,000	−89	188	16.0	37.8	46.2
DL-	2,000	−74	176	71.1	18.3	10.7

* The organism was grown in the basal medium for 24 hr at which time D-, L-, or DL-valine was added. After 48 hr additional incubation the actinomycin and mycelium were treated as described in Materials and Methods.

since the amount of mycelium produced in the presence of inhibitory concentrations of the D-isomer is about equal to or greater than that synthesized with no valine added (Table 1).

Although total synthesis of actinomycin is greatly depressed under these conditions, there is generally a limited but transitory increase in the amount of actinomycin IV in the mixture formed. This increase usually occurs after a few days of incubation in the presence of the D-isomer. In one experiment in which 500 μg of D-valine per ml were added to flasks, it was noted, after an additional 4 days of incubation, that actinomycin production was checked appreciably (89%) and that actinomycin IV represented 38% of the mixture elaborated. Following 2 more days of cultivation, antibiotic synthesis still was inhibited markedly (53%), whereas actinomycin IV represented only 18% of the actinomycin mixture.

2) L-Valine:—Production of all actinomycins increases after L-valine is added to the medium (Table 1, Fig. 2). For example, with 250 μg of L-valine per ml supplied, a 47% rise in the activity of culture filtrates was observed. Growth of the organism under these conditions is only slightly greater (about 12%) (Table 1). Similar results have been obtained in a number of other experiments.

In particular, enhanced formation of actinomycin IV occurs when suitable concentrations of L-valine are employed (Table 1); this increase is evident within 1 day of L-valine supplementation. However, within 2 days the exogenous supply of L-valine (1,000 μg per ml) is metabolized, and synthesis of the actinomycins then appears to revert to that occurring in control flasks. Actinomycin IV might represent 50 to 80% of the actinomycins produced after 3 days of incubation but only about 35 to 40% after 7 days of cultivation.

3) DL-Valine:—DL-Valine apparently has two independent but simultaneous effects upon actinomycin formation. The D-isomer of the racemic mixture markedly inhibits antibiotic synthesis (Table 1); the mixture formed, how-

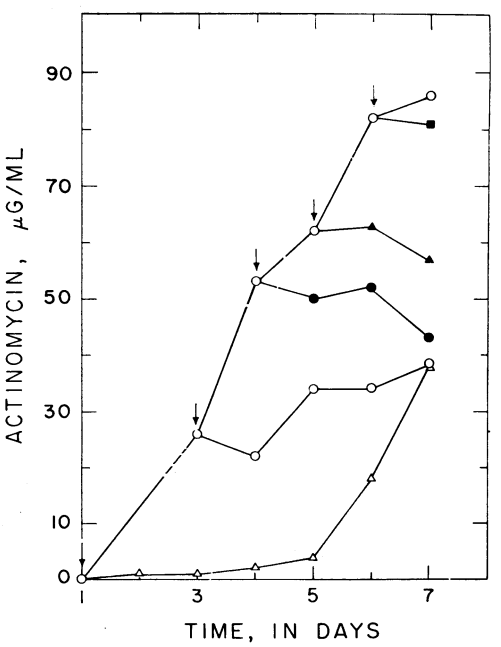

FIG. 3. *Effect of* D-*valine (500 μg per ml) addition at various intervals during actinomycin synthesis by Streptomyces chrysomallus. Arrow* (↓) *denotes time of* D-*valine addition.*

ever, consists predominantly of actinomycin IV, suggesting that the L-isomer selectively stimulates its production.

Addition of D- or L-valine at different times during actinomycin synthesis. D-Valine is an effective inhibitor of actinomycin synthesis when added prior to or during antibiotic formation (Fig. 3). At a concentration of 500 μg per ml, the D-isomer, supplied to cultures of *S. chrysomallus* after 3, 4, 5, or 6 days of incubation, essentially checked further synthesis of actinomycin. When D-valine was supplied prior to antibiotic formation, synthesis was blocked for about 5 days. Subsequently, rapid production occurred and, by 7 days, the titer of culture filtrates was approximately 45% of that attained in control flasks.

L-Valine was found to increase over-all antibiotic production and to selectively stimulate synthesis of actinomycin IV, whether it was supplied before or during antibiotic formation. The maximal percentage of actinomycin IV, attained in a given mixture after L-valine (1,000 μg per ml) supplementation, ranged from a high of 89.1% (addition at time of inoculation) to a low of 45.4% (addition at 5 days).

Influence of D-valine on actinomycin synthesis in the presence of L-valine. Actinomycin IV invariably constitutes a greater percentage of the mixture synthesized when DL-valine is employed rather than the L- or D-isomer (at one-half the DL-concentration) (Table 1). Since a limited increase in synthesis of actinomycin IV has been observed with only the addition of D-valine to the basal medium, it was of interest to determine the extent to which it might contribute to enhanced formation of actinomycin IV in the presence of L-valine.

TABLE 2. *Synthesis of actinomycin IV by Streptomyces chrysomallus in presence of mixtures of D- and L-valine*

Expt	L-Valine concn	D-Valine concn	Actinomycin IV in mixture at 3 days
	μg/ml	μg/ml	%
1	0	0	15.1
	250	0	19.1
	0	100	20.3
	250	100	35.7
2	0	0	17.9
	1,000	0	69.9
	0	10	15.4
	1,000	10	76.8
	0	50	18.3
	1,000	50	85.0

TABLE 3. *Reversal of D-valine inhibition of actinomycin synthesis by L-valine*

D-Valine concn	L-Valine concn	Stimulation or inhibition of actinomycin synthesis at 3 days
μg/ml	μg/ml	%
0	0	0*
0	1,000	+52
10	0	−21
10	1,000	+48
20	0	−35
20	1,000	+28
200	0	−94
200	1,000	−46

* Actinomycin titer at 3 days, 47 μg per ml.

It was seen that combinations of L- and D-valine (ratio L:D from 100:1 to 1:2), in comparison to the use of either L- or D-valine alone, does result in somewhat greater yields of actinomycin IV in the mixture elaborated. However, it was also noted that inhibition of actinomycin synthesis becomes more pronounced as the ratio of D- to L-valine increases. The results of certain of these experiments are presented in Table 2.

Reversal of D-valine inhibition by L-valine. L-Valine reverses the inhibition of actinomycin synthesis due to D-valine (Table 3). The reversal does not appear to be the result of increased growth followed by greater antibiotic production. Mycelium formation in the various experimental series did not exceed that produced in control flasks by more than 10 to 15%; moreover, enhanced growth of the organism occurred in flasks containing D-, L-, or mixtures of both isomers.

Influence of isoleucine and threonine isomers on actinomycin formation. 1) Isoleucine:—In certain experiments we observed that L-isoleucine, at concentrations of 500 μg per ml or more, selectively stimulated actinomycin VII synthesis, whereas formation of actinomycins IV and VI was markedly curtailed (Table 4, Fig. 4). In addition, the component, actinomycin E_1, which represented about 20 to 25% of the mixture, was produced. In other studies, conducted under

TABLE 4. *Influence of isoleucine and threonine isomers on actinomycin formation by Streptomyces chrysomallus*

Amino acid isomer	Concn	Actinomycin titer	Actinomycin component in mixture				
			IV	VI	VII	E_1	E_2
	μg/ml	*μg/ml*	%	%	%	%	%
None	0	37	12.9	43.6	43.6	—	—
L-Isoleucine	50	33	10.2	37.0	47.7	5.2	—
	500 (expt 1)	20	3.4	11.6	64.0	20.9	—
	500 (expt 2)	16	1.4	6.0	46.1	36.1	10.4
D-Isoleucine	10	21	8.0	24.4	34.6	20.4	12.7
	50	8	3.2	12.3	24.3	28.0	32.3
	500	6	3.7	8.9	23.2	34.5	29.7
DL-Isoleucine	100	8	4.9	13.2	33.1	28.7	20.1
	1,000	15	3.1	6.3	37.9	37.1	15.7
D-Alloisoleucine	100	7	8.7	24.6	39.6	21.2	5.9
	250	3	5.0	8.2	21.7	36.7	28.4
L-Threonine	1,000	—	7.4	31.5	57.7	3.4	—
D-Threonine	1,000	—	3.2	19.3	63.3	11.9	2.3

similar conditions, significant production of actinomycins E_1 and E_2 took place, but only a slight increase in actinomycin VII synthesis occurred.

Two new actinomycins (which appear to be the same substances as those formed in the presence of L-isoleucine) are synthesized when the organism is grown with D-isoleucine (Table 4). These components constitute significant amounts of the actinomycin mixture elaborated even at comparatively low levels of the D-isomer (10 μg per ml); optimal synthesis of these actinomycins (30 to 35% of each component) occurred when 50 to 100 μg D-isoleucine per ml were employed (Fig. 4).

The results obtained with DL-isoleucine (Table 4) are intermediate between those found with L- and D-isoleucine. Actinomycin VII synthesis is less than that observed with L-isoleucine but greater than that seen with D-isoleucine. Production of actinomycin E_1 is always greater than actinomycin E_2 formation at all concentrations of the DL-isomer (10 to 2,000 μg per ml) employed.

D-Alloisoleucine, found in actinomycins VI and VII, fails to stimulate production of either actinomycin, however. Instead, results comparable to those reported for D-isoleucine were obtained (Table 4).

The inhibition of synthesis with isoleucine was observed. The antibiotic potency of certain filtrates from cultures grown in media containing any of the isoleucine isomers was reduced by as much as 50 to 90% (Table 4). Since growth of the organism was not inhibited under these conditions, the apparent inhibition of antibiotic production may be due to decreased potency of the new actinomycins synthesized or to an actual depression of actinomycin formation or both. As the microbiological activity of the new actinomycins has not been reported to date, it is not possible to decide which factor is responsible for the reduced antibiotic titers.

2) Threonine:—The relationship of threonine to isoleucine biosynthesis in *Neurospora crassa* and *Escherichia coli* is well established (Abelson, 1954; Adelberg, 1954, 1955*a*, *b*). Both L- and D-threonine were observed, in studies with *S. chrysomallus*, to have an effect comparable to that obtained with L-isoleucine (Table 4). Actinomycin VII production is considerably greater

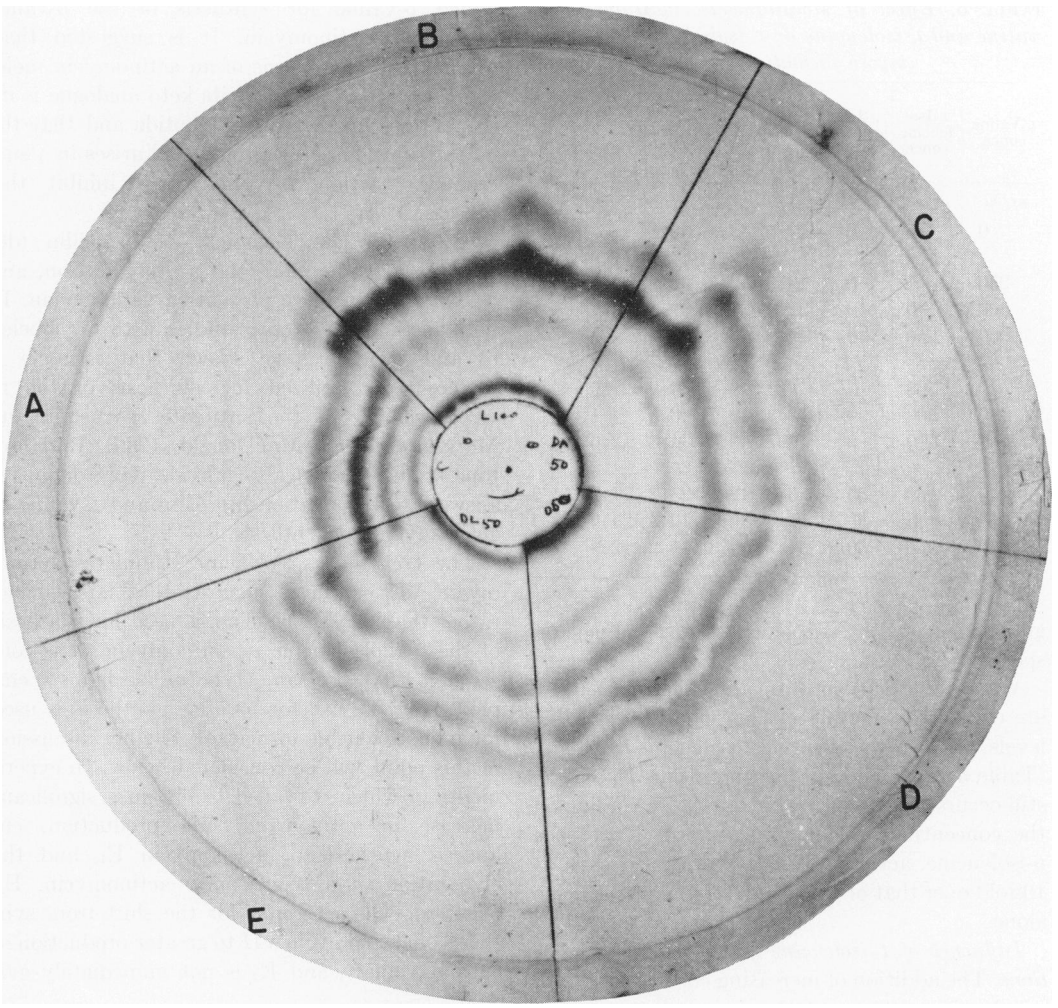

FIG. 4. *Synthesis of actinomycins by Streptomyces chrysomallus in the presence of various isomers of isoleucine. S. chrysomallus was grown in the basal medium to which a given isomer of isoleucine was added 12 hr after inoculation. The actinomycin mixture synthesized was isolated 3 days later and was separated by circular paper chromatography as described in Materials and Methods. Sequence of actinomycins is from center to periphery; in all cases, the first zone just beyond the origin constitutes biologically inactive, colored material. A, basal medium: actinomycins IV, VI, and VII. B, basal medium plus* L*-isoleucine, 1,000 μg per ml: actinomycin IV (trace), VI, VII, E_1 and E_2. C, basal medium plus* D*-alloisoleucine, 500 μg per ml: actinomycin IV (trace, not evident) VI, VII, E_1 and E_2. D, basal medium plus* D*-isoleucine, 500 μg per ml: actinomycin IV (trace, not evident), VI, VII, E_1, and E_2. E, basal medium plus* DL*-isoleucine, 500 μg per ml: actinomycin IV (trace, not evident), VI, VII, E_1, and E_2.*

than that occurring in control flasks and there is synthesis of actinomycins E_1 (D- and L-threonine) and E_2 (D-threonine).

Influence of simultaneous addition of L*-valine and* L*-isoleucine or* D*-isoleucine.* The simultaneous addition of both L-valine and L-isoleucine to the medium reduces, to some extent, the effectiveness of either compound upon actinomycin biosynthesis (Table 5). Thus, with both amino acids present, actinomycin E_1 synthesis declines appreciably and there is much less production of actinomycin IV in comparison with cultures

TABLE 5. *Effect of simultaneous additions of* L-*valine and* L-*isoleucine or* D-*isoleucine on actinomycin formation*

L-Valine concn	L-Isoleucine concn	D-Isoleucine concn	Actinomycin component in mixture				
			IV	VI	VII	E_1	E_2
μg/ml	*μg/ml*	*μg/ml*	%	%	%	%	%
0	0	0	12.1	42.1	45.8	—	—
250	0	0	19.8	44.3	35.9	—	—
500	0	0	22.5	37.9	39.6	—	—
1,000	0	0	49.2	23.8	26.9	—	—
0	500	0	4.4	9.8	63.9	21.9	—
500	500	0	12.3	42.7	40.9	4.1	—
1,000	500	0	19.7	43.3	34.3	2.6	—
0	0	50	5.0	12.0	23.2	27.2	32.5
250	0	50	16.6	16.2	19.3	24.9	22.9
1,000	0	50	54.7	12.3	11.2	12.9	9.7

grown only with L-isoleucine or L-valine, respectively.

When combinations of L-valine and D-isoleucine are employed, the former compound, at suitable levels, reverses the effect of D-isoleucine markedly (Table 5). Production of actinomycins E_1 and E_2 still occurs but is sharply curtailed. Depending on the concentration of L-valine supplied with the D-isoleucine, actinomycin IV synthesis rises 3- to 10-fold over that of cultures receiving D-isoleucine alone.

Influence of L-*isoleucine and* D-*isoleucine additions.* The addition of increasing concentrations of L-isoleucine (100 to 1,000 μg per ml) to a given amount of D-isoleucine (50 to 250 μg per ml) results in greater synthesis of actinomycin VII (from 23 to 43%) and a decline in actinomycin E_2 production (32 to 11%); actinomycin E_1 formation remains virtually unchanged (32 to 37%).

DISCUSSION

The observation that D-valine is an effective inhibitor of actinomycin synthesis by *S. chrysomallus*, whereas L-valine stimulates its formation and reverses the inhibition due to the D-enantiomorph, is similar to that reported previously for *S. antibioticus* (Katz, 1960). L-Valine, in particular, enhances actinomycin IV synthesis. These data provide further evidence for the hypothesis that L-valine is used in preference to added D-valine for synthesis of the D-valine present in actinomycin. It is suggested that, during the biosynthesis of an actinomycin molecule, the L-amino acid or its keto analogue is incorporated into a growing peptide and that the D-configuration of the amino acid arises in a succeeding reaction. D-Valine may inhibit this process.

The penicillamine moiety of penicillin (derived from valine) has the D-configuration, and D-valine residues are present in valinomycin. In both instances, isotopic studies with C^{14}-labeled D- and L-valine have shown that L-valine is preferentially utilized for synthesis of the D-valine moiety of the antibiotic (Arnstein and Margreiter, 1958; MacDonald, 1960). Radioisotope studies should also provide more definitive answers to the relationship of D- and L-valine to actinomycin biosynthesis.

The fact that L-isoleucine stimulates actinomycin VII synthesis suggests that L-isoleucine, rather than D-alloisoleucine, is used for synthesis of the D-alloisoleucine in actinomycin. It is conceivable that the same type of mechanism employed by the cell for D-valine synthesis is used for D-alloisoleucine formation. Further discussion of this point will be considered below. In experiments in which we failed to obtain a significant increase in actinomycin VII production, enhanced synthesis of actinomycin E_1, and the elaboration of a second new actinomycin, E_2, occurred. The reason(s) for the shift from synthesis of actinomycin VII to greater production of actinomycin E_1 and E_2 is not immediately evident.

We propose a tentative hypothesis (illustrated in Fig. 5) to explain the results which have been obtained with the various isoleucine and threonine isomers.

L-Isoleucine and D-alloisoleucine yield the same keto analogue, L-α-keto-β-methylvaleric acid (Meister, 1951, 1952). Cell-free extracts of *S. chrysomallus* catalyze transamination between L-isoleucine and α-ketoglutaric acid to yield L-glutamic acid (*unpublished results*) and, presumably, L-α-keto-β-methylvaleric acid. The latter compound, once formed, might then undergo transamination or reductive amination to yield D-alloisoleucine (Meister, 1952).

D- and L-threonine are attacked by specific D- and L-threonine deaminases in *Escherichia coli* (Pardee and Prestidge, 1955; Umbarger and

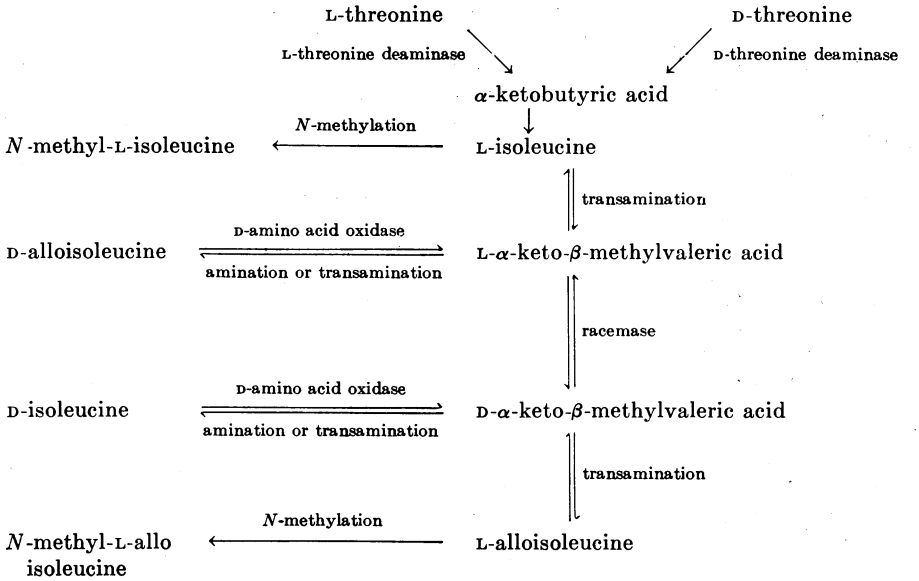

FIG. 5. *Schematic diagram showing the possible interconversions of isoleucine and threonine isomers to amino acids found in actinomycin.*

Brown, 1956), giving rise to α-ketobutyric acid, which lies directly on the pathway of L-isoleucine biosynthesis (Willson and Adelberg, 1957). The conversion of D- and L-threonine to L-isoleucine via α-ketobutyric acid may occur by the same mechanism in *S. chrysomallus* and thus explain why both isomers of threonine exhibit an effect similar to that observed with L-isoleucine.

The configuration of the β-carbon atom of methylisoleucine found in actinomycin E_1 and E_2 has not yet been reported (Schmidt-Kastner, 1956 *a,b*; Brockmann, 1960). The order of effectiveness of the various amino acids tested as "precursors" for actinomycin E_1 and E_2 synthesis is D-isoleucine > D-alloisoleucine > L-isoleucine > D-threonine > L-threonine. If one can relate the effectiveness of a compound to its proximity to the final reaction product, this order of activity suggests that the *N*-methylamino acid is the L-alloisoleucine form. Oxidation of D-isoleucine would yield D-α-keto-β-methylvaleric acid (Meister, 1951) which, in turn, might undergo transamination to form L-alloisoleucine. Methylation of the latter compound would give rise to *N*-methyl-L-alloisoleucine (Fig. 5). One additional reaction, the racemization of L-α-keto-β-methylvaleric acid to the D-α-keto acid (Meister, 1952), would be required to convert D-alloisoleucine to *N*-methyl-L-alloisoleucine. The rapid metabolism of L-isoleucine, plus its conversion to D-alloisoleucine, would reduce the effectiveness of this amino acid for actinomycin E_1 and E_2 formation as our data reveal. Finally, L- and D-threonine, by virtue of loss through metabolism as threonine and subsequently, after conversion, as isoleucine, probably would make the least suitable precursor compounds. Conceivably, *N*-methyl-L-isoleucine, rather than *N*-methyl-L-alloisoleucine, is the amino acid incorporated into actinomycins E_1 and E_2. However, this possibility seems to us to be the less likely one.

ACKNOWLEDGMENT

This investigation was supported by grants E-2280 and E-3232, from the National Institutes of Health, U. S. Public Health Service.

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