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Relations between tryptophan configuration and actinomycin biosynthesis

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SUMMARY

Addition of D-tryptophan to the growth media of Streptomyces *V-187 results in an inhibition of actinomycin production, while growth of the microorganism appears to be unaffected. L-tryptophan overcomes the inhibition brought about by the D-stereoisomer.*

D-[benzene ring-^{14}C]-*tryptophan is taken up by the cells of* Streptomyces *V-187 and incorporated into proteins and actinomycin almost to the same extent of L*-[benzene ring-^{14}C]-*tryptophan, though at a slower rate. The benzene ring of both stereoisomers contributes only to the phenoxazinone residue of the actinomycin molecule. Indeed, D, L*-[methylene-^{14}C]-*tryptophan is incorporated into actinomycin only to a very small extent. Other possible precursors of the chromophoric residue of actinomycin,* [benzene ring-^{14}C]-*indolepyruvic acid,* [benzene ring-^{14}C]-*benzoic acid and* [uniformly-^{14}C]-*shikimic acid, were not taken up by the cells in significant amounts.*

INTRODUCTION

One characteristic feature of many peptide antibiotics is the presence in their molecule of D-amino acids. When such D-amino acids are added to the growth medium of the antibiotic producing micro-organisms, the synthesis of the antibiotic is specifically inhibited. Since the L-forms of these amino acids were found to reverse the inhibition brought about by the D-isomers, it has been postulated that the L-isomers are the precursors of the D-isomers present in the peptide molecules.

Conclusive evidence for such a hypothesis has been demonstrated in the case of the D-valine in valinomycin [1], actinomycin [2,3], and penicillin [4], of the D-leucine in polymyxin D[5], of the D-ornithine in bacitracin [6], of the D-alloisoleucine in actinomycin [7].

The phenoxazinone chromophore of actinomycin (Fig. 1) may arise from the benzene ring of tryptophan [8]; however, no effect on the biosynthesis of the antibiotic was observed when stereoisomers of tryptophan and some related compounds were added to the growth media [9].

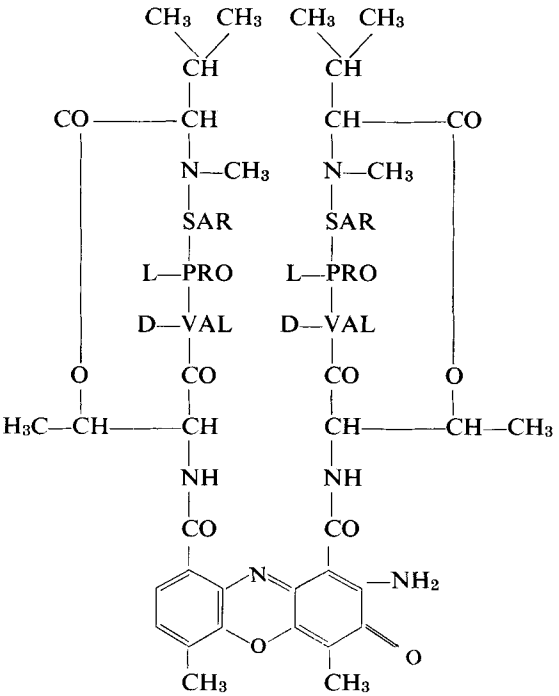

FIG. 1 — *Actinomycin D.* Sequence of amino acids: L-threonine, D-valine, L-proline, sarcosine, *N*-methyl-L-valine. Actinomycin A_1: one molecule of proline is replaced by 1 molecule of hydroxyproline.

The present investigation concerns the effect of the addition of tryptophan stereoisomers on growth and actinomycin production by *Streptomyces* V-187 as well as the incorporation of labelled tryptophan stereoisomers into the chromophoric moiety of the antibiotic.

METHODS AND MATERIALS

Streptomyces V-187, a producer of actinomycin D (actinomycin C_1, actinomycin IV) and actinomycin A_1 (actinomycin I) [10] was employed throughout the investigation. The cultural conditions as well as the procedures for extraction, determination and degradation of the antibiotic have been described previously [7, 11].

Actinocinin [12] was obtained by hydrolyzing the antibiotic in 20% HCl under reflux for a period (6 hr in general) sufficient for the solution to become dark green [11]. The solution was cooled for three hours and the dark green crystals of actinocinin that separated on acidification with HCl, were recovered by centrifuging, dried and counted.

Proteins from the cells grown in the presence of radioactive tryptophan were purified by the procedures already reported [11]. To determine the distribution of the radioactivity in the amino acids, proteins from cells grown in the presence of D-[*benzene ring*-^{14}C]-tryptophan were fractionated as follows. After hydrolysis in 2 N NaOH under reflux for 8 hours, the solution was neutralized with 1 N HCl and tryptophan extracted by shaking the solution several times with *n*-butanol [13] until no radioactivity was extracted in the organic solvent. The butanol was dried *in vacuo*, the residue taken up in a few ml of 1.5 N HCl and applied to a 60×2.5 cm column packed with Dowex 50 (H^+ form). The amino acids were eluted with HCl of increasing normality (from 1.5 to 4) and localized by nynhydrin assay. Radioactivity was determined on the nynhydrin positive peaks. Further purification and identification of the labelled amino acid obtained from the column was achieved by paper chromatography in *n*-butanol-acetic acid-water (52 : 14 : 34 by volume).

Uptake of radioactive amino acids by growing cells was determined as reported previously [11]. For the incorporation of radioactive compounds into actinomycin, the labelled substrates were added to duplicate flasks, containing 100 ml of minimal medium each, 24 hr after inoculation with a cell suspension of *Streptomyces* V-187.

All cultures were incubated at 28 °C for 72 hr on a reciprocating shaker.

D, L-[*benzene ring*-^{14}C]-tryptophan (specific activity 3.81 mC/mMole), D, L-[*methylene*-^{14}C]-tryptophan (specific activity 10.3 mC/mMole) and [*benzene ring*-^{14}C]-benzoic acid (specific activity 5.12 mC/mMole) were purchased from the Radiochemical Centre, Amersham, England. [*Uniformly*-^{14}C]-shikimic acid (specific activity 4 mC/mMole) from the New England Nuclear Corporation. D-[*benzene ring*-^{14}C]-tryptophan and L-[*benzene ring*-^{14}C]-tryptophan were prepared from D, L-[*benzene ring*-^{14}C]-tryptophan by treating with L-amino acids oxidase from *Ancistrodon piscivorus* (Ross Allen's Reptile Institute, Silver Springs, Florida) or *Crotalus adamanteus* (Sigma) and with D-amino acid oxidase from hog kidney (British Drug House Ltd.). Purification of the resolved enantiomorph was achieved as already reported [5, 7]. Radioactive indolepyruvic acid from the oxidation of D- or L-tryptophan was recovered by evaporating the ether phase and purified by paper chromatography using the *n*-butanol-acetic acid-water system already reported.

Radioactive samples were counted under conditions of negligible self absorption in a S.E.L.O. (Società Elettronica Lombarda) mica window counter tube and corrected for background activity and counting efficiency.

RESULTS

Effect of D-amino acids on actinomycin production

As shown in Table I, the production of actinomycin is strongly inhibited by the D-isomers of the amino acids which are present in the polypeptide chains of the actinomycin either in the D-form (D-valine, D-alloisoleucine, D-isoleucine) or in the L-form (L-threonine). On the other hand, the addition to the culture medium of D-leucine, D-alanine or D-phenylalanine, amino acids presumably not involved in the biosynthesis of the antibiotic, appears to slightly stimulate actinomycin production.

TABLE I. Effect of D-amino acids on the production of actinomycin by *Streptomyces* V-187

Amino acid	Amount added	Actinomycin production	% inhibition	% stimulation
	μg/ml	μg/ml		
None		71.4	0	0
Amino acids present in, or related to the antibiotic molecule :				
D-valine	100	41.3	43	0
D-alloisoleucine	100	32.4	55	0
D-threonine	100	42.3	41	0
D-isoleucine	100	37.6	48	0
D-tryptophan	100	48.9	32	0
Amino acids unrelated to the antibiotic molecule :				
D-leucine	100	96.5	0	35
D-alanine	100	94.2	0	31
D-phenylalanine	100	104.0	0	46

The D-isomers were added at the time of inoculation of the synthetic medium and the incubation was protracted for 72 hours. All data are the average of three experiments.

Such results are of special interest in the case of tryptophan, insofar as this amino acid contributes only a portion of its molecule (the benzene ring) to the antibiotic. Whereas the addition of L-tryptophan stimulates actinomycin production, addition of the D-isomer results in a striking inhibitory effect (Tables I and II). The degree of inhibition depends on the concentration of the amino acid in the medium; thus concentrations ranging from 5 to 100 γ/ml reduced actinomycin synthesis from 13 to 42% of that of control experiments (Table III).

The addition of large amounts of tryptophan results in the production of

TABLE II. Influence of D-tryptophan and L-tryptophan on the synthesis of actinomycin by *Streptomyces* V-187

Amino acid added μg/ml	Spectrophotometric assay			Microbiological assay		
	μg/ml	% inhib.	% stimulat.	μg/ml	% inhib.	% stimulat.
None	67.6	0	0	74.8	0	0
D-tryptophan 20	48.2	29	0	53.1	30	0
50	45.3	23	0	50.4	33	0
100	36.2	47	0	41.2	45	0
L-tryptophan 20	76.2	0	12	82.2	0	11
50	79.4	0	17	84.8	0	13
100	101.6	0	50	109.6	0	46
D-tryptophan 50 +						
+ L-tryptophan 20	69.8	0	3	75.2	0	0.5
+ L-tryptophan 50	70.2	0	4	82.4	0	10
+ L-tryptophan 100	99.7	0	47	106.1	0	41

Culture conditions as in Table I except that the concentration of tryptophan was varied as shown. Each result is the average of three experiments.

TABLE III. Effect of the addition of D-tryptophan on growth of and actinomycin production by *Streptomyces* V-187

D-tryptophan μg/ml	Antibiotic production		Growth	
	μg/ml	% inhibition	% mycelial volume	% inhibition
0	66.8	0	4.4	0
2	71.2	0	4.4	0
5	58.4	13	4.0	9
10	51.0	23	4.7	0
20	53.6	20	4.8	0
50	50.4	25	4.7	0
100	39.2	42	4.7	0

Culture conditions as in Table I except that the concentration tryptophan was varied as shown. Each result is the average of three experiments.

highly coloured cultural liquids; therefore, actinomycin production was assayed both microbiologically and colorimetrically [14]. As reported in Table II, no significant difference was observed by employing the two assay methods. Such results indicate that the coloured products (possibly produced by degradation of the tryptophan supplied to the culture) have absorption maxima different from that of actinomycin. Therefore the colorimetric assay, which is simpler and more rapid than the biological assay, may be used even in the experiments employing tryptophan. The results of Table II also indicate that addition of L-tryptophan may overcome the inhibitory effect brought about by the D-enantiomorph.

As in the case of other amino acids, D-tryptophan seems to inhibit only antibiotic synthesis since the growth of the organism is not affected by concentrations of D-tryptophan that strongly inhibit actinomycin synthesis (Table III).

Incorporation of L-, D- and D, L-[benzene ring-^{14}C]*-tryptophan into the actinomycin chromophore*

As shown in Table IV, if D-[*benzene ring*-^{14}C]-tryptophan is supplied to the culture at concentrations that do not inhibit antibiotic synthesis, the amino acid is incorporated into actinomycin to the same extent as the racemic mixture. However, the incorporation of the D-[*benzene ring*-^{14}C]-tryptophan is less than that of the L-form. This is probably due to a slower uptake by the cells of the D-isomer. This is verified by a study of the rate of uptake of the two

TABLE IV. Incorporation of L-[*benzene ring*-^{14}C]-tryptophan, D, L-[*benzene ring*-^{14}C]-tryptophan and D-[*benzene ring*-^{14}C]-tryptophan into actinomycin

Radioactive amino acid	Amount added		Antibiotic recovered	Radio-activity incorporated	Specific activity	% incor-poration
	μC	μ Moles	μg	counts/min $\times 10^{-3}$	counts/min/μg	
L-[*benzene ring*-^{14}C]-tryptophan	2	0.52	1.984	1.113	560	50.1
D, L-[*benzene ring*-^{14}C]-tryptophan	2	0.52	1.848	866	460	38.7
D-[*benzene ring*-^{14}C]-tryptophan	2	0.52	1.954	860	440	39.1

The radioactive amino acids were added after 24 hours of incubation to duplicate flasks at a concentration of 1 μC per 100 ml of culture. The cultures were incubated for a further 48 hours. At the end of this period, the mycelium was removed by centrifugation and the supernatant used for extraction, purification and determination of actinomycin.

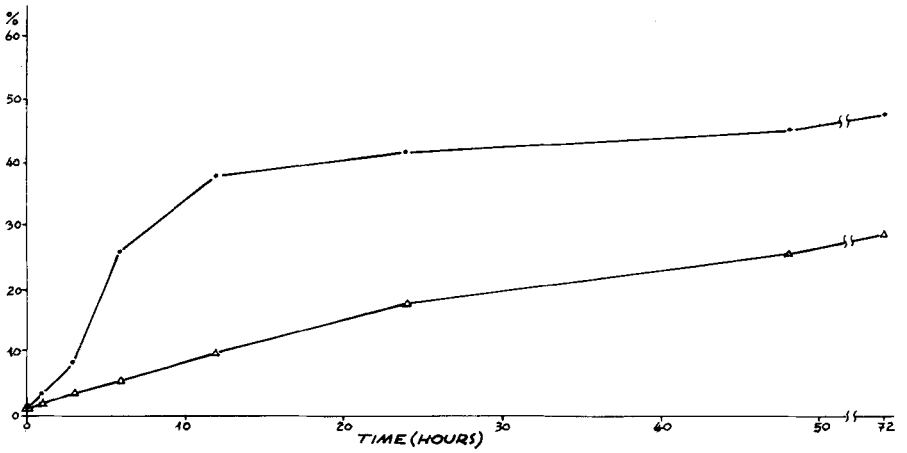

FIG. 2 — *Uptake of L-[benzene ring-^{14}C]-tryptophan and D-[benzene ring-^{14}C]-tryptophan by Streptomyces V-187.* 1 μC (0.26 μMoles) of each isomer was added to 100 ml of culture medium at the time of inoculation. At the shown time intervals, 5 ml aliquots were removed and the cells recovered by centrifugation. The supernatant was extracted with ethyl acetate to remove actinomycin and counted to determine the percentage of radioactive amino acid taken up by the cells. Each result is the average of two experiments.

•———• L-[*benzene ring*-^{14}C]-tryptophan
Δ———Δ D-[*benzene ring*-^{14}C]-tryptophan

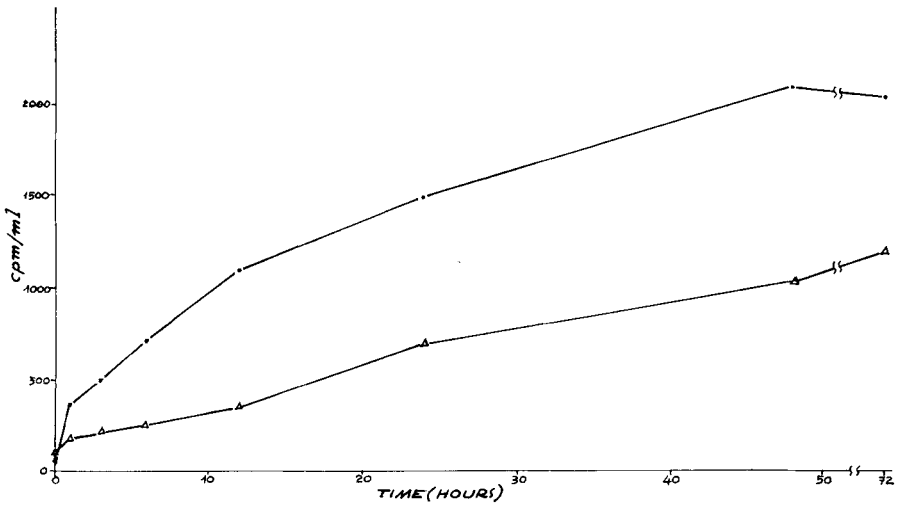

FIG. 3 — *Incorporation of L-[benzene ring-^{14}C]-tryptophan and D-[benzene ring-^{14}C]-tryptophan into the actinomycin synthesized by Streptomyces V-187.* The radioactivity incorporated into actinomycin was determined on aliquots of ethyl acetate extracts of the experiments reported in figure 2. Each result is the average of two experiments.

•———• L-[*benzene ring*-^{14}C]-tryptophan
Δ———Δ D-[*benzene ring*-^{14}C]-tryptophan

isomers by the cells and their incorporation into actinomycin (Figs. 2 and 3). Indeed, in the early stages of fermentation, L-[*benzene ring*-^{14}C]-tryptophan is taken up and incorporated into actinomycin more rapidly than the D-isomer. However, after 72 hr of incubation the amount of amino acid taken up by the cells and incorporated into the antibiotic is almost identical for both enantiomorphs. When the actinomycin synthesized in the presence of D-[*benzene ring*-^{14}C]-tryptophan and L-[*benzene ring*-^{14}C]-tryptophan was purified and degraded, almost all the radioactivity recovered was localized in the chromophoric moiety of the antibiotic whereas only traces were evident in the amino acids which make up the peptide chains of the antibiotic (Table V).

TABLE V. Degradation of actinomycin and distribution of the radioactivity incorporated

Amino acid added	Radioactivity (counts/min)		
	Hydrolysed actinomycin	Amino acids of the peptide chain	Actinocinin
L-[*benzene ring*-^{14}C]-tryptophan	105,050	955 (0.9%)	61,168 (58%)
D-[*benzene ring*-^{14}C]-tryptophan	108,100	171 (0.1%)	83,312 (77%)

Aliquots of radioactive actinomycins corresponding to approximately 120,000 cpm were hydrolysed together with 5 mg of unlabelled antibiotic. After determination of the radioactivity present in the hydrolysates, the samples were fractionated and purified as reported under Material and Methods.

Incorporation of L-[benzene ring-^{14}*C*]*-tryptophan and D-*[benzene ring-^{14}*C*]*-tryptophan into protein*

Approximately the same amount of labelled tryptophan was incorporated into cellular protein irregardless of the isomer used. Indeed, the radioactivity incorporated in the presence of L-, D- and D, L-[*benzene ring*-^{14}C]-tryptophan was of 32,131, 29,725, and 25,420 counts/min per mg of cellular protein, respectively.

However, incorporation of the labelled D-isomer into protein takes place at a later stage than that of the L-isomer, which is consistent with the results obtained for the uptake by the cells. As shown in Fig. 4, incorporation of D-[*benzene ring*-^{14}C]-tryptophan into hot TCA-insoluble material takes place more slowly than incorporation of L-[*benzene ring*-^{14}C]-tryptophan. The former reaches a maximum after 72 hr of incubation whereas in the latter case maximal incorporation is achieved approximately after 24 hr of incubation.

To confirm that D-[*benzene ring*-^{14}C]-tryptophan is incorporated into proteins without extensive degradation, a sample of the cellular proteins from

the experiments employing the D-isomer was hydrolysed and the distribution of the radioactivity in the amino acids determined. By column chromatography it was established that almost all the radioactivity (up to 76%) present in the hydrolysate was recovered in a single peak which was eluted in the position of tryptophan. Separation by paper chromatography of the amino acids present in the radioactive peak showed that all the radioactivity was associated with a single component, indistinguishable from tryptophan.

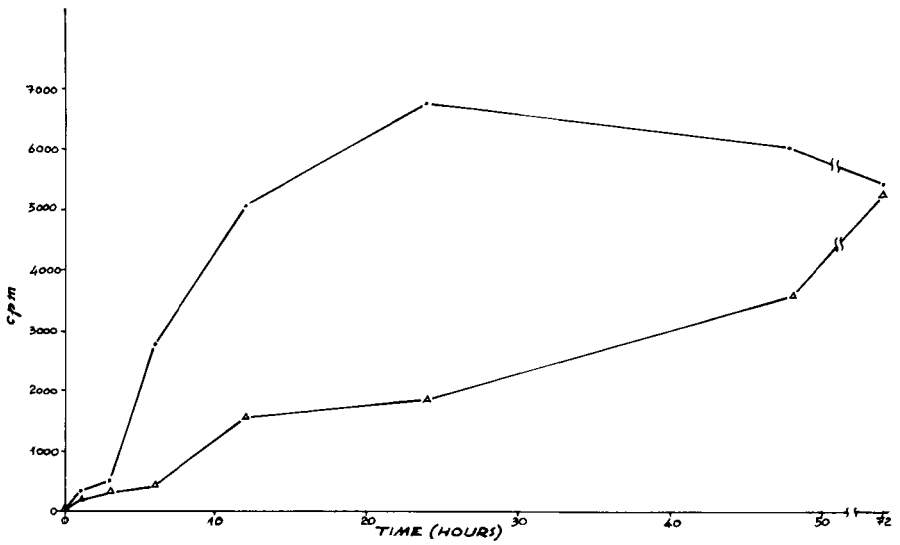

FIG. 4 — *Incorporation of L-[benzene ring-^{14}C]-tryptophan and D-[benzene ring-^{14}C]-tryptophan into the proteins of Streptomyces V-187.* The cells recovered by centrifugation in the experiments reported in figure 2, were treated as already reported [11] in order to determine the radioactivity incorporated into proteins. Each result is the average of three experiments. Values in ordinate report the amount of radioactivity recovered from the protein of 1 ml of culture suspension.

•———• L-[*benzene ring*-^{14}C]-tryptophan
Δ———Δ D-[*benzene ring*-^{14}C]-tryptophan

Incorporation into actinomycin of possible precursors of the chromophoric moiety

The high level of radioactivity incorporated into the actinocinin of actinomycin from [*benzene ring*-^{14}C]-tryptophan gives strong presumptive evidence for the role of the benzene ring residue of the amino acid in the biogenesis of the chromophoric moiety of the antibiotic. Such a hypothesis is substantiated by the findings that tryptophan labelled in the side chain is only slightly incorporated into actinomycin (Table VI). The incorporation of D, L-[*methylene*-^{14}C]-tryptophan was too low to permit degradation of the antibiotic in order to determine localization of the label but it seems possible

TABLE VI. Incorporation of D, L-[*benzene ring*-^{14}C]-tryptophan, D, L-[*methylene*-^{14}C]-tryptophan and related compounds into the actinomycin

Radioactive substance	Amount added		Specific activity of purified actinomycin	% incorporation
	μC	μ Moles	counts/min/μg	
D, L-[*benzene ring*-^{14}C]-tryptophan	4	1.04	311	28.5
D, L-[*methylene*-^{14}C]-tryptophan	4	1.04	1.18	1.09
[*benzene ring*-^{14}C]-indolepyruvic acid	4.8	1.24	1.22	< 1.0
[*benzene ring*-^{14}C]-benzoic acid	5	0.9	1.08	< 1.0
[*uniformly*-^{14}C]-shikimic acid	4	1.0	< 1.0	< 1.0

2 μC of D, L-[*benzene ring*-^{14}C]-tryptophan, D, L-[*methylene*-^{14}C]-tryptophan and [*uniformly*-^{14}C]-shikimic acid, 2.4 μC of [*benzene ring*-^{14}C]-indolepyruvic acid and 2.5 μC of [*benzene ring*-^{14}C]-benzoic acid were added to each 100 ml of culture 24 hours after inoculation. Radioactive benzoic acid was dissolved in 1 ml of ethyl alcohol prior to addition to the culture. Fermentations were carried for a further 48 hours. At the end of this period, the mycelium was removed by centrifugation and the supernatant used for extraction, purification and determination of actinomycin. Production of actinomycin was approximately identical in all the experiments reported in the Table.

that the radioactivity incorporated was not localized in the chromophoric residue but rather in some other part of the antibiotic molecule.

The search for other possible precursors of the chromophoric residue was unsuccessful insofar as [*benzene ring*-^{14}C]-indolepyruvic acid, [*benzene ring*-^{14}C]-benzoic acid and [*uniformly*-^{14}C]-shikimic acid were not taken up by the cells to a considerable extent. In all cases uptake curves of these compounds revealed that less than 1 percent of the compound supplied to the culture disappeared from the medium after 72 hr of incubation.

DISCUSSION

Addition of the D-amino acids present in the molecule of actinomycin to the growth media of *Streptomyces* V-187, results in a specific inhibition of the production of the antibiotic. Such results confirm previous reports on the effect of D-valine and D-alloisoleucine [2, 15] on actinomycin production and further them by showing that D-threonine is also inhibitory to actinomycin production, even if the amino acid is present in the antibiotic molecule as the L-form. D-isoleucine, whose L-form appears to be the precursor of the D-alloisoleucine of actinomycin [7], is equally inhibitory of the biosynthesis of the antibiotic.

As for tryptophan, an amino acid which contributes only a portion of its molecule (the benzene ring) to the antibiotic, the addition of the D-form to

a chemically defined medium results in a specific inhibition of the synthesis of the antibiotic. As in the case of the other amino acids, the concomitant addition of an excess of the L-stereoisomer abolishes the inhibitory effect. It has been reported [9] that L-tryptophan reverses the inhibition of the synthesis of actinomycin brought about by the addition of a series of methyl analogs of tryptophan.

Contrary to what has been found for most other D-amino acids, when D-tryptophan is added to the culture media at concentrations too low to inhibit actinomycin synthesis, the D-isomer is incorporated in the antibiotic to the same extent and in the same position as the L-isomer. D-tryptophan appears to be taken up more slowly by the cells than L-tryptophan. Therefore the incorporation of D-[*benzene ring*-^{14}C]-tryptophan into actinomycin and into protein proceeds at a slower rate than that of the L-isomer. However, after 72 hr of incubation the same level of incorporation is reached for both isomers. Such results indicate that the difference between the two isomers lies rather in their entry (or active transport) into the cells than in their utilization after entrance into the cellular pool. A racemase for D-tryptophan could not be found in cell-free extracts of *Streptomyces* V-187. This of course does not rule out the possibility that the enzyme may be inactivated during preparation of the cell-free extracts. As an alternative hypothesis it is possible that D-tryptophan is oxidized to an intermediate devoid of optical configuration, *e.g.* indolepyruvic acid or anthranilic acid. From this intermediate, the L-tryptophan necessary for protein could be synthesized as well as the 4-methyl-3-hydroxyanthranilic acid which appears to be the precursor of the actinomycin chromophore [16]. The pathway could be similar to that postulated for the incorporation of D-tryptophan into ergot alkaloids [17] and it is of a certain interest that for the incorporation of D-tryptophan into ergot alkaloids VINING and TABER [18] stated " that utilization of the D-form takes place after its slow conversion by the organism to a more acceptable precursor. " The same conclusion may be drawn in the case of actinomycin and *Streptomyces* V-187, with the only difference being that the rate limiting step appears to be the entry of the D-amino acid into the cells rather than its conversion to a precursor.

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