STUDIES OF ETAMYCIN BIOSYNTHESIS EMPLOYING SHORT-TERM EXPERIMENTS WITH RADIOLABELED PRECURSORS

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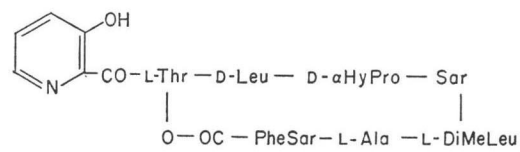
The biosynthesis of etamycin by *Streptomyces griseoviridus* was investigated with the precursors, ^{14}C-L-leucine and $^{14}CH_3$-L-methionine, during short-term incubations. Both radioisotopes are rapidly incorporated into the antibiotic without significant lag. D-Leucine, but not L-leucine, inhibits etamycin formation. The kinetics of incorporation of ^{14}C-L-leucine as well as the inhibitor studies suggest that L-leucine is the direct precursor of the D-enantiomer present in the antibiotic peptide. Chloramphenicol has been shown to inhibit protein synthesis by *S. griseoviridus* without reduction of etamycin formation, indicating that a non-ribosomal mechanism of synthesis is involved in etamycin biogenesis. When L-ethionine was employed, both the formation of antibiotic and protein were blocked.

Etamycin (Fig. 1) is a peptidolactone antibiotic produced by *Streptomyces griseoviridus* and certain other *Streptomycete* species.[1] Radiotracer studies[1~8] have demonstrated that L-amino acids are the biosynthetic precursors of the etamycin molecule; for example, L-leucine is the substrate for the synthesis of D-leucine and N-,β-dimethyl-L-leucine moieties, and L-methionine provides the source of the methyl groups found in the sarcosine, phenylsarcosine and dimethylleucine residues. Despite this information, there has been little insight into the biochemical mechanism of synthesis of the antibiotic. We have, therefore, initiated an investigation to examine the biogenetic origin of certain amino acid components present in the antibiotic employing short-term incubations with radiolabeled precursors. Some of these experiments are described in the present communication.

Fig. 1. Structure of etamycin.



Materials and Methods

Organism and Conditions of Cultivation

S. griseoviridus was kindly provided by Dr. LEO VINING, Dalhousie University, Halifax, Nova Scotia. The organism was maintained on glucose-yeast extract-malt extract agar slants.[1] Vegetative growth was prepared as follows: 5 ml of glucose-yeast extract-malt extract (GYM) liquid medium were added to a slant of the organism; the spores and mycelium were scraped off and the contents transferred to a 250 ml Erlenmeyer flask containing 75 ml of GYM liquid medium. Incubation was carried out for 24 hours at 28°C on a gyratory shaking incubator (Model G-25, New Brunswick Scientific Co., New Brunswick, N. J.) at 240 rpm. For production of etamycin, 5 ml of the GYM culture were transferred to a 250 ml Erlenmeyer flask containing 75 ml of a glucose-nitrate-mineral salts medium.[1] Incubation for various periods of time was carried out at 28°C under the conditions described for vegetative growth of *S. griseoviridus*.

Radiochemicals

L-Leucine-U-^{14}C (227 mCi/mMole) and L-methionine-$^{14}CH_3$ (57.5 mCi/mMole) were purchased

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from New England Nuclear Corp., Boston, Mass.

Chemicals

All chemicals and reagents were obtained from commercial sources.

Analytical Methods

Etamycin in culture filtrates was assayed by a modification of the disk diffusion method, using *Bacillus subtilis* ATCC 6633 as the test organism. The antibiotic etamycin, employed as a reference standard, was kindly supplied by Dr. H. DION, Parke, Davis & Co., Detroit, Mich. Analyses revealed that the preparation retained 78% of its biological potency. A spectrophotometric procedure[1] did not correlate well with the bioassays and, consequently, was not used for measurement of the etamycin concentration in culture filtrates.

Mycelial dry weight was determined by the procedure of KATZ *et al.*[4] Data are expressed as an increase in dry weight per ml of culture medium during a 24- hour incubation. Protein was assayed by the procedure of LOWRY *et al.*[5]

Radioactivity incorporated into etamycin was determined as described by KATZ and WEISSBACH[6] for actinomycin. Following incubation of *S. griseoviridus* with a ^{14}C-labeled substrate on a Dubnoff metabolic shaker at 28°C, 5 ml of the culture were rapidly filtered through glass wool. Two ml of the culture filtrate plus 4 ml of benzene were transferred to a ground-glass-stoppered centrifuge tube and shaken vigorously. After centrifugation, the benzene layer plus an equal volume of water were added to a second centrifuge tube, shaken and centrifuged as before. One ml of the benzene layer was then transferred to a counting vial to which 10 ml of BRAY's liquid scintillation fluid[7] were added. Data are expressed as mμmoles of ^{14}C-labeled amino acid incorporated into etamycin per 5 ml incubation. After solubilization of protein, according to the procedure of KATZ and WEISSBACH,[6] radioactivity incorporated into protein (cpm per mg) was determined in BRAY's scintillation fluid. Radioactive samples were counted in a Mark I Nuclear Chicago refrigerated liquid scintillation spectrometer.

Autoradiography and radiostrip scanning of thin-layer and paper chromatograms revealed that 90~100% of the radioactivity incorporated cochromatographed with authentic etamycin. Moreover, examination of hydrolysates demonstrated that the anticipated amino acid moieties of etamycin were specifically labeled with a ^{14}C-precursor.

Results

Influence of D- and L-Leucine on Etamycin Biosynthesis

Although etamycin possesses a residue of D-leucine, the addition of the D-enantiomer to the culture medium markedly inhibited antibiotic formation (Fig. 2). The extent of inhibition was dependent on the concentration of D-leucine supplied and the length of the incubation. By contrast, L-leucine, up to a level of 250 μg per ml, did not affect etamycin biosynthesis significantly. In some experiments we did observe a 10~20% inhibition of synthesis after 1 day's incubation with the L-isomer (at 100~500 μg per ml). Subsequently, however, the rate of antibiotic synthesis was similar to that noted in control flasks and, in some cases, a slight stimulation of etamycin formation was seen following 72-hours' incubation. Inhibition of etamycin formation by D-leucine appears to be directly related to antibiotic production as no reduction in mycelium synthesized was found during the incubation. These results are quite comparable to those reported previously for actinomycin,[8] polymyxin,[9] penicillin[10] and certain other peptide antibiotics possessing D-amino acids.[11]

Incorporation of ^{14}C-L-Leucine and $^{14}CH_3$-L-Methionine into Etamycin as a Function of Time

The addition of either ^{14}C-labeled leucine or methionine to cultures of *S. griseoviridus* resulted in an immediate and rapid incorporation of the ^{14}C-precursor into etamycin (Fig. 3). No lag was observed and the rate of incorporation was linear for approximately 30 minutes. Similar data were obtained by us in experiments with the other precursors of the etamycin molecule (*e.g.*, L-threonine,

Fig. 2. Inhibition of etamycin synthesis by D-leucine.

After growth for 14 hours in chemically-defined medium, D- or L-leucine, at various concentrations, was added to flasks and the incubation was resumed. Aliquots of the culture medium were then taken after an additional 24-, 48- and 72-hour intervals for microbiological assays.

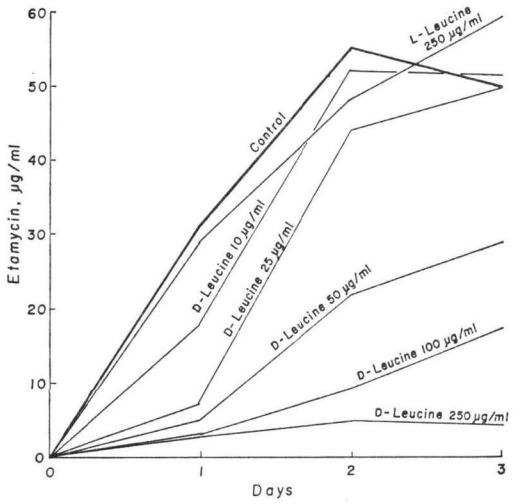

Fig. 3. Incorporation of ^{14}C-L-leucine or $^{14}CH_3$-L-methionine into etamycin as a function of time.

After 24 hours' growth in glucose-nitrate medium, 5 ml aliquots of the culture were transferred to 50 ml Erlenmeyer flasks containing ^{14}C-radioisotope (^{14}C-L-leucine, 20.3 mμmoles, 1.67×10^5 cpm, 8,230 cpm/mμmole, or $^{14}CH_3$-L-methionine, 21.7 mμmoles, 1.78×10^5 cpm, 8,175 cpm/mμmole). Incubation was carried out in a DUBNOFF metabolic shaker at 28°C for various times. Radioactive assays were performed as described in Materials and Methods.

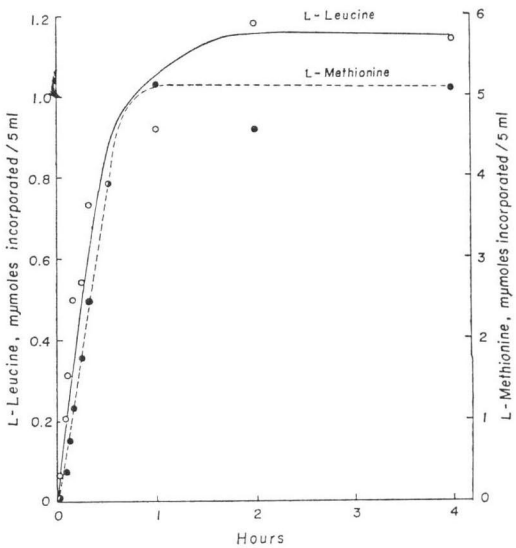

Table 1. Incorporation of L-methionine-$^{14}CH_3$ and L-leucine-^{14}C(U) into etamycin

Radioactive precursor	Length of incubation (min.)	Etamycin			
		μmoles isolated	Total radioactivity (cpm)	mμmoles incorporated	% incorporation
L-Methionine-$^{14}CH_3$	60	5.04	3.1×10^6	379	23.3
L-Leucine-^{14}C(U)	120	5.17	7.16×10^5	87	5.7

Radioactive precursor	Specific radioactivity, cpm/μmole				
	Etamycin	N-,β-Dimethyl-leucine	Sarcosine	Phenylsarcosine	D-Leucine
L-Methionine-$^{14}CH_3$	6.15×10^5	2.38×10^5	1.65×10^5	1.71×10^5	—
L-Leucine-^{14}C(U)	1.38×10^5	5.0×10^4	—	—	8.3×10^4

S. griseoviridus was cultivated in GYM medium for 24 hours and then inoculated into glucose-nitrate medium.[1] After 24 hours' incubation, a given radioisotope (L-methionine-$^{14}CH_3$, 7.5 μCi, 1.33×10^7 cpm, 1,631 mμmoles, 8,174 cpm/mμmole, or L-leucine-^{14}C(U), 7.5 μCi, 1.25×10^7 cpm, 1,523 mμmoles, 8,226 cpm/mμmole) was added (5 flasks, 1.5μCi per flask). Incubation was resumed for the times designated. Etamycin was isolated from culture filtrates by extraction with benzene, hydrolyzed in 6 N HCl and the amino acids in hydrolysates separated by high voltage electrophoresis. The individual amino acids were assayed with the Beckman-Spinco automatic amino acid analyzer, Model 120C. Radioactivity was determined as described in Materials and Methods.

glycine, L-proline, L-lysine, L-alanine and L-phenylalanine). Examination of hydrolysates derived from ^{14}C-labeled etamycin have confirmed that ^{14}C-L-leucine is incorporated specifically into the D-leucine and N-, β-dimethyl-L-leucine residues and that the methyl group of $^{14}CH_3$-L-methionine is present in sarcosine, phenylsarcosine and N-, β-dimethylleucine (Table 1). Although there are 2 'leucyl' residues for each four methyl equivalents present in the antibiotic, the ^{14}C-radiolabel from methionine was utilized more efficiently (5-to-6-fold) than the corresponding ^{14}C-label from L-leucine. Such differences may reflect differences in the size of the endogenous amino acid pool due to the specific requirements for protein synthesis and the catabolic activities of the organism.

Table 2. The effect of L- or D- leucine on the incorporation of ^{14}C-labeled L-methionine into etamycin by *S. griseoviridus*.

Leucine		$^{14}CH_3$-L-Methionine	
L μg/ml	D μg/ml	Etamycin mμmoles incorporated/5 ml	Inhibition or stimulation %
—	—	2.12	—
5	—	2.02	−5
10	—	2.37	+12
25	—	2.27	+7
50	—	2.86	+35
100	—	2.43	+15
—	5	2.29	+8
—	10	0.62	−71
—	25	0.31	−85
—	50	0.25	−88
—	100	0.19	−91

S. griseoviridus was cultivated in GYM medium for 24 hours and then inoculated into glucose-nitrate medium. After incubation for 12 hours, L- or D-leucine was added; after an additional period of 12 hours' cultivation, 5 ml of each culture were transferred to a 50 ml Erlenmeyer flask containing $^{14}CH_3$-L-methionine (21.7 mμmoles, 1.61×10^5 cpm, 7,430 cpm/mμmole). Incubation was carried out in a DUBNOFF metabolic shaker at 28°C for 30 minutes. Radioactive assays were performed as described in Materials and Methods.

Influence of D- or L-Leucine on Incorporation of ^{14}C-Labeled Precursors into Etamycin

As demonstrated in Table 2, D-leucine markedly blocked the incorporation of $^{14}CH_3$-methionine into etamycin, whereas L-leucine generally enhanced the rate of synthesis. The experiments presented here as well as those reported previously[1~3] provide evidence that L-leucine is the direct precursor of the D-enantiomer present in the antibiotic molecule.

Differential Effect of Chloramphenicol upon Etamycin and Protein Synthesis

In vitro and *in vivo* investigations have shown that the biosynthesis of a number of peptide antibiotics proceeds by a mechanism distinct from that described for protein synthesis.[11] We anticipated that suitable experiments would reveal that etamycin formation also occurs in a manner analogous to other antibiotic peptides. HOOK and VINING,[1] however, have demonstrated that biosynthesis of

Table 3. Differential effect of chloramphenicol on protein and antibiotic synthesis by *S. griseoviridus*.

^{14}C-Radioisotope	Chloramphenicol	Protein cpm/mg	Inhibition %	Etamycin mμmoles incorporated/5 ml	Stimulation %
L-Leucine	−	4.3×10^4	—	0.7	—
	+	5.7×10^3	87	1.1	+51
L-Methionine	−	1.94×10^4	—	3.3	—
	+	3.5×10^3	82	3.2	−5

Radioisotopic precursors (^{14}C-L-leucine, 20.5 mμmoles, 1.74×10^5 cpm, 8,500 cpm/mμmole, or $^{14}CH_3$-L-methionine, 21.7 mμmoles, 1.56×10^5 cpm, 7,200 cpm/mμmole) were used with or without chloramphenicol (30 μg/ml, final concentration). Incubation was for 30 minutes in a DUBNOFF metabolic shaker at 28°C. Radioactive assays and protein determinations were carried out as described in Materials and Methods.

etamycin and the growth of *S. griseoviridus* occur simultaneously in a glucose-nitrate medium. Short-term experiments (30 minutes) revealed that etamycin biogenesis proceeds in the absence of protein synthesis (Table 3). In fact, an enhanced incorporation of leucine into the etamycin molecule occurred with chloramphenicol present.

Inhibition of Growth, Protein Synthesis and Etamycin Formation by L-Ethionine

L-Ethionine is the ethyl analogue of L-methionine and has been employed extensively as a biochemical probe for investigations of protein synthesis,[12] methylation reactions[13] and antibiotic formation.[14,15]

Initially, an experiment was carried out similar to the one described in Fig. 2; L-ethionine, at various concentrations, was supplied to *S. griseoviridus* in glucose-nitrate medium. Although etamycin production was reduced markedly as a function of ethionine concentration, cell growth was severely inhibited as well. The effect of the analogue on the incorporation of ^{14}C-radioisotopes was next explored during short-term experiments (Table 4). Both etamycin and protein synthesis are blocked by ethionine; indeed, incorporation of the ^{14}C-labeled precursors into protein proved to be somewhat more sensitive to the action of the analogue.

Table 4. Inhibition of etamycin and protein synthesis by L-ethionine.

L-Ethionine	^{14}C-L-Leucine incorporated into			
	Etamycin		Protein	
μg/ml	mμmoles /5 ml	Inhibition %	cpm $\times 10^4$/mg	Inhibition %
0	0.82	—	4.84	—
5	0.78	4	4.23	13
10	0.74	10	4.03	17
25	0.65	20	3.02	38
100	0.43	47	2.28	53
250	0.33	59	1.70	65
500	0.20	76	1.09	77

S. griseoviridus was cultivated in GYM and then in glucose-nitrate medium. After 24 hours' incubation in chemically-defined medium, 5 ml aliquots of the culture were transferred to 50 ml Erlenmeyer flasks containing ^{14}C-radioisotope (^{14}C-L-leucine, 20.3 mμmoles, 1.78×10^5 cpm, 8,775 cpm/mμmole), plus or minus L-ethionine as shown above. Incubation conditions were similar to those in Table 3.

Discussion

Etamycin is classified as a heteromeric heterodetic cyclic peptide; other members of this group include such antibiotics as pyridomycin, griselimicin, staphylomycin S, doricin and vernamycin.[16] Radioisotope experiments, carried out with etamycin[1~8] and pyridomycin,[17] have defined the biosynthetic precursors of these two antibiotics and revealed that natural L-amino acids, employed for protein synthesis, are utilized for the biogenesis of the antibiotic peptides.

Due to certain similarities in the chemical structure of etamycin with actinomycin,[18] we anticipated that the mechanism of their biogenesis would also exhibit marked parallels. Both antibiotics possess L-threonine, D-amino acid, imino acid and N-methylamino acid residues. Further, the threonine residue in both substances participates (a) in an amide linkage with a non-amino acid component, (b) in lactone formation with an N-methylamino acid residue and (c) in a peptide with a D-amino acid residue in the antibiotic molecule. The present radioisotope experiments establish that the biosynthesis of etamycin as noted with actinomycin[6] can be investigated during short-term experiments with labeled precursors of the antibiotic; the ^{14}C-labeled compounds are rapidly and efficiently incorporated without any significant lag. The studies with D- and L-leucine and the kinetics of incorporation of ^{14}C-L-leucine into etamycin suggest that the D-leucine moiety in the antibiotic probably is formed *via* epimerization of its L-amino acid.[1] Such findings have been noted previously in an investigation of D-valine synthesis during actinomycin biosynthesis.[6]

Chloramphenicol has been shown to inhibit protein synthesis by *S. griseoviridus* without significant inhibition of etamycin formation. These results also compare favorably with those described pre-

viously for actinomycin[4] and a number of other peptide antibiotics,[11] indicating that a non-ribosomal mechanism of synthesis is involved in etamycin peptide formation. By contrast, when L-ethionine was employed as a methionine analogue, both the formation of antibiotic and protein were blocked.

We suggest that the utilization of short-term experiments can provide a reliable, rapid procedure for studying antibiotic biogenesis *in vivo*. The classical methods[1] generally employed have involved several days' incubation, large amounts of radiolabeled precursor of relatively low specific radioactivity and millimolar amounts of product for analyses. Moreover, the analyses themselves require the expenditure of considerable time and effort, some of which can be avoided by the use of micromolar amounts of material (precursors, products) of high specific radioactivity. We would also like to point out that the synthesis of an antibiotic of high specific radioactivity obtained by the general procedures described here are useful not only for studies of precursor-product relationships, but may also be of value for pharmacologic and mechanism of action investigations.

Acknowledgments

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