Biosynthesis of the Quinoxaline Antibiotic, Triostin, by *Streptomyces* s-2-210L*

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ABSTRACT: The biosynthesis of triostin, a cyclodepsipeptide antibiotic produced by *Streptomyces* s-2-210L, was studied using cultures grown in a sodium nitrate–maltose medium. Radioisotope experiments revealed that the various constituents of the triostin molecule were derived from L-amino acids (L-serine, L-valine, L-cystine, and L-alanine); methionine served as a source of the *N*-methyl group of *N*-methylvaline and *N,N'*-dimethylcystine and tryptophan was an efficient precursor of the quinoxaline moiety. Short-term experiments were employed to study the incorporation of the precursor amino acids into triostin and cellular proteins. Biosynthesis of the antibiotic was maximal during the late logarithmic phase of growth at which time protein synthesis had markedly declined. Chloramphenicol was found to block protein synthesis while stimulating amino acid incorporation into triostin. This finding suggests that triostin synthesis occurs by a mechanism distinct from that of protein synthesis.

Triostin (Shoji and Katagiri, 1961) and quinomycin (Yoshida *et al.*, 1961), quinoxaline antibiotics, belong to a class of heterodetic cyclodepsipeptides. These antibiotics generally are synthesized as a mixture of closely related peptides which differ at a single amino acid site in the molecule. Modifications of the nutritional environment of the producing organism may influence the biosynthesis of the antibiotics (Katz and Goss, 1958; Yoshida and Katagiri, 1967; Yoshida *et al.*, 1968).

Recent investigations have revealed that the mechanism of biosynthesis of peptide antibiotics appears to differ markedly from the synthesis of proteins (Attardi, 1967), *e.g.*, actinomycin (Katz and Weissbach, 1962), tyrocidines (Mach *et al.*, 1963), polymyxin B (Paulus and Gray, 1964), gramicidin S (Eikhom *et al.*, 1964), and edeine (Borowska and Tatum, 1966). Since the biosynthesis of antibiotic peptides remains obscure we decided to investigate the biosynthesis of triostin. The triostins (Figure 1) were selected for study because (1) all the amino acid residues present in the peptide are recovered intact by acid hydrolysis (Otsuka and Shoji, 1965) and (2) the triostins are easily purified by recrystallization (Shoji and Katagiri, 1961). We also examined the relationship of peptide antibiotic formation to cellular growth and protein synthesis by the producing organism.

Materials and Methods

Orgnaism and Conditions of Cultivation. Streptomyces s-2-210L was employed throughout the investigation. The strain, isolated from a single colony of *Streptomyces* s-2-210 (Shoji and Katagiri, 1961), was transferred monthly on slants of Bennett's agar (Waksman, 1961). Procedures for cultivation of *Streptomyces* s-2-210L have been described in an earlier publication (Yoshida and Katagiri, 1967).

The basal medium for production of triostin was modified as follows. The basal medium (sodium nitrate–maltose medium) contained $NaNO_3$ (2 g), D-maltose (20 g), K_2HPO_4 (1 g), $MgSO_4 \cdot 7H_2O$ (0.25 g), $ZnSO_4 \cdot 7H_2O$ (0.125 g), $CaCl_2 \cdot 2H_2O$ (0.05 g), and deionized water (1000 ml). Sodium nitrate and K_2HPO_4 were dissolved in 500 ml of deionized water (A) and the pH was adjusted to 7.0 with 2 N HCl. D-Maltose and the inorganic salts were dissolved separately in 500 ml of deionized water (B). Both solutions were autoclaved for 15 min at 15 psi and 120°. Solutions A and B were combined after cooling.

Chemicals. Quinoxaline-2-carboxylic acid was synthesized according to the method of Keller-Schierlein and Prelog (1957). All other compounds were obtained from commercial sources.

Radiochemicals. [1-^{14}C]Glycine (5.2 mCi/mmole) and L-[U-^{14}C]cystine (190 mCi/mmole) were purchased from New England Nuclear Corp., Boston, Mass. L-[U-^{14}C]Alanine (20.2 mCi/mmole), L-[U-^{14}C]isoleucine (8.7 mCi/mmole), L-[*Me*-^{14}C]methionine (25 mCi/mmole), L-[U-^{14}C]phenylalanine (405 mCi/mmole), L-[U-^{14}C]serine (17 mCi/mmole), L-[U-^{14}C]threonine (127 mCi/mmole), DL-[*Bz*-U-^{14}C]tryptophan (19.5 mCi/mmole), and L-[U-^{14}C]valine (6.9 mCi/mmole) were obtained from the Radiochemical Centre, Amersham, England; L-[^{35}S]cystine (35.6 mCi/mmole) was purchased from Schwarz Bio-Research Inc., Orangeburg, N. Y.

Methods. Incorporation of radioactivity into the triostins was assayed as follows. Incubations were carried out at 28° in a Taiyo incubator K-II (110 strokes/min). A radioactive amino acid was added to cultures of suitable age; at specified intervals, 5 ml of the culture medium was transferred to a centrifuge tube. Extraction of the triostins from cells was accomplished by shaking intermittently with 5 ml of acetone. The mixture was centrifuged at 2500 rpm for 5 min; 4 ml of the supernatant was removed and evaporated to dryness under reduced pressure. The residue was redissolved in 3 ml of water which was then transferred to a fresh centrifuge tube containing ethyl acetate (5 ml). After extraction, the aqueous layer was removed by suction; the ethyl acetate fraction was washed successively with 1.5 ml of a 2% sodium bicarbonate

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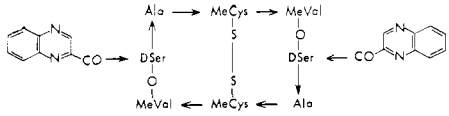

FIGURE 1: Structure of triostin A. In triostin C both *N*-methyl-L-valine residues are replaced by *N*-γ-dimethyl-L-alloisoleucine. Abbreviations: DSer, D-serine; Ala, L-alanine: MeVal, *N*-methyl-L-valine: MeCys, *N*-methyl-L-cysteine.

solution and 4 ml of water. An aliquot of the ethyl acetate fraction was used for determination of radioactivity.

The concentration of the triostins was determined spectrophotometrically. An aliquot of the ethyl acetate fraction was dried *in vacuo*, dissolved in a known volume of methanol, and the absorbancy of the antibiotic was measured at 243 mμ with a Beckman DB spectrophotometer. The extinction coefficient of triostin is $E_{243\,m\mu}$ 70,800.

Identification of the radioactive product as triostin was determined by paper chromatography using the solvent system: dibutyl ether–*sym*-tetrachloroethane–10% sodium *o*-cresotinate (2:1:3, v/v). A typical paper chromatogram is shown in Figure 2. The mobility of the radioactive compounds was identical with that of the triostins formed endogenously. Thin-layer chromatography also was employed to identify the radioactive principle as triostin. The solvent systems used were (1) the lower phase of an ethyl acetate–*sym*-tetrachloroethane–water (3:1:3, v/v) system with aluminum oxide GF_{254} (Merck) employing a circular technique (Shoji, 1967) and (2) methyl ethyl ketone with silica gel GF_{254} by the ascending method. Triostins A and C were visualized with an ultraviolet light (Mineralight, Ultra-Violet Products, Inc.). The individual regions were scraped off the plate and the antibiotics were eluted with chloroform–methanol (2:1, v/v). The solvents were removed by evaporation under vacuum; the residues were redissolved in methanol and the specific radioactivity of the antibiotics was determined. Triostins A and C represented more than 95% of the radioactivity originally found in the ethyl acetate extract. In some experiments, radioactive triostin A was recovered as described and recrystallized from chloroform–methanol mixtures following addition of carrier triostin A.

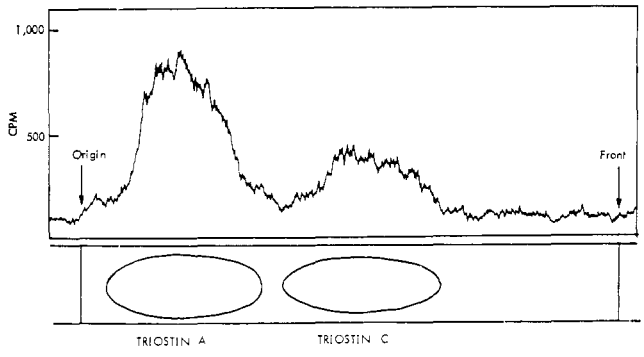

FIGURE 2: Identification of triostins isolated from *Streptomyces* s-2-210L incubated with L-[*Me*-^{14}C]methionine. Radioactive triostin was chromatographed with the solvent system; 10% *o*-cresotinate–dibutyl ether–tetrachloroethane (3:1:2, v/v) as described in Materials and Methods. Triostin A and C were located by a bioautographic technique using *Bacillus subtilis* (Yoshida and Katagiri, 1967).

Incorporation of [^{14}C]amino acids into cellular proteins and the intracellular pool was examined also. After incubation of the organism with a ^{14}C-labeled amino acid, 5 ml of the culture medium was filtered on a Millipore filter membrane (type HA) and the mycelium was washed with 10 ml of ice-cold deionized water. The mycelium was transferred to a centrifuge tube with 10 ml of 50% aqueous acetone. After standing for 1 hr at room temperature, the tubes were centrifuged, and the supernatant solution was removed; 5 ml of 5% trichloroacetic acid was added to the precipitate which was heated at 100° for 20 min. The precipitate was sedimented by centrifugation, washed twice with alcohol–ether (1:1, v/v), and finally with ether. After drying, the precipitate was digested in 0.5 N NaOH at 100° for 30 min. Aliquots were taken for determination of radioactivity and for assay of protein by the method of Lowry *et al.* (1951) using bovine serum albumin as standard. The supernatant solution (intracellular pool) was reduced in volume to remove acetone. After transfer to a centrifuge tube, the supernatant solution was extracted once with ethyl acetate to remove triostin. After removal of the ethyl acetate layer, the aqueous phase was acidified to pH 2 with 1 N HCl and reextracted with ethyl acetate once. The aqueous solution was desalted on a column (0.9 × 1.5 cm) of Dowex 50-X8 (H^+ form). After washing the column with water, amino acids were eluted with 0.5 N NH_4OH. The eluate was evaporated to dryness and the residue was taken up in 1 ml of water. Aliquots were counted to determine the incorporation of radioactivity into the intracellular pool. In some cases, an aliquot of the pool was examined by paper chromatography.

Triostin was hydrolyzed in 6 N HCl at 105° for 20 hr (Otsuka and Shoji, 1967). Hydrolysates were diluted with water and extracted with ether. The aqueous phase (amino acids) was concentrated to dryness *in vacuo;* the residue, taken up in water, was examined by paper chromatography. The ether extract (quinoxaline-2-carboxylic acid) was evaporated to dryness. The residue was dissolved in methanol and the quinoxaline moiety was separated by thin-layer chromatography with silica gel (Merck).

The R_F values of the amino acids (derived from triostin A) in the solvent system: 1-butanol–acetic acid–water (4:1:2, v/v) on Toyo filter paper no. 50 were: *N*,*N*′-dimethylcystine, 0.18; D-serine, 0.26; alanine, 0.36; and *N*-methylvaline, 0.60. When a mixture of triostin A and C was hydrolyzed, *N*-γ-dimethylalloisoleucine (R_F 0.74) was separated in addition to the above amino acids.

Identification of the radioactive amino acids in hydrolysates also was determined by paper chromatography using phenol saturated with water and benzyl alcohol saturated with water.

The individual amino acids were eluted from the paper with 0.05 N HCl. Quantitative assays for serine and alanine were carried out by the chromatographic procedure of Naftalin (1948).

Radioactive measurements were made in a Nuclear-Chicago liquid scintillation spectrometer Model 725 with a naphthalenedioxane solution (Bray, 1960). The location of radioactive components on chromatograms was determined by scanning with an Aloka paper chromatogeam scanner Model PCS-4.

TABLE I: Incorporation of ^{14}C-Labeled Amino Acids into Triostin and Protein by *Streptomyces* s-2-210L.[a]

Amino Acid	Triostin dpm/ml	Triostin % Incorpd	Protein[b] (dpm/mg)
DL-[*Bz*-U-^{14}C]Tryptophan	4,560	12.0	22,030
L-[*Me*-^{14}C]Methionine	8,950	40.5	8,190
L-[U-^{14}C]Serine	1,975	8.8	22,400
L-[U-^{14}C]Valine	1,440	6.4	32,700
L-[U-^{14}C]Alanine	1,090	5.0	7,820
L-[U-^{14}C]Cystine	2,105	9.6	4,580
L-[U-^{14}C]Isoleucine	605	2.9	19,780
L-[U-^{14}C]Threonine	585	2.6	35,640
[1-^{14}C]Glycine	855	3.5	29,970
L-[U-^{14}C]Phenylalanine	90	0.5	23,200

[a] ^{14}C-Labeled amino acids (5 μCi/μmole) were added at 2 mμmoles per ml of culture a 5 days, except for DL-[^{14}C]-tryptophan which was supplied at 4 mμmoles/ml. Incubation was for 2 hr. [b] The protein concentration was 0.44 mg/ml.

Results

Triostin Synthesis during the Growth of Streptomyces s-2-210L. When *Streptomyces* s-2-210L was grown in a sodium nitrate–maltose medium, it was observed that triostin synthesis lagged somewhat behind the growth of the organism (Figure 3). Antibiotic activity was first demonstrable after 2-days incubation and increased rapidly during the late stages of growth. Although maximal yields of triostin varied in different cultures (40–70 μg/ml), it was always detected initially at the same time. The organism produced triostins A and C simultaneously; under the conditions employed the relative percentage of triostin A in the antibiotic mixture was found to be 70–80% after 5-days incubation.

The extent of incorporation of [^{14}C]serine into triostin and cellular protein was examined with cultures of various ages. As shown in Figure 4, triostin biosynthetic activity was very low until 2 days; thereafter, it increased rapidly reaching a peak at 5 days, after which it declined. By contrast, protein synthesis (as measured by incorporation of [^{14}C]serine) was maximal in young cultures and decreased continuously throughout the incubation period. On the basis of these results, cultures grown for 5 days were used routinely for short-term experiments.

Incorporation of Various Amino Acids into Triostin and Protein. The extent of incorporation of various amino acids into triostin during a 2-hr incubation is presented in Table I. Of the amino acids tested, L-[^{14}C]methionine was the most efficiently (40.6%) utilized for triostin synthesis; [^{14}C]tryptophan also was incorporated into triostin extensively (21%). The amino acids, serine, alanine, valine, and cystine which are present in the peptide portion of triostin A, were incorporated to a lesser extent (5–10%). Isoleucine, threonine, glycine, and phenylalanine, which do not normally occur in triostin, were not employed to any significant extent for antibiotic synthesis although these amino acids were incorporated effectively into cellular proteins. Different concentrations of endogenously formed amino acids in the intracellular pool make it difficult to determine the net efficiency of utilization of these amino acids.

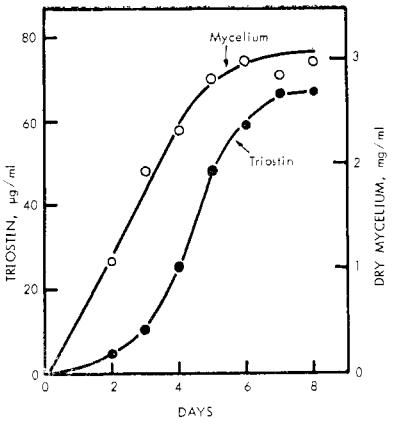

FIGURE 3: Relationship of triostin synthesis to mycelial growth of *Streptomyces* s-2-210L. The organism was grown in a sodium nitrate–maltose medium as described in Materials and Methods.

The time course of incorporation of ^{14}C-labeled serine, methionine, and tryptophan into triostin and protein is shown in Figure 5. Amino acid incorporation into triostin was linear for a 20–30-min period without any discernible lag. Incorporation of L-[*Me*-^{14}C]methionine into protein was less than that of L-[U-^{14}C]serine; by contrast; methionine was more efficiently utilized for triostin synthesis.

The distribution of radioactivity in triostins A and C was determined by a paper chromatographic technique. It was determined that the ratio of ^{14}C label in triostin A as compared with triostin C was virtually identical (86:14) when tryptophan, methionine, or serine was employed as precursor. These results compare favorably with the data obtained measuring the relative amounts of endogenously formed triostin A and triostin C (78:22). When [^{14}C]valine was used,

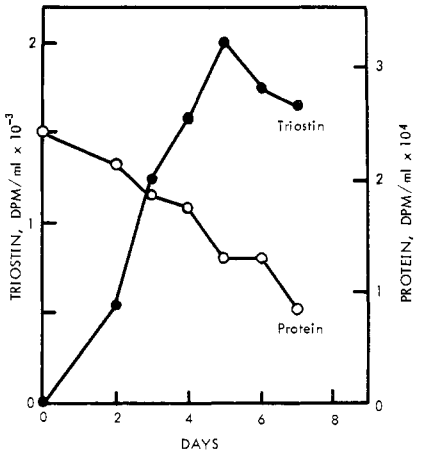

FIGURE 4: Incorporation of L-[U-^{14}C]serine into triostin and cellular protein during growth of *Streptomyces* s-2-210L. At different intervals an aliquot of the culture was harvested and incubated with L-[U-^{14}C]serine: (5μCi/μmole, 2 mμmoles/ml) for 1 hr. Experimental procedures employed are described under Materials and Methods.

TABLE II: Effect of Chloramphenicol on Triostin and Protein Synthesis by *Streptomyces* s-2-210L.[a]

	^{14}C Incorporation into					
	Triostin			Protein		
^{14}C Substrate	−CM (dpm/ml)	+CM (dpm/ml)	% Stimulation	−CM (dpm/mg)	+CM (dpm/mg)	% Inhibn
Tryptophan	10,525	14,525	38	15,250	2,290	85
Serine	2,050	3,140	53	12,900	2,450	81
Alanine	1,490	2,040	37	7,590	1,975	74
Valine	1,530	2,570	68	24,550	4,420	82
Cystine	4,500	4,230	−6	3,610	830	77
Methionine	13,325	14,260	7	7,690	1,460	81

[a] Chloramphenicol (CM, 10 μg/ml) and ^{14}C substrates (5 μCi/μmole, 2 mμmoles/ml) were added simultaneously. Incubation was terminated at 1 hr.

the radiolabel was present almost exclusively in triostin A (>99%). Triostin A contains *N*-methylvaline whereas triostin C possesses instead *N*-γ-dimethyl-L-alloisoleucine.

Effect of Chloramphenicol on the Biosynthesis of Triostin and Protein Synthesized by Streptomyces s-2-210L. The preceding studies revealed that triostin synthesis follows active growth and protein synthesis by *Streptomyces* s-2-210L. On the basis of these observations a study of the relationship between the biosynthesis of protein and triostin was undertaken using chloramphenicol. Figure 6 shows that the incorporation of L-[^{14}C]serine into cellular proteins was markedly inhibited by chloramphenicol. By contrast, incorporation of ^{14}C label into triostin was not blocked; in fact, antibiotic formation was greatly enhanced. A stimulation of ^{14}C incorporation into triostin was also observed with tryptophan, valine, and alanine but not with methionine or cystine (Table II). The stimulatory effect of chloramphenicol does not appear to be a direct effect of the compound on triostin synthesis but rather a secondary effect following inhibition of protein synthesis. As seen in Figure 7 when protein synthesis was blocked there was a greater accumulation and a slower utilization of the ^{14}C-labeled intracellular pool. Therefore, the stimulation of [^{14}C]triostin synthesis is presumably due to the availability of the intracellular pool exclusively for antibiotic synthesis. This finding indicates that triostin biosynthesis proceeds by a mechanism different from that of protein synthesis although both processes compete for the same amino acid pool.

As shown in Figure 8, triostin biosynthesis continued at an accelerated pace for 3–4 hr after the inhibition of protein synthesis; thereafter, antibiotic synthesis gradually declined. Even after 10-hr exposure to chloramphenicol, the organism was synthesized triostin at 60% of the normal rate.

Precursors of the L-*Alanine and* D-*Serine Residues in Triostin.* Radioactive triostin was synthesized during an incubation with L-[^{14}C]alanine. The ^{14}C-labeled triostin A was isolated by thin-layer chromatography using silica gel and recrystal-

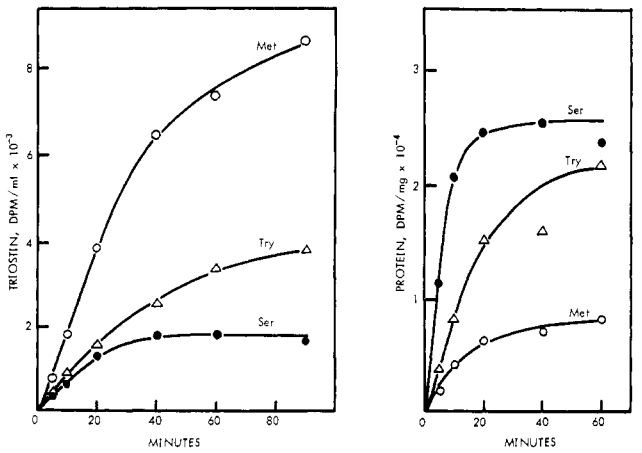

FIGURE 5: Time course of incorporation of L-[*Me*-^{14}C]methionine (○), DL-[*Bz*-U-^{14}C]tryptophan (△), and L-[U-^{14}C]serine (●) into triostin and cellular protein by *Streptomyces* s-2-210L. The experimental conditions are the same as those given in the legend of Table I.

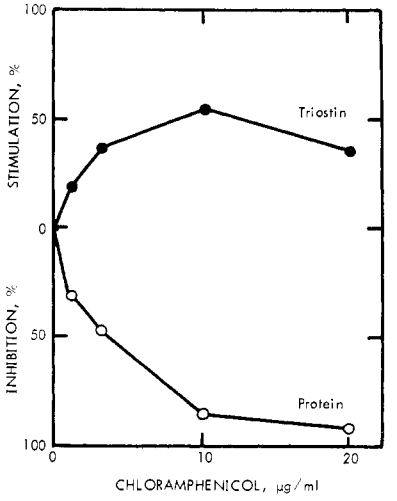

FIGURE 6: Triostin and cellular protein synthesis by *Streptomyces* s-2-210L in the presence of different concentrations of chloramphenicol. Chloramphenicol was supplied simultaneously with L-[U-^{14}C]serine (5 μCi/μmole, 2 mμmoles/ml), which was employed as a substrate. Incubation was terminated at 1 hr. Triostin (●) and protein (○).

TABLE III: Distribution of ^{14}C Label in Triostin A Synthesized with [^{14}C]Amino Acids as Precursors.

Amino Acid	Sp Act. of Substrate (mCi/mmole)	Amt Added (dpm)	^{14}C Incorpd[a] in Triostins (dpm)	%	Sp Act. (dpm/μmole) Triostin A	L-Alanine	D-Serine	Quinoxaline-2-carboxylic Acid
L-[U-^{14}C]Alanine	17.0	2.21×10^7	3.57×10^5	1.8	9.24×10^3	4.48×10^3	*b*	0
L-[U-^{14}C]Serine	20.2	2.26×10^7	3.94×10^5	1.6	1.03×10^4	*b*	5.16×10^3	0
DL-[*Bz*-U-^{14}C]Tryptophan	19.8	1.23×10^7	2.60×10^6	21.2	9.25×10^4	*b*		4.45×10^4

[a] Incubation time for 100 min. [b] Within the error (<50).

lized from chloroform–methanol mixtures as described in Materials and Methods. Radioautograms revealed that there was a single radioactive compound in triostin hydrolysates. This substance possessed the same R_F value as authentic L-alanine in a number of paper chromatographic systems. The specific radioactivity of the L-alanine was one-half the value obtained for triostin A (Table III) as would be expected since there are 2 moles of L-alanine/mole of triostin. When L-[U-^{14}C]serine was employed there was one or two additional radioactive compounds besides serine present on paper chromatograms of hydrolysates. The unknown compounds might represent degradation products arising from serine during acid hydrolysis (Ress, 1946). Serine, however, represents >60% of the radioactivity in the hydrolysates. Moreover, a comparison of the specific radioactivity of triostin A with serine revealed a 2:1 ratio clearly indicating that ^{14}C-labeled L-serine was incorporated exclusively into the D-serine residues of triostin.

Derivation of N-Methyl-L-valine Residues in Triostin A. When L-[U-^{14}C]valine was supplied as a radioactive precursor, radioautographic studies revealed that only *N*-methylvaline in triostin hydrolysates was labeled (Figure 9, top). This observation correlates well with the fact that [^{14}C]valine is selectively incorporated into triostin A. These results provide evidence that the carbon chain of *N*-methyl-L-valine is derived from L-valine during triostin biosynthesis.

Derivation of N,N'-Dimethyl-L-cystine Residue in Triostin. When ^{35}S- or ^{14}C-labeled L-cystine was employed as pre-

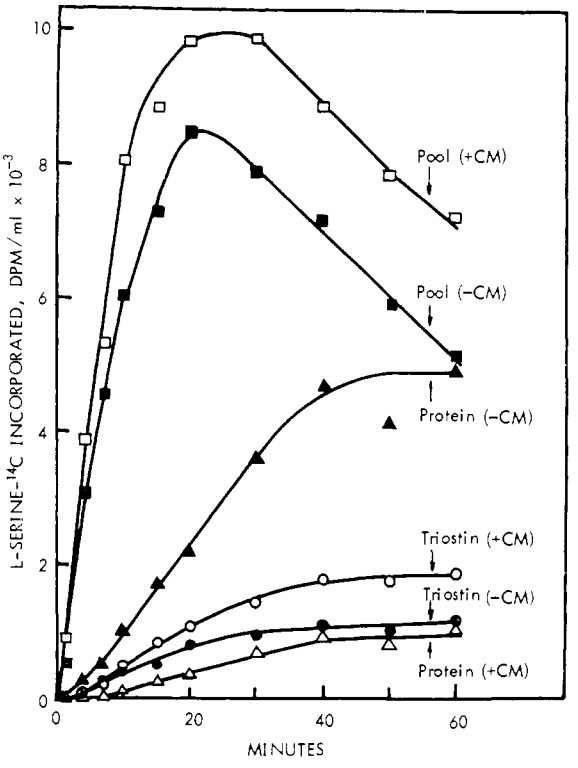

FIGURE 7: The effect of chloramphenicol on the incorporation of L-[U-^{14}C]serine into triostin, intracellular pool, and protein by *Streptomyces* s-2-210L. Aliquots of a 5-day culture were added to flasks containing L-[U-^{14}C]serine (5 μCi/μmole, 2 mμmoles/ml) with or without chloramphenicol (CM, 10 μg/ml, final concentration). Samples were removed at intervals for determination of radioactivity in triostin (●, ○), intracellular pool (■, □), and protein (▲, △) as described in Materials and Methods. Closed and open symbols refer to absence and presence of chloramphenicol, respectively.

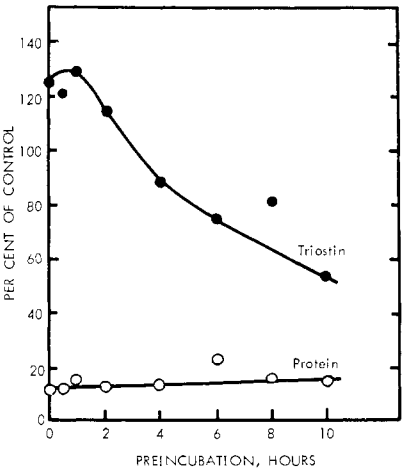

FIGURE 8: Synthesis of triostin by *Streptomyces* s-2-210L during prolonged incubation with chloramphenicol. L-[U-^{14}C]Serine was supplied to aliquots of the culture, which had been preincubated with chloramphenicol (10 μg/ml, final concentration) for 0, 0.5, 1, 2, 4, 6, 8, and 10 hr. Incubation with [^{14}C]serine was for 1 hr. Results were expressed as per cent of control. Triostin (●); protein (○).

TABLE IV: Identification of ^{14}C Compounds in Triostins Synthesized with L-[*Me*-^{14}C]Methionine as Precursor.[a]

Radioactive Component	R_F	Identification	Recov dpm	Recov %	%
I	0.18	*N,N'*-Dimethylcystine	10,870	36.3	
II	0.24	Not done	930	3.1	48.9
III	0.33	Methylamine	2,860	9.5	
IV	0.60	*N*-Methylvaline	12,490	41.7	
V	0.74	*N*-γ-Dimethylalloisoleucine	2,700	9.0	50.7

[a] Radioactivity of the hydrolysate as much as 29,930 dpm was subjected on paper and developed by a solvent system; butanol–acetic acid–water (4:1:2, *v/v*).

cursor, the incorporation of radioactivity into triostin was extensive (Table I). As determined by radioautography, virtually all of the radioactivity incorporated into triostin (^{14}C or ^{35}S) was present in *N,N'*-dimethylcystine (Figure 9, bottom).

Source of N-Methyl Group of N-Methylamino Acids in Triostin. No radiolabel was found in the quinoxaline-2-carboxylic acid moiety of triostin following an incubation with L-[*Me*-^{14}C]methionine. Paper chromatography did reveal that the radioactivity (*ca.* 99% of the ^{14}C label in hydrolysates) was distributed in five separate components (Table IV). Radioactive components I, IV, and V were identified as *N,N'*-dimethylcystine, *N*-methylvaline, and *N*-γ-dimethylalloisoleucine, respectively. Approximately half of the radioactivity (50.7%) was present in components IV and V. The ratio of radioactivity in *N*-methylvaline: *N*-γ-dimethylalloisoleucine was 82:18%, respectively; these values agree favorably with the ^{14}C ratio of triostin A to triostin C in the antibiotic mixture presented earlier.

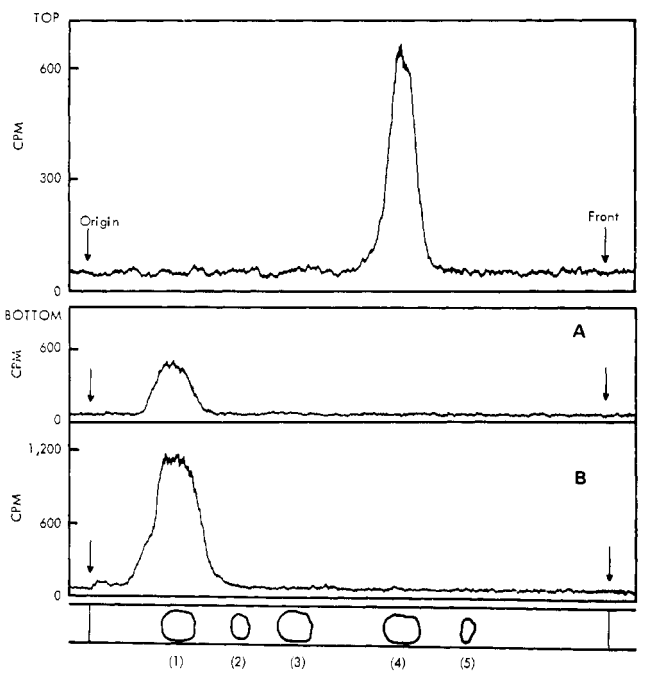

FIGURE 9: Paper chromatography of hydrolysates of radioactive triostins (triostins A and C) produced during incubations with L-[U-^{14}C]valine, L-[U-^{14}C]cystine, and L-[^{35}S]cystine, respectively. The hydrolysates were chromatographed, and paper strips were cut out and scanned as described in Materials and Methods. Amino acids in hydrolysates were visualized with ninhydrin. Radioactive substrates employed: top, L-[U-^{14}C]valine; bottom, L-[U-^{14}C]cystine (A) and L-[^{35}S]cystine (B). (1) *N,N'*-Dimethyl-L-cystine, (2) D-serine, (3) L-alanine, (4) *N*-methyl-L-valine, and (5) *N*,γ-dimethyl-L-alloisoleucine.

N,N'-Dimethylcystine (component I) contained 36% of the radioactivity in the hydrolysates. Presumably both *N*-methyl groups were derived from methionine. Components II and III represented 12.6% of the radioactivity. Component III has been identified as methylamine and was probably derived from *N,N'*-dimethylcystine during acid hydrolysis (M. Ebata, 1967, personal communication). Component II has not been identified as yet. It may also represent a degradation product derived from *N,N'*-dimethylcystine.

The separate experiment, in which radiolabeled triostin A was purified by removing triostin C from the antibiotic mixture and hydrolyzed, revealed that virtually half of the radioactivity (52.1%) was recovered from *N*-methylvaline (component IV). The rest of radiolabel (46.8%) was present in components I–III.

The preceding results indicate that valine and cystine are precursors of the carbon chain of *N*-methylvaline and *N,N'*-dimethylcystine, respectively, and that the methyl group of methionine, incorporated into triostins, exclusively provides 4 *N*-methyl equiv of the amino acids.

Precursor of Quinoxaline-2-carboxylic Acid in Triostin. An experiment was carried out to ascertain whether DL-[*Bz*-U-^{14}C]tryptophan was employed as precursor of the quinoxaline moiety of triostin. Only a single radioactive spot was observed on chromatograms of the ether extractable fraction of hydrolysates of ^{14}C-labeled triostin; the radioactive substance possessed the same R_F as quinoxaline-2-carboxylic acid in the system: 1-butanol saturated with 3% aqueous ammonia. The radioactive compound was purified further by thin-layer chromatography on silica gel. The specific radioactivities of triostin A and the quinoxaline-2-carboxylic acid derived from it are presented in Table III. It can be seen that the specific radioactivity of quinoxaline-2-Carboxylic acid is one-half that of triostin A as would be expected since there are 2 moles of the quinoxaline chromophore per mole of triostin. The data

indicate that the ring system of tryptophan is incorporated exclusively into the chromophoric moeity of triostin.

Discussion

A molecule of triostin consists of two residues of quinoxaline-2-carboxylic acid linked by amide bonds to a symmetric cyclodepsipeptide. Although the antibiotic contains D-serine, *N*-methyl-L-valine, *N,N'*-dimethylcystine as well as quinoxaline-2-carboxylic acid, radioisotopic experiments have revealed that triostin is derived from L-amino acids. These amino acids were incorporated efficiently into both triostin and cellular proteins by the growing organism; however, the efficiency of incorporation into triostin depended upon the age of the organism. Maximal incorporation into the antibiotic was attained at 5 days at which time growth of the organism had reached the late logarithmic phase of growth. Chloramphenicol was found to inhibit markedly (>80%) the incorporation of [^{14}C]amino acids into cellular proteins of *Streptomyces* s-2-210L. By contrast, triostin biosynthesis was not inhibited and, in fact, was enhanced in the presence of the antibiotic. Similar observations have been reported in the case of the actinomycins (Katz and Weissbach, 1963) and polymyxin (Daniels, 1968). These data provide further evidence that antibiotic peptide synthesis proceeds by a mechanism different from that of protein synthesis (Katz *et al.*, 1965; Yoshida *et al.*, 1966; Bhagavan *et al.*, 1966; Tomino *et al.*, 1967).

Normally, triostin and protein synthesis by the organism compete for the available amino acid pool. The stimulatory effect of chloramphenicol was probably due to the availability of the intracellular pool for antibiotic synthesis in the absence of protein synthesis. It was observed using ^{14}C-labeled serine as precursor that the intracellular pool (^{14}C) was larger and its utilization slower when chloramphenicol was present.

With the use of radiolabeled amino acids it was found that L-valine, L-alanine, L-serine, and L-cystine are biosynthetic precursors of the constituents in the peptide portion of triostin A. The biogenetic source of *N*-γ-dimethylalloisoleucine in triostin C was not fully elucidated. Although L-serine was rapidly utilized for the synthesis of the D-serine residues in triostin, it was not established whether the inversion of L-serine to its D enantiomorph occurs prior to or after incorporation of the L isomer into a peptide.

L-Methionine was demonstrated to be the source of the *N*-methyl groups in the *N*-methylvaline and *N,N'*-dimethylcystine residues of triostin A. The transfer of the methyl group from methionine to valine or cystine is probably mediated by *S*-adenosylmethionine. However, it is not clear whether the methylation reaction involves free valine and cystine or a peptide bound form of the amino acids. Kerridge (1966) suggested that a peptide form of lysine may be methylated to yield the ε-*N*-methyllysine found in flagella protein. Exogenously supplied ε-*N*-methyllysine did not influence the incorporation of either L-[*Me*-^{14}C]methionine or L-[U-^{14}C]lysine into the protein bound ε-*N*-methyllysine. On the other hand, direct methylation of amino acids may occur during actinomycin biosynthesis since it was shown that both [1-^{14}C]sarcosine and [*Me*-^{14}C]sarcosine were utilized to the same extent for synthesis of the antibiotic (Katz and Weissbach, 1963).

Previous studies had revealed that quinomycin formation was markedly stimulated when L-[^{12}C]tryptophan was supplied to *Streptomyces* sp. 732 (Yoshida and Katagiri, 1967). The participation of tryptophan as an efficient precursor of the quinoxaline moiety of triostin, however, was demonstrated for the first time in the present investigation by experiments using L-[*Bz*-U-^{14}C]tryptophan. Virtually all the radioactivity in the triostin synthesized was located in the quinoxaline-2-carboxylic acid moiety. Quinoxalines comprise a unique structure among natural products. Elucidation of the biosynthetic pathway of the quinoxaline moiety will be published shortly.

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