Origin of the Sarcosine Molecules of Actinomycins

By O. CIFERRI, A. ALBERTINI AND G. CASSANI

Department of Genetics, University of Pavia, Italy

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1. *Streptomyces* V-187 produces on minimal medium a mixture composed mainly of actinomycin C₁ (actinomycin D) and actinomycin A₁ (actinomycin I). If sarcosine is added to the medium, the micro-organism produces, in addition to actinomycins C₁ and A₁, actinomycin F₈ (actinomycin II) and actinomycin F₉ (actinomycin III), characterized by the substitution by sarcosine of one or both the proline molecules present in actinomycin C₁. 2. Exogenous sarcosine seems to be incorporated as such by *Streptomyces* V-187 only in the sarcosine molecule(s) that replace proline in the actinomycins of the F group, whereas, for the synthesis of the other sarcosine molecules, the amino acid is first demethylated to glycine. 3. The incorporation of sarcosine and glycine into actinomycin by *Streptomyces antibioticus* appears to follow a similar pattern, except that a portion of the methyl group produced in the degradation of sarcosine is utilized as a source of the methyl groups of the antibiotic. This explains the previously reported lack of cross-dilution between glycine and sarcosine observed in the incorporation of these amino acids into actinomycin.

Strains of *Streptomyces antibioticus* produce under suitable culture conditions one or more types of actinomycin. Chemically, the actinomycins are characterized by the presence of a chromophoric phenoxazinone residue and two pentapeptide chains (Fig. 1). All the actinomycins so far isolated contain the same chromophoric residue but differ in some of the amino acids that constitute the peptide portion of the antibiotic (Brockmann, 1960). In general, all the natural actinomycins have two molecules of sarcosine but, when some strains are cultured in chemically defined media in the presence of sarcosine, new types of actinomycins appear to be synthesized. These new actinomycins, designated actinomycins F₁ to F₄ (Schmidt-Kastner, 1956) and actinomycins II and III (actinomycins F₈ and F₉) (Katz & Goss, 1959), are characterized by the replacement of one or both of the molecules of proline by sarcosine.

It has been reported (Katz & Weissbach, 1963; Ciferri, Albertini & Cassani, 1964) that when sarcosine is supplied to growing cultures of *Streptomyces antibioticus* the amino acid is utilized as a source of the sarcosine moiety of the actinomycins. In addition, experiments with methyl-labelled sarcosine have indicated that sarcosine is incorporated without degradation. In the absence of sarcosine, glycine appears to be utilized as a precursor of the sarcosine of the antibiotic.

Such results, however, did not explain the fact

Fig. 1. Actinomycin C₁ (actinomycin D): L-threonine, D-valine (D-Val), L-proline (L-Pro), sarcosine (Sar), N-methyl-L-valine (Brockmann, 1960). In actinomycin A₁ 1 mol. of L-proline is replaced by 1 mol. of L-hydroxyproline. In actinomycin F₈ 2 mol. of L-proline is replaced by 2 mol. of sarcosine. In actinomycin F₉ 1 mol. of L-proline is replaced by 1 mol. of sarcosine. (*Denotes carbon atom having the L-configuration.)

that the specific activity of the actinomycin produced in the presence of labelled sarcosine was not decreased by the addition to the medium of a large excess of unlabelled glycine, nor was the incorporation of labelled glycine into actinomycin diminished by adding unlabelled sarcosine. Such discrepancies appear to be explained by the findings, reported in this paper, that exogenous sarcosine is incorporated as such only in the sarcosine that replaces the proline molecules in the actinomycins of the F group ('alternative' sarcosine). On the contrary, the sarcosine molecules present in the other actinomycins and the two analogous sarcosine residues of the actinomycins of the F group arise from glycine, even when the micro-organism is cultured in the presence of sarcosine.

EXPERIMENTAL

Materials. [^{14}C]Sarcosine (sp. activity 8mc/m-mole) was obtained from The Radiochemical Centre, Amersham, Bucks. L-[methyl- ^{14}C]Methionine (sp. activity 13.4mc/m-mole), [^{14}C]glycine (sp. activity 4.43mc/m-mole) and [^{14}C]sarcosine (sp. activity 0.65 and 2.04mc/m-mole) were obtained from New England Nuclear Corp., Boston, Mass., U.S.A.

Micro-organisms and media. In addition to *Streptomyces antibioticus* strain no. 1692 (Ciferri *et al.* 1964), an unidentified species, *Streptomyces* sp. V-187, was employed. *Streptomyces* V-187 was kindly supplied by Lepetit S.p.A., Milan, Italy. The latter culture had the advantage of producing actinomycin also on media containing ammonium sulphate as sole nitrogen source. In addition, *Streptomyces* V-187 produced substantial amounts of the actinomycins of the F group when cultured in the presence of amounts of sarcosine too small to stimulate the production of detectable amounts of these actinomycins by *S. antibioticus*.

Consequently, all fermentations carried out with *S. antibioticus* were in the galactose-glutamic acid medium (Katz & Goss, 1960) and Davis's minimal medium (Davis & Mingioli, 1950) was used for *Streptomyces* V-187. *S. antibioticus* grew well on Davis's medium but did not produce antibiotic. The possibility of growing *Streptomyces* V-187 in a medium devoid of any source of organic nitrogen was especially useful in the experiments designed to evaluate the cellular content of amino acids.

The maintenance of the cultures, the preparations of the inocula and all the cultural conditions were as reported by Ciferri *et al.* (1964).

Extraction and purification of the actinomycins. After centrifugation of the cells, the liquid medium was extracted with two 0.5 vol. of ethyl acetate. The organic solvent was washed twice with 5 vol. of water and then evaporated to dryness *in vacuo*. The residue was dissolved in acetone, the insoluble matter discarded and the acetone evaporated *in vacuo*. The actinomycins were then purified and separated by thin-layer chromatography. If the amount of antibiotic was sufficiently high (at least 10 mg.), a first separation was achieved by column chromatography (Roussos & Vining, 1956). A glass column (2 cm. $\times$ 25 cm.) was packed with silicic acid (Mallinckrodt) washed and suspended in benzene; the column was packed by applying to the top a slight posi-

tive pressure. The actinomycin mixture was dissolved in benzene and applied to the column. After adsorption, the column was washed with 200 ml. of benzene and the elution was begun first with 600 ml. of ethyl acetate followed by 250 ml. of the same solvent containing 2% (v/v) of methanol.

Elution of the actinomycins was followed by spectrophotometric determination of extinction at 440 m μ and, when feasible, by determination of the radioactivity. The procedure afforded a good separation of the actinomycins D, A₁ and the F group. The fractions containing each group of actinomycins were pooled and freed from the solvents by evaporation. Further separation of the individual components was obtained by thin-layer chromatography.

Separation and purification of actinomycins by thin-layer chromatography. Acetone solutions of the actinomycin

Fig. 2. Separation of actinomycins by thin-layer chromatography. (a) Separation of actinomycins A₁, C₁ and F group (F₈ + F₉). Approx. 100 μg . of the actinomycins extracted from the fermentations on minimal medium and minimal medium plus 50 μg . of sarcosine/ml. were applied to an alumina plate and developed in the dark for 1 hr. in the ethyl acetate-sym-tetrachloroethane-water system. Left to right: minimal medium, minimal medium + sarcosine, actinomycin A₁ (50 μg .), actinomycin C₁ (50 μg .), actinomycins F₈ + F₉ (40 μg .). (b) Separation of actinomycins F₈ and F₉. Approx. 50 μg . of the purified F mixture from thin-layer chromatography on alumina plates was applied to a plate of silica gel and developed in the dark for 30 min. in the ethyl acetate-methanol-water system. Left to right: F mixture, F₈ (25 μg .), F₉ (25 μg .).

mixtures were applied to 20 cm. $\times$ 20 cm. plates and developed for 2 hr. in the dark with ethyl acetate-*sym*-tetrachloroethane-water (3:1:3, by vol.) (Cassani, Albertini & Ciferri, 1964). Actinomycins C and A₁ separated quite well (Fig. 2a), but actinomycins F₈ and F₉ moved as a single band and were fractionated by chromatography on plates of silica gel (Merck) with ethyl acetate-methanol-water (20:1:20, by vol.). In this system, actinomycin F₈ had R_F 0.21, whereas actinomycin F₉ showed R_F 0.16 (Fig. 2b). The actinomycins were recovered as already reported (Cassani *et al.* 1964).

Identification of the actinomycins produced by Streptomyces V-187. The actinomycins were identified by quantitative determination of the amino acids of the peptide chains. A purified sample of each actinomycin (1.2–1.3 mg.) was hydrolysed in 10 ml. of 6N-HCl in a sealed tube at 110° for 30 hr. (Hanger, Howell & Johnson, 1958). The hydrolysate was evaporated to dryness *in vacuo* over P₂O₅ and NaOH and the residue dissolved in 2 ml. of 0.2N-sodium citrate-HCl buffer, pH 2.2. The melanin-like material produced during the hydrolysis was removed by centrifugation and the amino acids present in the supernatant were separated and determined quantitatively in a Beckman Spinco Amino Acid Analyzer 120-B. Control experiments with 1 mg. samples of actinomycin C₁ (actinomycin D) gave the following amounts (μ moles) of amino acids (figures in parentheses are percentages of the theoretical): sarcosine 1.64 (102%); valine 1.41 (71%); proline 1.59 (99%); threonine 1.54 (95%). *N*-Methyl-L-valine, present in all peptide chains, was disregarded since it gave very low results even in standard runs with a pure sample of the imino acid. The amino acid contents of the actinomycins produced by *Streptomyces V-187* in the minimal medium and in the presence of sarcosine (50 μ g./ml.) are given in Table 1.

It may be added that other actinomycins, present in lesser amounts, were evident when the micro-organism was grown in minimal medium and in minimal medium plus sarcosine. No effort has been made to identify such minor components. Since these minor components had different mobility on thin-layer chromatography (Fig. 2a) they were not recovered by elution and neglected.

Determination of the amino acid content of the cellular pools. Triplicate flasks, containing 100 ml. of minimal medium

alone or with 50 μ g. of sarcosine/ml. were removed at different time-intervals and the cells recovered by centrifuging. The cells were then washed twice with 5 vol. of water and suspended in 50 ml. of water. Samples of the suspensions were removed for the determination of the dry weight and to the remaining suspension was added trichloroacetic acid to a final concentration of 5% (w/v) (Ciferri *et al.* 1964). After extraction for 1 hr. at room temperature the suspension was filtered by suction and the mycelial cake extracted on the filter with 0.1 vol. of 5% trichloroacetic acid. Trichloroacetic acid was removed by repeated extraction with ethyl ether and the aqueous solution dried in a rotary evaporator. The residue was dissolved in 6 ml. of 0.2N-sodium citrate-HCl, pH 2.2. Samples (from 0.5 to 5 ml.) of these solutions were analysed for their amino acid content on a Beckman Spinco Amino acid Analyzer 120-B. Attempts to identify sarcosine in the cellular pools of the cells grown in the minimal medium were made on samples of the solutions ten times as great as those employed for the determination of the other amino acids.

Determination of the composition of the actinomycin mixtures produced by Streptomyces antibioticus and Streptomyces V-187. The actinomycins present in Davis's minimal medium containing different amounts of sarcosine (for the experiments of Table 2) or containing radioactive precursors (for the experiments reported in Tables 7 and 8) were purified as already described and separated into the main groups by two successive runs on thin-layer chromatography. The bands containing the actinomycin groups were eluted and the content of the antibiotic was determined spectrophotometrically. When feasible, the radioactive content was also determined.

Uptake of sarcosine by growing cells of Streptomyces V-187. The experiment was performed as reported by Ciferri *et al.* (1964) by measuring the disappearance of the amino acid from the liquid medium after removal of the cells by centrifugation and of the antibiotic produced by extraction with ethyl acetate.

Analytical methods. Actinomycin in the cultural liquids was assayed microbiologically (Goss & Katz, 1957); in the purified preparations it was determined spectrophotometrically (Katz & Weissbach, 1963) with crystalline preparations of actinomycin C₁ and actinomycin F₁ as standards.

Table 1. *Actinomycins produced by Streptomyces V-187 on minimal medium and minimal medium plus sarcosine*

Approximately 1 μ mole (1.2–1.3 mg.) of each actinomycin was hydrolysed and the amino acid content determined on an Amino Acid Analyzer (for details see the text). Values in parentheses give the percentage recovery as compared with theoretical values. Sar, sarcosine; a-Ile, alloisoleucine.

Medium	R_F on thin-layer chromatography		Amino acid content (moles/mole of antibiotic)							Identification
	On alumina	On silica gel	Thr	Sar	Pro	Hyp	Val	a-Ile		
Minimal	0.47	—	1.9 (95%)	1.8 (90%)	1.0 (100%)	0.9 (90%)	1.7 (70%)	0	Actinomycin A ₁ (I)	
Minimal and sarcosine (50μg./ml.)	0.40	—	1.9 (95%)	1.6 (80%)	2.0 (100%)	0	1.7 (70%)	0	Actinomycin C ₁ (D)	
		0.21	1.9 (95%)	4.0 (100%)	0	0	1.7 (70%)	0	Actinomycin F ₈ (II)	
Minimal and sarcosine (50 μg./ml.)	0.26	0.16	2.1(105%)	2.9 (97%)	1.0 (100%)	0	1.7 (70%)	0	Actinomycin F ₉ (III)	

Table 2. *Influence of the addition of sarcosine on the biosynthesis of actinomycin by Streptomyces V-187*

Sarcosine was added to the synthetic medium at the time of inoculation and the fermentations were carried on for 72 hr. The production of antibiotic was determined microbiologically and the percentage inhibition referred to the production of antibiotic in the control experiment. Each result is the average of duplicate experiments. The antibiotic was purified and the actinomycins were separated by thin-layer chromatography. The percentage composition of the actinomycin mixture was determined spectrophotometrically.

Concn. of sarcosine ($\mu\text{g./ml.}$)	Actinomycin synthesized ($\mu\text{g./ml.}$)	Percentage inhibition	Percentage composition of actinomycin components		
			A ₁	C ₁	F ₃ + F ₉
0 (control)	62.4	0	35	65	0
2.5	54.2	14	27	65	8
5	53.1	15	22	64	14
25	52.4	17	25	58	17
50	48.6	23	24	58	18
250	41.2	34	23	57	20

Isotope measurement. In addition to the methods reported by Ciferri *et al.* (1964) samples of low radioactivity were counted with a Tracerlab windowless flow counter. All counts were corrected for counting efficiency. Specific activity was expressed as counts/min./ $\mu\text{g.}$ of antibiotic.

RESULTS

Effect of the addition of sarcosine to the fermentation medium of Streptomyces V-187. As found with *S. antibioticus* (Ciferri *et al.* 1964), the addition of sarcosine to the fermentation medium of *Streptomyces V-187* results in a decrease in the total amount of actinomycin synthesized (Table 2). Thus at a concentration of sarcosine of 2.5 $\mu\text{g./ml.}$, the antibiotic production is reduced by 14%, whereas concentrations of 50 and 250 $\mu\text{g.}$ of amino acid/ml. further decrease the antibiotic yield by 23 and 34% respectively. Also, in *Streptomyces V-187*, the addition of sarcosine to the fermentation medium stimulates the production of actinomycins of the F group (Fig. 2). As compared with *S. antibioticus*, *Streptomyces V-187* had the advantage of producing fairly high amounts of the actinomycins of the F group even when the micro-organism was grown in the presence of low concentrations of sarcosine. Indeed, when sarcosine was added at a concentration of 2.5 $\mu\text{g./ml.}$, the production of the actinomycins of the F group was already about 4 $\mu\text{g./ml.}$ (Table 2).

The uptake by *Streptomyces V-187* of sarcosine added to the medium was measured in a fashion similar to that employed for *S. antibioticus* (Ciferri *et al.* 1964) (Table 3). The former micro-organism removed sarcosine from the medium at a somewhat lower rate than the latter; so that approx. 40% of the sarcosine present in the medium was taken up after 96 hr. of incubation, whereas the same

Table 3. *Uptake of sarcosine during growth of Streptomyces V-187*

[3-¹⁴C]Sarcosine (3 μC , 1.47 μmoles) together with 50 μmoles of [¹²C]sarcosine were added at the time of inoculation to triplicate flasks containing 100 ml. of medium each. Samples (3 ml.) were quickly removed at intervals, centrifuged to remove the mycelium and extracted with 1 vol. of ethyl acetate. The ethyl acetate was removed and washed with 1 vol. of water. The two aqueous portions were combined and samples plated for counting. Percentage of the labelled amino acid remaining was referred to the control samples taken at 0 hr. Each result is the average of three experiments.

Time after addition of the amino acid to the medium (hr.)	Sarcosine in the medium (%)
0	100
6	97
12	96
18	93
24	90
36	88
48	80
96	58

percentage was taken up by *S. antibioticus* after approximately 30 hr. incubation.

Incorporation of [1-¹⁴C]glycine into the actinomycins. Both *Streptomyces* incorporate labelled glycine into the actinomycins C, A₁ and F (Table 4, Expt. 1; Table 5, Expt. 1). The specific activity of the purified actinomycins is practically identical for both the actinomycins C and A₁ and the actinomycins F. Such results would indicate that the 'alternative' sarcosine, i.e. the sarcosine residues that are incorporated in place of proline, are not

Table 4. Incorporation of [1-¹⁴C]glycine, [1-¹⁴C]sarcosine and [3-¹⁴C]sarcosine into the actinomycins synthesized by *Streptomyces antibioticus* 1692

In Expt. 1, 10 μ C of [1-¹⁴C]glycine (2.25 μ moles) was added to 200 ml. of the synthetic medium alone or together with 25 μ g. of unlabelled sarcosine/ml. to stimulate the production of actinomycins of the F group. In Expt. 2, 10 μ C of [1-¹⁴C]sarcosine (1.23 μ moles) with 25 μ g. of unlabelled sarcosine/ml. and in one case with 100 μ g. of unlabelled glycine/ml. were added to 400 ml. of medium. In Expt. 3, to each 200 ml. of medium were added 5 and 10 μ C of [3-¹⁴C]sarcosine (7.5 and 15 μ moles) with 25 μ g. of unlabelled sarcosine/ml. at the time of inoculation and after 24 hr. respectively. In Expt. 4, 10 μ C of [3-¹⁴C]sarcosine (15 μ moles) was added to each 400 ml. of medium together with 25 μ g. of unlabelled sarcosine/ml. and, in one case, with 1000 μ g. of methionine/ml. All fermentations were carried out for 72 hr. from the time of inoculation and terminated by separation of the mycelium and extraction of the antibiotic from the fermentation liquid. The radioactive antibiotic was purified and determined spectrophotometrically to calculate the specific activity. The actinomycin groups were then separated by thin-layer chromatography.

Expt. no.	Addition to the synthetic medium	Partially purified actinomycin mixture						
		Amount of amino acid added		Antibiotic recovered (mg.)	10 ⁻³ $\times$ Radio-activity in antibiotic (counts/min.)	Sp. activity of antibiotic (counts/min./ μ g.)	Sp. activity of purified actinomycin groups	
		(μ C)	(μ moles)				C	F
1	[1- ¹⁴ C]Glycine	10	2.25	2.925	76.5	26.1	33.6	—
	[1- ¹⁴ C]Glycine and	10	2.25	2.613	83.2	31.8	33.6	24
	[¹² C]sarcosine	—	56					
2	[1- ¹⁴ C]Sarcosine and	10	1.23	3.420	123.8	36.2	4.96	49.6
	[¹² C]sarcosine	—	112					
	[1- ¹⁴ C]Sarcosine,	10	1.23	3.651	128.1	35.1	3.36	49.6
	[¹² C]sarcosine and	—	112					
3	[¹² C]glycine	—	533.2					
	[3- ¹⁴ C]Sarcosine and	5	7.5	2.832	10.5	3.7	0.84	28.5
	[¹² C]sarcosine (time of	—	56					
	addition: 0 hr. after							
4	inoculation)							
	[3- ¹⁴ C]Sarcosine and	10	15	1.990	29.0	14.5	1.6	204
	[¹² C]sarcosine (time of	—	56					
	addition: 24 hr. after							
5	inoculation)							
	[3- ¹⁴ C]Sarcosine and	10	15	4.382	20.3	4.63	0.67	27.2
	[¹² C]sarcosine	—	112					
	[3- ¹⁴ C]Sarcosine,	10	15	2.500	2.08	0.83	0.11	29.3
6	[¹² C]sarcosine and	—	112					
	[¹² C]methionine	—	2680					

derived from the labelled glycine but rather from the unlabelled sarcosine present in the medium. If such had not been the case, the specific activity of the actinomycins F would have been higher than that of the actinomycins C and A₁, whereas in our experiments the specific activity was lower (24 versus 33.6 for *S. antibioticus* and 46.4 versus 56 for *Streptomyces* V-187).

Incorporation of [1-¹⁴C]sarcosine into actinomycins. In these experiments (Table 4, Expt. 2; Table 5, Expt. 2) the specific activity of the actinomycins F was considerably higher than that of the actinomycins C and A₁ (about 10-fold with *Streptomyces antibioticus* and 20–30-fold with *Streptomyces* V-187). Such results are to be expected if sarcosine is incorporated as such in the 'alternative' sarcosine of the

actinomycins F. The radioactivity present in the actinomycins C and A₁ is probably the result of the incorporation of carboxyl-labelled glycine produced by demethylation of sarcosine (Ciferri *et al.* 1964).

Incorporation of [3-¹⁴C]sarcosine and [methyl-¹⁴C]methionine into actinomycins. The addition of methyl-labelled sarcosine to the growth medium results in an incorporation of the label in both types of actinomycins in *S. antibioticus* (Table 5, Expt. 3) and only in the actinomycins F in *Streptomyces* V-187 (Table 5, Expt. 3). In *S. antibioticus*, the label incorporated into the actinomycins C is probably the result of demethylation of sarcosine and the incorporation of the methyl group into the 'regular' sarcosine, the *N*-methylvaline and the chromophore as reported for the methyl groups of

Table 5. *Incorporation of [1-¹⁴C]glycine, [1-¹⁴C]sarcosine, [3-¹⁴C]sarcosine and [methyl-¹⁴C]methionine into the actinomycins synthesized by Streptomyces V-187*

In Expt. 1, 6 μ C of [1-¹⁴C]glycine (1.35 μ moles) was added to 200 ml. of the synthetic medium alone or together with 5 μ g. of unlabelled sarcosine/ml. In Expt. 2, 6 μ C of [1-¹⁴C]sarcosine (0.75 μ mole) with 5 μ g. of unlabelled sarcosine/ml. and, in the second part of the experiment, with 100 μ g. of unlabelled glycine/ml. were supplied to 200 ml. of the medium. In Expt. 3, 6 μ C of [3-¹⁴C]sarcosine (2.94 μ moles) with 5 μ g. of unlabelled sarcosine/ml. was added to each 200 ml. of medium at the time of inoculation, after 24 hr. and after 36 hr. In Expt. 4, 6 μ C of [3-¹⁴C]sarcosine (2.94 μ moles) was added to 200 ml. of culture together with 5 μ g. of unlabelled sarcosine/ml. and 50 or 500 μ g. of methionine/ml. respectively. In Expt. 5, 6 μ C of [methyl-¹⁴C]methionine (0.45 μ moles) alone or together with 5 μ g. and 100 μ g. of sarcosine/ml. was added to 200 ml. of synthetic medium. All fermentations were carried for 72 hr. from the time of inoculation and the antibiotic was purified and determined as reported in Table 4.

Expt. no.	Addition to the synthetic medium	Partially purified actinomycin mixture						
		Amount of amino acids added		Antibiotic recovered (mg.)	10 ⁻³ $\times$ Radio-activity in antibiotic (counts/min.)	Sp. activity of antibiotic (counts/min./ μ g.)	Sp. activity of actinomycin groups	
		(μ C)	(μ moles)				C ₁ + A ₁	F
1	[1- ¹⁴ C]Glycine	6	1.35	11.7	683	58.4	57.6	—
	[1- ¹⁴ C]Glycine and	6	1.35	11.25	666	59.2	56	46.4
	[¹² C]sarcosine	—	11.22					
2	[1- ¹⁴ C]Sarcosine and	6	0.75	12	752	62.7	29.6	712
	[¹² C]sarcosine	—	11.22					
	[1- ¹⁴ C]Sarcosine,	6	0.75	11.85	720	60.8	28.6	691
	[¹² C]sarcosine and	—	11.22					
3	[¹² C]glycine	—	266.6					
	[3- ¹⁴ C]Sarcosine and	6	2.94	11.7	332	28.4	0	396
	[¹² C]sarcosine (time of	—	11.22					
	addition: 0 hr. after							
4	inoculation)							
	[3- ¹⁴ C]Sarcosine and	6	2.94	12.16	289	23.8	0	324
	[¹² C]sarcosine (time of	—	11.22					
	addition: 24 hr. after							
5	inoculation)							
	[3- ¹⁴ C]Sarcosine,	6	2.94	12.22	291	23.8	0	377
	[¹² C]sarcosine and	—	11.22					
	[¹² C]methionine	—	67.0					
	[3- ¹⁴ C]Sarcosine,	6	2.94	12.81	291	22.7	0	352
	[¹² C]sarcosine and	—	11.22					
5	[¹² C]methionine	—	670					
	[methyl- ¹⁴ C]Methionine	6	0.45	12.24	2166	177	268	—
	[methyl- ¹⁴ C]Methionine	6	0.45	11.62	2091	180	242	289
	and [¹² C]sarcosine	—	11.22					
5	[methyl- ¹⁴ C]Methionine	6	0.45	8.04	1455	181	254	289
	and [¹² C]sarcosine	—	224					

methionine (Birch, Cameron, Holloway & Rickards, 1960; Sivak, Meloni, Nobili & Katz, 1962; Ciferri *et al.* 1964). Indeed the addition of an excess of unlabelled methionine (Table 4, Expt. 4) reduces the specific activity of the actinomycin C to one-sixth of that obtained in the presence of sarcosine labelled only, whereas it does not affect the specific activity of the actinomycins F.

The results obtained with *Streptomyces* V-187 are clearer (Table 5, Expts. 3 and 4), since no radio-activity is detectable in the actinomycins C and A₁. As in *S. antibioticus*, no significant decrease in the

specific activity of the actinomycin F was produced by adding unlabelled methionine together with labelled sarcosine. Further, in *Streptomyces* V-187, the addition of unlabelled sarcosine to fermentations containing methyl-labelled methionine (Table 5, Expt. 5) did not affect the specific activity of the actinomycins.

Composition of the amino acid pool of Streptomyces V-187. The amounts of the amino acids which are direct precursors of the peptide chains of actinomycin were determined in cells of *Streptomyces* V-187 grown in minimal medium and in minimal

Table 6. *Free amino acids related to actinomycin present in cells of Streptomyces V-187*

Cells from triplicate flasks containing 100 ml. of synthetic medium alone or with the addition of 50 μ g. of sarcosine/ml. were recovered by centrifugation, washed once with water and extracted with 5% (w/v) of trichloroacetic acid. The acid was removed by ethyl ether extractions and the samples of the aqueous solution were analysed for their amino acid content (for details see text). N.D., Not detected; < 0.1 means that the amino acid was detected on the chromatograms but the amount was too low to permit an accurate quantitative determination.

Medium	...	...	Amino acid (μ moles/100 ml. of culture)													
			Minimal							Minimal+sarcosine						
Time (hr.)	...	...	6	18	24	36	48	72	96	6	18	24	36	48	72	96
Sarcosine			N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	0.14	1.03	1.29	0.80	0.89	1.33	1.44
Threonine			<0.1	0.15	0.19	0.32	0.31	0.38	0.39	0.12	0.46	0.57	0.65	0.57	0.70	0.75
Proline			<0.1	<0.1	0.18	0.17	0.81	0.20	0.26	<0.1	0.26	0.40	0.42	0.31	0.47	0.53
Hydroxyproline			N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Glycine			<0.1	0.19	0.33	0.49	0.46	0.49	0.46	0.14	0.54	0.79	0.86	0.78	1.04	0.94
Valine			<0.1	0.27	0.40	0.53	0.49	0.35	0.45	0.24	0.68	1.01	0.95	0.99	1.01	1.20
Isoleucine			<0.1	0.12	0.16	0.18	0.10	0.09	0.10	0.11	0.40	0.53	0.46	0.44	0.45	0.58
Methionine			<0.1	0.13	0.15	0.35	0.49	0.24	0.10	—	0.11	0.19	0.25	0.42	0.30	—
Dry wt. of culture (mg.)			31	63	61	73	107	108	127	16	50	52	100	115	130	138

medium plus sarcosine (Table 6). It is evident that sarcosine appears to be present in substantial amounts in the cells only when the micro-organism is grown in the presence of the amino acid, that is under conditions which allow the production of the actinomycins of the F group. In the cells grown in minimal medium, sarcosine was not detected, thus substantiating the hypothesis that the sarcosine molecules of actinomycins C and A₁ arise from glycine. In general, the addition of sarcosine to the medium results in an increase in the concentration of the amino acids in the cells. Such an increase is probably the result of the degradation of a portion of the sarcosine taken up from the medium. Indeed it has been reported (Ciferri *et al.* 1964) that sarcosine is incorporated into protein after demethylation.

Hydroxyproline, which occurs in actinomycin A₁, was not detected in the amino acid pool of the cells grown in minimal medium and in minimal medium plus sarcosine. Katz, Prockop & Udenfriend (1962) have reported that hydroxyproline added to the medium may be incorporated as such in the hydroxyproline of actinomycin A₁ (actinomycin I); however, no information has been given on the presence of this amino acid in the cells of *S. antibioticus*. It may be that either hydroxyproline is present in the cells in a concentration too low to be detected, but sufficient for the synthesis of actinomycin A₁, or it arises, as in mammalian systems (Stetten, 1949), by hydroxylation of peptide-bound proline. Such a mechanism would be comparable with the postulated origin of the 'regular' sarcosine, that is by methylation of a

peptide-bound glycine (Katz & Weissbach, 1963; Ciferri *et al.* 1964).

DISCUSSION

The results presented suggest that sarcosine supplied exogenously is incorporated as such only in the sarcosine molecules that replace proline in the peptide chains of the actinomycins of the F group (Tables 7 and 8). The other sarcosine of actinomycins F, as well as all the sarcosine of the actinomycins C and A₁, arises from glycine. When the micro-organisms are grown in the presence of sarcosine, a part of the amino acid is demethylated to glycine and used as a precursor of the sarcosine of the actinomycins C, A₁ and of the 'regular' sarcosine of actinomycins F. Indeed, when the actinomycins produced in the presence of methyl-labelled sarcosine were separated into the C, A₁ and F groups, most, if not all, of the label was recovered in the actinomycins of the F group, even if these actinomycins constitute only a small fraction (5–10%) of the total antibiotic produced (Tables 7 and 8). The reported incorporation of methyl-labelled sarcosine into the actinomycins C synthesized by *S. antibioticus* (Ciferri *et al.* 1964) is presumably the result of a failure to identify and separate the actinomycins of the F group, which were produced only in traces, and, in part, to the utilization by *S. antibioticus* of the methyl group of sarcosine as a source of the methyl groups for the chromophore and for *N*-methyl-L-valine. When, in the present investigation, the fermentation of *S. antibioticus* was repeated in the presence of

Table 7. *Composition and radioactivity of the actinomycin mixture synthesized by Streptomyces antibioticus 1692*

Samples of the actinomycins of the C and F groups from the experiments reported in Table 4 were purified by thin-layer chromatography on alumina plates. The actinomycins were recovered by elution, determined spectrophotometrically and counted.

Expt. no.	Addition	Percentage composition		Percentage of radioactivity	
		C group	F group	C group	F group
1	[1- ¹⁴ C]Glycine	100	0	100	0
	[1- ¹⁴ C]Glycine and [¹² C]sarcosine	94	6	96	4
2	[1- ¹⁴ C]Sarcosine and [¹² C]sarcosine	94	6	35	65
	[1- ¹⁴ C]Sarcosine, [¹² C]sarcosine and [¹² C]glycine	95	5	28	72
3	[3- ¹⁴ C]Sarcosine and [¹² C]sarcosine (time of addition: 0 hr.)	94	6	25	75
	[3- ¹⁴ C]Sarcosine and [¹² C]sarcosine (time of addition: 24 hr.)	95	5	15	85
4	[3- ¹⁴ C]Sarcosine and [¹² C]sarcosine	94	6	25	75
	[3- ¹⁴ C]Sarcosine, [¹² C]sarcosine and [¹² C]methionine	97	3	13	87

Table 8. *Composition and radioactivity of the actinomycin mixture synthesized by Streptomyces V-187*

Samples of the actinomycins of the C₁+A₁ and F groups from the experiments of Table 5 were purified by thin-layer chromatography on alumina plates. The actinomycins were recovered by elution, determined spectrophotometrically and counted.

Expt. no.	Addition	Percentage composition		Percentage of radioactivity	
		C ₁ +A ₁ group	F group	C ₁ +A ₁ group	F group
1	[1- ¹⁴ C]Glycine	100	0	100	0
	[1- ¹⁴ C]Glycine and [¹² C]sarcosine	92	8	94	6
2	[1- ¹⁴ C]Sarcosine and [¹² C]sarcosine	95	5	40	60
	[1- ¹⁴ C]Sarcosine, [¹² C]sarcosine and [¹² C]glycine	93	7	31	69
3	[3- ¹⁴ C]Sarcosine and [¹² C]sarcosine (time of addition: 0 hr.)	92	8	0	100
	[3- ¹⁴ C]Sarcosine and [¹² C]sarcosine (time of addition: 24 hr.)	91	9	0	100
4	[3- ¹⁴ C]Sarcosine, [¹² C]sarcosine and [¹² C]methionine (50 µg./ml.)	91	9	0	100
	[3- ¹⁴ C]Sarcosine, [¹² C]sarcosine and [¹² C]methionine (500 µg./ml.)	91	9	0	100
5	[methyl- ¹⁴ C]Methionine	100	0	100	0
	[methyl- ¹⁴ C]Methionine and [¹² C]sarcosine (5 µg./ml.)	93	7	91	9
	[methyl- ¹⁴ C]Methionine and [¹² C]sarcosine (100 µg./ml.)	89	11	88	12

enough unlabelled sarcosine to stimulate the production of detectable amounts of actinomycins F, or when we used a strain such as *Streptomyces V-187*, which produces fair amounts of actinomycins F even at low concentrations of sarcosine, most, if not all, of the methyl-labelled sarcosine was recovered in the actinomycins F.

Glycine is incorporated equally well into both types of actinomycins and the addition of a large excess of unlabelled sarcosine does not interfere with glycine incorporation (Tables 7 and 8). Such results indicate that, in the actinomycins F, glycine

is the precursor of the 'regular' sarcosine molecules even in the presence of an excess of sarcosine. Similarly, sarcosine, as such, is incorporated only in the 'alternative' sarcosine molecules, and its incorporation is not prevented by the addition of large amounts of glycine. The previously unexplained lack of cross-dilution between these two amino acids (Ciferri *et al.* 1964) occurs because the two amino acids are incorporated into different amino acid moieties of the peptide chains of the antibiotic. Of course, as reported by Ciferri *et al.* (1964), part of the sarcosine is demethylated to glycine and

used as a source of this amino acid. Indeed, with sarcosine labelled in the carboxylic acid, a portion of the radioactivity (up to 31%) is recovered in the sarcosine moiety of the actinomycins C and A₁. In these experiments, the specific activity of the actinomycins F is about tenfold that of the actinomycins C and A₁, as would be expected since the actinomycins F should be labelled in the 'regular' sarcosine, via glycine, and in the 'alternative' sarcosine directly by sarcosine.

The hypothesis that sarcosine is demethylated to glycine and incorporated in place of the sarcosine molecules of the actinomycins C and A₁ and of the 'regular' sarcosine molecules of actinomycins F is further substantiated by demonstrating that methionine, whose methyl group is incorporated into the methyl groups of sarcosine, *N*-methylvaline and the chromophore (Sivak *et al.* 1962; Ciferri *et al.* 1964), even when added in large excess to the fermentations of *Streptomyces* V-187, does not dilute the radioactivity of the methyl-labelled sarcosine which is incorporated in the actinomycins F. However, in *S. antibioticus* 1692, which appears to incorporate a portion (up to 25%) of the methyl group of sarcosine into the actinomycins C, addition to the growth medium of a large excess of unlabelled methionine reduces the specific activity of the actinomycins C to approximately one-sixth, whereas it does not affect the specific activity of the actinomycins F. Such results indicate that, for the synthesis of the actinomycins C by *S. antibioticus*, the methyl group of sarcosine may be utilized in part as a source of methyl groups.

The direct incorporation of sarcosine into the 'alternative' molecules of sarcosine of the actinomycins F indicates that this amino acid competes in the cells with proline rather than with glycine, thus inhibiting the biosynthesis of the actinomycins containing two molecules of proline. Such a hypothesis is tenable if one thinks of the structural analogies that may exist between sarcosine and the cyclic imino acids and of the findings that other imino compounds, such *N*-methylalanine (Bossi, Hütter, Keller-Schierlein, Neipp & Zähler, 1958), azetidine-2-carboxylic acid or pipercolic acid (Katz & Goss, 1959; Katz, 1960) may replace proline and/or hydroxyproline in the peptide chains of the actinomycins. Indirect support for this hypothesis is given by the findings that proline is present in comparatively small amounts in the cellular pools of *Streptomyces* V-187, whereas glycine is present in fairly large amount. When the micro-organism is grown in the presence of sarcosine, the amount of this amino acid present at any stage in the cellular

pool may compete effectively with proline and less so with glycine. In addition, the synthesis of the actinomycins F is inhibited by the presence in the growth medium of an excess of proline (Katz & Goss, 1959); in our experiments the addition of a fourfold excess of glycine did not affect the biosynthesis of the actinomycins F, stimulated by sarcosine.

The effect of sarcosine on the incorporation of proline into actinomycins may be explained by assuming that sarcosine is a substrate for the enzyme(s) that incorporate proline into the peptide chains of the antibiotic and not for the enzyme(s) that incorporate glycine. This hypothesis is tenable if it is assumed that, as found with *Streptomyces* V-187, sarcosine never occurs in substantial amounts as a free amino acid in the cellular pools of cells grown in the absence of sarcosine and originates at a later stage of the biosynthesis of the antibiotic, e.g. by methylation of a precursor peptide containing glycine. Indeed neither strain produces detectable amounts of the actinomycins of the F group when grown in media devoid of sarcosine.

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