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Controlled Biosynthesis of Actinomycin with Sarcosine

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The actinomycins represent a family of chromopeptide antibiotics which differ solely in the nature of the amino acids present in the peptides of the molecule (Fig. 1). It has been established that an actinomycin-producing organism generally synthesizes a mixture of these substances. For example, *Streptomyces antibioticus* forms a mixture consisting of actinomycins I-V; occasionally trace amounts of a sixth component are produced (Katz & Goss, 1958). *Streptomyces chrysomallus* produces actinomycins IV, VI and VII (Schmidt-Kastner, 1956b).

The quantitative and qualitative nature of the actinomycin mixture synthesized can be modified to a considerable extent; in particular, the nitrogen source supplied has been shown to have a profound influence on its composition. Actinomycin IV increased from 10 to 83 % of the mixture produced by *S. chrysomallus* when DL-valine was added to the medium (Schmidt-Kastner, 1956b); hydroxy-L-proline brought about an increase in synthesis of actinomycin I from 6-7 to 31 % of the actinomycins produced by *S. antibioticus* (Katz & Goss, 1958).

New actinomycins were formed by *S. chrysomallus* when DL-isoleucine or sarcosine was added to the medium (Schmidt-Kastner, 1956a). In our Laboratory it has been determined that *S. antibioticus* forms at least one new actinomycin with sarcosine and several new compounds when DL-pipecolic acid is used.

During an investigation of the role of amino acids on actinomycin synthesis by *S. antibioticus*, it was observed that sarcosine selectively stimulated the production of actinomycins II and III (Fig. 2). These components, normally synthesized in trace amounts, represented approximately 60 % of the actinomycin mixture formed under the nutritional conditions employed. By paper-chromatography techniques, it was established that actinomycin II contains threonine, valine, sarcosine, and N-methylvaline, whereas actinomycin III possesses, in addition, one-half the amount of proline found in actinomycin IV (Katz, Goss & Pugh, 1958). Recently Johnson & Mauger (1959) obtained quantitative data showing that 4 moles of sarcosine and no proline are present in actinomycin II, and

that 3 moles of sarcosine and only 1 mole of proline are in actinomycin III. They proposed for actinomycins II and III the structures indicated in the legend of Fig. 1.

Schmidt-Kastner (1956b) has suggested that exogenous sarcosine interfered with the incorporation of proline into certain actinomycin peptides. The results obtained in our studies provide evidence for the view that sarcosine competes with proline and replaces it in certain actinomycin peptides.

EXPERIMENTAL

Culture. *Streptomyces antibioticus*, strain 3720, was used throughout the investigation. The procedure for preparation of an inoculum has been described previously (Goss & Katz, 1957). An N-Z amine medium consisting of N-Z amine A (a pancreatic digest of casein prepared by Sheffield

Chemical Co., Norwich, N.Y., U.S.A.), 25 g., Bacto beef extract 10 g., tap water 1 l., pH 7.0, was employed for growth of the organism.

Production medium. A chemically defined medium containing L-glutamic acid 2.0 g., D-galactose 10.0 g., K_2HPO_4 1.0 g., $MgSO_4 \cdot 7H_2O$ 0.025 g., $CaCl_2 \cdot 2H_2O$ 0.025 g., $ZnSO_4 \cdot 7H_2O$ 0.025 g., $FeSO_4 \cdot 7H_2O$ 0.025 g., distilled water 1 l., pH 7.2–7.3, was used for actinomycin production. Amounts (100 ml.) of medium were distributed into 250 ml. Erlenmeyer flasks and sterilized at 15 lb./in.² pressure and 121° for 15 min. D-Galactose was autoclaved separately in a similar manner and added to the medium just before inoculation. Production of actinomycin was carried out in shaken cultures (240 rev./min.) at 28°. Aqueous solutions of sarcosine and other amino acids generally were added at the onset of actinomycin synthesis (1–3 days after inoculation), unless specified otherwise.

Determination of actinomycin potency. The antibiotic titre of culture filtrates was determined by a paper-disk method of bioassay (Goss & Katz, 1957).

Fig. 1. Actinomycin IV. Sequence of amino acids: L-threonine, D-valine (D-Val), L-proline (L-Pro), sarcosine (Sar), N-methyl-L-valine (Brockmann *et al.* 1956; Bullock & Johnson, 1957). Actinomycin I. 1 mole of proline is replaced by 1 mole of hydroxyproline (Brockmann & Pampus, 1955). Actinomycin II. 2 moles of proline are replaced by 2 moles of sarcosine (Johnson & Mauger, 1959). Actinomycin III. 1 mole of proline is replaced by 1 mole of sarcosine (Johnson & Mauger, 1959). Actinomycin V. 1 mole of proline is replaced by 1 mole of 4-oxoproline (Brockmann & Manegold, 1958). Actinomycin VI. 1 mole of D-valine is replaced by 1 mole of D-alloisoleucine (Brockmann *et al.* 1956). Actinomycin VII. 2 moles of D-valine are replaced by 2 moles of D-alloisoleucine (Brockmann *et al.* 1956).

Fig. 2. Circular paper chromatogram showing the actinomycin mixtures produced by *Streptomyces antibioticus*. Solvent system: 10% aqueous solution of sodium *o*-cresotinate-di-*n*-butyl ether-*s*-tetrachloroethane (4:3:1, by vol.). Sequence of actinomycins is from centre to periphery; in all cases, the first zone just beyond the origin constitutes biologically-inactive coloured material. A, Actinomycin mixture produced in the glutamic acid-galactose medium; actinomycins I, II (trace), III (trace), IV and V; B, actinomycin mixture produced in the glutamic acid-galactose medium plus sarcosine (250 µg./ml.); actinomycins I, II, III, IV, an unidentified component and V; C, actinomycin mixture produced in the glutamic acid-galactose medium plus L-valine (1000 µg./ml.); same as in A.

Extraction of actinomycin. After a given period of incubation, the actinomycin in 500 ml. of a fermentation broth from a replicate series of flasks was extracted three times with butan-1-ol [20, 10, and 5% (v/v) respectively]. The extracts were combined and the butan-1-ol was removed by distillation *in vacuo*. The crude actinomycin residue was recovered in a small volume of acetone and used directly for circular paper chromatography. If necessary, the material could be recovered by evaporation of the acetone and then stored in the dry state in the dark until used.

Extraction of actinomycin from the mycelium of *S. antibioticus* was accomplished in the following manner: the mycelium in a sample of broth was collected by suction filtration and washed with distilled water until the wash water was free of antibiotic. The mycelium was then suspended in a small volume of Sørensen's m/15-phosphate buffer, pH 7.0, placed in a Raytheon 50 w, 9 k-cyc. magnetostrictor oscillator (Raytheon Manufacturing Co., Waltham, Mass., U.S.A.) and disrupted by treatment for 15–20 min. The actinomycin in the cell-free material was then treated as previously described.

Chromatographic separation of actinomycin mixtures. Chromatographic separation of actinomycin mixtures was accomplished by a circular-paper technique (Roussos & Vining, 1956; Katz, Pienta & Sivak, 1958). The solvent system consisted of equal volumes of a 10% (w/v) aq. solution of sodium *o*-cresotinate or *m*-cresotinate and a mixture of di-*n*-butyl ether and *s*-tetrachloroethane (3:1, v/v). The solvent system containing *o*-cresotinate was used for most of the experiments.

Determination of the percentages of components in an actinomycin mixture. The determination of the percentages of components in an actinomycin mixture was carried out spectrophotometrically (Goss & Katz, 1957). The effects observed with sarcosine represent shifts in synthetic patterns. As a consequence, increased formation of actinomycins II and III is accompanied by decreased production of actinomycin IV. The total yield of actinomycin remains essentially the same, however.

Production of actinomycin by washed suspensions of *S. antibioticus*. The organism was first grown in N-Z amine medium and a mycelial suspension was prepared. A portion

of the washed mycelium was then inoculated into flasks of glutamic acid–galactose medium and allowed to grow for a 48 hr. period. The mycelium was harvested by centrifuging, washed twice in 0.09% NaCl soln. and finally suspended in water; the final amount of mycelium was 8 mg./ml. A 20 ml. amount of this suspension was inoculated into each of a duplicate series of 250 ml. Erlenmeyer flasks containing 20 ml. of m/15-phosphate buffer, 30 ml. of water and 10 ml. of an aqueous solution of sarcosine or L-glutamic acid (final concentration, 1 mM). In place of substrate, 10 ml. of water was added to the control flasks. The flasks were shaken at 240 rev./min. at 28°. The antibiotic titre of culture filtrates was determined daily by the disk-assay method. After 72 hr. incubation the actinomycin mixture was extracted from culture fluids and the percentage of components in the mixture was determined. No appreciable increase in cell weight occurred during the incubation period. Sterile conditions were maintained throughout the experiment.

RESULTS

Actinomycin synthesis in the presence and absence of sarcosine. The results of a study of actinomycin formation by *S. antibioticus* in the glutamic acid medium with and without sarcosine are presented in Figs. 3 and 4. Production of actinomycin mixtures began 24 hr. after inoculation of the organism and continued until the tenth day. Without sarcosine, actinomycin V was the major component produced initially, whereas actinomycin IV represented the chief constituent in the later stages of synthesis; actinomycins II and III were produced in trace amounts. With sarcosine present, actinomycins II and III constituted major components of the mixture within one day of its addition; moreover, the percentage of these substances in the mixture increased to some extent during the production period.

Fig. 3. Synthesis of an actinomycin mixture by *Streptomyces antibioticus* in glutamic acid medium. Five samples (500 ml.) of fermentation medium were taken daily, the actinomycin was extracted and the percentage of the components in a mixture determined. ○, Actinomycin I; △, actinomycin II; □, actinomycin III; ●, actinomycin IV; ▲, actinomycin V.

Fig. 4. Synthesis of an actinomycin mixture by *Streptomyces antibioticus* in glutamic acid medium plus sarcosine (250 µg./ml.). Sarcosine was added after 34 hr. incubation. Five samples (500 ml.) of fermentation medium were taken daily, the actinomycin was extracted and the percentage of the components in a mixture determined. ○, Actinomycin I; △, actinomycin II; □, actinomycin III; ●, actinomycin IV; ▲, actinomycin V.

Concentration of sarcosine. In Table 1 are presented data from a study in which different concentrations of sarcosine were added to the glutamic acid medium. A two- to three-fold increase in the amount of actinomycin III synthesized was observed when a concentration of 2.5 $\mu\text{g.}$ of sarcosine/ml. was employed. Optimum formation of actinomycin II and III occurred, however, when sarcosine was supplied at concentrations of 250–500 $\mu\text{g.}/\text{ml.}$

Nature of the actinomycin mixture produced in mycelium. Under the different nutritional conditions employed, it was of interest to determine whether the actinomycin mixture present in the mycelium of *S. antibioticus* was similar to or different from that found in the culture medium. Approximately 5–10% of the actinomycin synthesized was present in the mycelium; in addition, the actinomycin mixture was qualitatively and quantitatively similar to that found in the broth. Actinomycins II and III represented 35–40% of the mycelial actinomycin when the organism was grown in the presence of sarcosine; only trace quantities of these components could be found in cells grown in the absence of this amino acid.

Specificity of sarcosine. Compounds related to sarcosine biochemically or structurally were examined to ascertain whether they might influence synthesis of actinomycin in a similar manner. Choline, betaine, dimethylglycine, DL-phenylsarcosine, sarcosine anhydride, glycine, N-acetyl glycine, glycine anhydride, glycyglycine, L-serine and L-methionine at concentrations ranging from 0.001 to 0.20% were tested. The addition of glycine, choline, betaine and dimethylglycine, at concentrations of 0.1–0.2%, increased the synthesis of actinomycin III approximately three- to four-fold; however, certain unrelated substances, e.g. L-threonine and L-aspartic acid, at similar concentrations produced the same effect. In no case was there increased formation of actinomycin II.

Influence of time of addition of sarcosine. Initially sarcosine was added to growing cultures at the onset of actinomycin production. To determine its effect on actinomycin formation at different times during synthesis, sarcosine (250 $\mu\text{g.}/\text{ml.}$) was added to a series of cultures growing in the glutamic acid medium at 0, 1, 3, 5, 7 and 9 days after inoculation. At these periods, except for the initial set, the actinomycin mixture synthesized was extracted from a parallel series of cultures and the percentage of the components in a given mixture determined. After 10 days' incubation all experimental flasks were harvested and the nature of the actinomycin mixture in each set was determined. Thus the composition of the mixture produced before and after the addition of sarcosine was established.

Sarcosine was most effective when supplied during the early phases of actinomycin formation (Table 2). Optimum synthesis of actinomycins II and III occurred when sarcosine was added 24 hr. after inoculation of the organism—the point at which production of the antibiotic was just starting. When incorporated at the time of inoculation, it was almost as effective; subsequently the influence of sarcosine diminished as synthesis of the antibiotic decreased.

Single competing with multiple additions of sarcosine. Since the sarcosine (250 $\mu\text{g.}/\text{ml.}$) fed as a single addition disappeared from the medium within 2–3 days of its incorporation, it was likely that part of the exogenous supply was metabolized

Table 1. *Influence of sarcosine concentration on actinomycin biosynthesis*

Sarcosine was added to glutamic acid medium after 30 hr. incubation of *S. antibioticus*. After an additional 6 days' cultivation, the actinomycin mixture synthesized was harvested by extraction with butan-1-ol. After removal of the butanol the actinomycin mixture was separated by means of circular paper chromatography, and the percentage of actinomycin components in a mixture determined by a spectrophotometric method (Goss & Katz, 1957).

Concn. of sarcosine ($\mu\text{g.}/\text{ml.}$)	Percentage of actinomycin components				
	I	II	III	IV	V
0.0	6.2	2.1	2.8	80.4	8.4
2.5	6.5	1.9	7.3	70.8	13.6
10.0	5.3	9.2	11.9	63.5	10.1
50.0	6.5	21.2	30.8	35.9	5.6
100.0	8.1	22.5	33.1	30.8	5.5
250.0	8.9	25.4	35.3	24.6	5.7
500.0	9.1	25.6	35.0	24.4	5.9

Table 2. *Influence of time of addition of sarcosine on synthesis of actinomycins II and III*

Sarcosine (250 $\mu\text{g.}/\text{ml.}$) was added to glutamic acid medium at the times indicated. Actinomycin mixtures were harvested on the tenth day of cultivation; final antibiotic titre 101 $\mu\text{g.}$ of actinomycin/ml. The data represent the percentages of actinomycins II and III in the sarcosine-stimulated actinomycin mixture less the percentage of these actinomycins in the actinomycin mixture produced in the absence of sarcosine.

Age of culture at time of sarcosine addition (days)	Actinomycin titre at time of sarcosine addition ($\mu\text{g.}/\text{ml.}$)	Percentage of components after 10 days' incubation	
		Actinomycin II	Actinomycin III
0	0	23.7	25.9
1	1	26.4	32.6
3	23	16.3	27.1
5	73	11.7	18.8
7	88	6.9	13.1
9	92	0.0	4.1

by the organism. Consequently the maximum effect possible with this compound could not be attained. To compensate for the metabolic loss, attempts were made to increase the level of sarcosine in the medium. When given as a single addition, however, it was evident that levels beyond the optimum concentration noted previously were somewhat inhibitory to antibiotic synthesis, the final antibiotic titre being greatly decreased without any appreciable increase in the amount of actinomycin II and III formed. A given concentration of the amino acid was slightly but consistently more effective, however, when given in small portions over several days.

Production of actinomycin by washed suspensions of S. antibioticus. The study carried out with washed suspensions of *S. antibioticus* should be regarded as preliminary since many experimental details require further investigation. For example, the actinomycin titre in control flasks was approximately the same as that in the experimental groups. Similar results were obtained initially in studies on penicillin formation by washed suspensions of *Penicillium chrysogenum* (Demain, 1956). This effect was attributed to the high endogenous reserves in the mycelium which the organism used for production of the antibiotic. The differences observed in the nature of the actinomycin mixture synthesized in the presence and absence of sarcosine, however, appear to be significant.

In the absence of substrate, *S. antibioticus* produced approximately equal amounts of actinomycins IV and V; with glutamate, actinomycin IV was the chief constituent, representing 52% of the actinomycins formed. When sarcosine was employed, actinomycin III increased from 5.3 to 27.3% of the mixture synthesized. No change occurred in the amount of actinomycin II formed under these conditions.

Reversal of sarcosine effect by L-proline. Exogenous sarcosine may influence synthesis of actinomycin mixtures either directly, by its incorporation into certain actinomycin peptides, or indirectly, by modifying the metabolism and consequently the antibiotic-producing activities of the organism. The data obtained in this investigation suggest that exogenous sarcosine is directly incorporated into actinomycins II and III by competing with endogenous forms of proline for the 'proline' site in certain actinomycin peptides. Actinomycin II contains no proline, and actinomycin III only 1 mole of proline. Both possess additional sarcosine (Johnson & Mauger, 1959). If sarcosine does compete with proline and replaces it in certain peptides, it should be possible to reverse the effect of sarcosine by supplementing endogenous proline with an exogenous source. Conversely, it should be possible to abolish the activity

of the added proline by increasing the concentration of sarcosine in the medium.

The results of a series of experiments provide evidence in support of these conclusions (Table 3). Suitable concentrations of L-proline considerably diminished the effect of sarcosine. When an additional concentration of sarcosine was added to the medium, exogenous proline was no longer able to reverse the effect of sarcosine to any appreciable extent.

Addition of DL-pipecolic acid. Additional support for the hypothesis that sarcosine competes with proline was obtained by using an analogue of proline, DL-pipecolic acid. This compound might also compete because of its close structural relationship to proline. If this were so, new actinomycins containing pipecolic acid or its derivatives might be formed.

According to the concentrations of DL-pipecolic acid employed, 1-5 new actinomycins were formed by *S. antibioticus*. Fig. 5 shows the composition of the mixture produced with 100, 250, 500, 1000 and 2000 $\mu\text{g.}$ of DL-pipecolic acid/ml. in the medium. These new compounds represented approximately 48% of the actinomycin mixture synthesized. In addition, by paper chromatography of acid hydrolysates, pipecolic acid was shown to be present in certain of the new actinomycin components.

Because of the close similarity between proline and pipecolic acid, it appeared likely that the analogue would compete more favourably with endogenous proline than sarcosine for the 'proline' site. The results of an experiment confirmed this view (Table 4). When simultaneous additions of sarcosine and pipecolic acid were made, the effect of sarcosine decreased as the concentration of DL-pipecolic acid increased.

Table 3. *Percentage of components II and III in actinomycin mixtures synthesized in the presence of sarcosine and L-proline*

Sarcosine and proline were added to glutamic acid medium after 24 hr. incubation. After 6 more days' cultivation the actinomycin mixture synthesized was harvested and the percentage of components determined.

Concn. of sarcosine ($\mu\text{g./ml.}$)	Concn. of L-proline ($\mu\text{g./ml.}$)	Actinomycins II and III (%)	Actinomycins I, IV and V (%)
Expt. 1			
100	0	62.4	37.6
100	500	59.2	40.8
100	1000	44.6	55.4
100	2000	18.2	81.8
Expt. 2			
500	0	57.0	43.0
500	500	55.5	44.4
500	1000	51.2	49.8
500	2000	47.1	53.0

DISCUSSION

It appears likely that an exogenous amino acid (a) is incorporated directly into certain actinomycin peptides, or (b) is first partly modified by the organism before its incorporation, or (c) acts indirectly by altering the metabolic activities of the organism and subsequently influencing antibiotic synthesis as well. We shall first consider the mechanism of synthesis of an actinomycin mixture under the normal conditions of cultivation.

Johnson (1956) suggested that an actinomycin molecule is formed through the oxidative condensation of 2 molecules of 3-hydroxy-4-methylanthranilic acid peptide. The peptides involved may or may not be identical. If the anthranilic acid peptide is the final intermediate assembled before synthesis of a molecule of the antibiotic, some specific enzymic site must exist in the cell to synthesize this compound. The structural units of the antibiotic (amino acids, 3-hydroxy-4-methylanthranilic acid), possibly formed elsewhere in the cell, must be transported to this enzymic site. There, a process comparable with patternization may occur (Gale, 1958) so that the various molecules are arranged in proper sequence before the formation of bonds between adjacent amino acid residues and the anthranilic acid moiety. It appears reasonable to assume that such a specific synthetic site is involved and that some kind of sequential arrangement of the amino acid residues and the anthranilic acid moiety precedes synthesis of the 3-hydroxy-4-methylanthraniloyl peptide.

The amount of anthranilic acid peptide formed would depend, in part, on the presence of each of the amino acids and 3-hydroxy-4-methylanthranilic acid at this site at the appropriate time. A lack of one of the constituents would result in

little, if any, synthesis; a delay in appearance of one of the compounds would limit synthesis of the anthranilic acid peptide and ultimately, of course, of actinomycin.

Since the only difference in the actinomycin peptides produced by *S. antibioticus* occurs at the 'proline' position, it is likely, under conditions of synthesis, that competition exists between proline, hydroxyproline, 4-oxoproline and, to a slight extent, sarcosine for the 'proline' spot on the surface of this specific enzymic site. If there exists a greater affinity for proline than for any of

Fig. 5. Circular paper chromatogram showing the actinomycin mixtures produced by *Streptomyces antibioticus*. Solvent system: 10% aqueous solution of sodium o-cresotinate-di-n-butyl ether-s-tetrachloroethane (4:3:1, by vol.). Sequence of actinomycins is from centre to periphery; actinomycin I has not emerged from the first biologically-inactive coloured zone just beyond the origin. In all cases it appears that actinomycins I to V were formed. A, Glutamic acid-galactose medium; actinomycins I to V and a sixth unidentified component; B, glutamic acid-galactose medium plus DL-pipecolic acid, 100 µg./ml.; actinomycins I-V and three additional components; C, glutamic acid-galactose medium plus DL-pipecolic acid, 250 µg./ml.; actinomycins I-V and four additional components; D, glutamic acid-galactose medium plus DL-pipecolic acid, 500 µg./ml.; actinomycins I-V and five additional components; E, glutamic acid-galactose medium plus DL-pipecolic acid, 1000 µg./ml.; actinomycins I-V and four additional components; F, glutamic acid-galactose medium plus DL-pipecolic acid, 2000 µg./ml.; actinomycins I-V and four additional components.

Table 4. Influence of sarcosine and DL-pipecolic acid on the synthesis of actinomycins II and III

Sarcosine and DL-pipecolic acid were added to glutamic acid medium after 36 hr. incubation. On the seventh day of cultivation the actinomycin mixture synthesized was harvested and the percentage of components determined.

Concentration (µg./ml.) of		Percentage of components in actinomycin mixture	
DL-Pipecolic acid	Sarcosine	Actinomycins II and III	Pipecolic acid components
0	0	6.8	0
0	250	67.2	0
5	250	62.7	5.4
10	250	57.1	14.4
25	250	38.8	18.9
100	250	16.2	44.5
100	0	6.1	48.2

the other compounds mentioned, or if the concentration of proline at this site is greater than that of the other substances, we can expect it to be incorporated into the actinomycin peptides much more frequently than are the other amino acids. When condensation of two molecules of anthranilic acid peptide occurs, it should take place most frequently with peptides containing proline (actinomycin IV, 60–80%) (Goss & Katz, 1957). Condensation of a peptide containing proline with one containing hydroxyproline (actinomycin I, 5–8%), 4-oxoproline (actinomycin V, 5–15%) or sarcosine (actinomycin III, 3–4%) will occur much less frequently. Condensations of two peptides with hydroxyproline or 4-oxoproline or sarcosine (actinomycin II, 2–3%) probably take place also, but the frequency of this event would be of a very low order. Components produced in such trace amounts would be extremely difficult to detect with the chromatographic systems available at present.

If one modifies the nutritive environment, e.g. by changing the nitrogen source (Katz, Pienta & Sivak, 1958), adding a second one (Schmidt-Kastner, 1956*a, b*; Katz & Goss, 1958) or changing the C/N ratio (Martin & Pampus, 1956), different equilibria may arise between the competing amino acids of a given system. In this newly established condition a different actinomycin component may become the major one of a mixture or it may at least increase in amount relative to the other components.

If one introduces an exogenous supply of one of the amino acids in a competing system, the opportunity to shift the equilibrium becomes even greater, as was observed with hydroxyproline and actinomycin I (Katz & Goss, 1958), sarcosine and actinomycins II and III with *S. antibioticus*, and DL-valine and actinomycin IV with *S. chrysomallus* (Schmidt-Kastner, 1956*b*). An exogenous compound may be altered first by the organism; the modified compound then competes with a naturally synthesized amino acid for a particular site in the peptide. For example, *S. chrysomallus* converts isoleucine into *N*-methylisoleucine and the methylated amino acid competes with endogenous *N*-methylvaline (Schmidt-Kastner, 1956*a, b*). The condensation of an anthranilic acid peptide containing the former amino acid with a natural one, or the condensation of two such peptides, would result in synthesis of new actinomycins.

Finally, the addition of a substance closely related to an amino acid found in actinomycin may result in competition between the endogenously synthesized substance and the exogenous compound; incorporation of such related substances into the peptides will probably occur with new actinomycins ultimately synthesized. Pipecolic

acid appears to be incorporated directly into actinomycin molecules in this manner.

To what extent can the actinomycin peptides be modified? Can one, for example, replace any one of the amino acids in these peptides? Can one replace two or more amino acids simultaneously? Also, what is the specificity of the various amino acid receptors present on the surface of the peptide-synthesizing site? The information available concerns chiefly the 'proline' position in the various peptides; it appears that the specificity requirements of the 'proline' receptor site are not of a particularly high order. In some cases the exogenous compound bears a close structural relationship to the endogenous substance it competes with (hydroxyproline, pipecolic acid competing with proline); in other instances, only a portion of the exogenous molecule is found to be similar to a fragment of the natural amino acid (sarcosine competing with proline). Since proline, hydroxyproline, oxoproline, pipecolic acid and sarcosine probably all compete for the same location in the peptide, a compound must have the structural configuration $\text{—H}_2\text{C}\cdot\text{NH}\cdot\text{CH}(\text{CO}_2\text{H})\text{—}$ to compete successfully with proline for this position. It is possible to predict, therefore, that *N*-methyl amino acids, proline analogues and certain substituted forms of these substances should also compete favourably with proline.

The number of new compounds synthesized in any given situation will vary with the amino acid, the concentration used, the affinity of the surface of the specific synthesizing site etc. On theoretical grounds, one would expect several new compounds to be produced in each instance where incorporation takes place. For example, with pipecolic acid it should be possible to find actinomycin peptides containing (1) pipecolic acid/pipecolic acid, (2) proline/pipecolic acid, (3) hydroxyproline/pipecolic acid and (4) oxoproline/pipecolic acid. In addition, isomers of the actinomycins containing unsymmetrical peptides are probably synthesized. For sarcosine, one should find sarcosine/sarcosine and sarcosine/proline peptides and, in addition, sarcosine/hydroxyproline and sarcosine/ketoproline peptides. Isomers of the last three combinations should also exist.

Controlled biosynthesis can be defined as the technique of predetermining the structure of a new antibiotic by furnishing specific chemical precursors to the antibiotic-producing micro-organism (Woodruff & McDaniel, 1958). This technique has been successfully applied to the formation of modified forms of actinomycin. It should be possible to utilize this procedure with other antibiotic-producing micro-organisms for developing antibiotics with new or changed properties.

SUMMARY

1. Increased synthesis of actinomycins II and III occurred when sarcosine was added to the medium of growing cultures of *Streptomyces antibioticus*. The amount of actinomycins II and III formed depends, in part, on the concentration of sarcosine and on the time and the number of additions of this amino acid. Mycelial actinomycin, as well as that produced in the medium, contains considerable amounts of actinomycins II and III.

2. The effect observed with sarcosine is highly specific; compounds structurally and biochemically related were ineffective.

3. Exogenous L-proline reversed the effect of a given concentration of sarcosine; larger amounts of sarcosine abolished the activity of proline.

4. Incorporation of DL-pipecolic acid, a proline analogue, into the medium resulted in synthesis of several new actinomycins.

5. When washed suspensions of *S. antibioticus* were incubated in the presence of 1 mM-sarcosine, there was a fivefold increase in synthesis of actinomycin III, but no change in the percentage of actinomycin II.

6. The results obtained support the view that exogenous sarcosine and pipecolic acid compete with and replace endogenous proline in certain actinomycin peptides.

7. A generalized theory of actinomycin biogenesis is proposed.

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The Formation of Mercapturic Acids

3. N-ACETYLATION OF S-SUBSTITUTED CYSTEINES IN THE RABBIT, RAT AND GUINEA PIG*

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In the previous papers (Barnes, James & Wood, 1959; Bray, Franklin & James, 1959) evidence was given which supported the hypothesis that, in the formation of mercapturic acids *in vivo*, glutathione and glutathionase are involved in the synthesis of S-substituted cysteines. The subsequent acetylation of these compounds to mercapturic acids

remained to be examined. During this work it was observed that, although guinea-pig liver had greater glutathionase activity than had the livers of either the rabbit or the rat, the guinea pig excreted unexpectedly small amounts of mercapturic acids when given compounds which were readily converted into mercapturic acids by the rabbit and rat. A possible explanation of the failure of the guinea pig to excrete mercapturic acid

* Part 2. Bray, Franklin & James (1959).