light petroleum or carbon tetrachloride, but was extracted into butanol, in which the colour was stable for several days. The product of bufotenin oxidation remained in the aqueous phase with all the above-mentioned solvents including butanol. When a concentrated solution of serotonin was oxidized by concentrated denaturing oxyhaemoglobin, the filtrate was a turbid brownish pink which formed a black precipitate overnight. The addition of ascorbic acid to the pink product causes a rapid loss of colour, but the fluorescence does not return.

The product was tested for possible biological activity on the heart of the clam *Venus mercenaria*, with essentially the bioassay procedure of Twarog & Page (1953). No activity was found for the oxidized products of serotonin, bufotenin, 5-hydroxytryptophan or 5-HIAA.

DISCUSSION

The present results show that during the denaturation of oxyhaemoglobin there is a strong peroxidase-like activity which can attack 5-hydroxyindole compounds, as well as glutathione and ascorbic acid, as was found by Numata (1940). Rodnight (1958) has also noted that under certain conditions oxyhaemoglobin can destroy serotonin and he suggested that the process was similar to the oxidation of ascorbic acid and glutathione by denaturing oxyhaemoglobin. The product of this oxidation may be similar to a compound first observed by Cromartie & Harley-Mason (1957), who found that when oxygen is bubbled through a solution of 5:6-dihydroxy-2:3-dimethylindole, a pink product develops and the ultraviolet-absorption spectrum changes. The absorption spectrum of their product is similar to the spectrum of the oxidized product formed by the action of oxyhaemoglobin undergoing denaturation on serotonin (Fig. 4). It is thus possible that in the reaction with oxyhaemoglobin the 5-hydroxyindoles are first hydroxylated and then further oxidized. The formation of a pink product from serotonin is reminiscent of the oxidation of adrenalinesolutions by atmospheric oxygen,

and there may be some similarity in the structure of the serotonin oxidation product and compounds like adrenochrome (Harley-Mason, 1950; Beaudet, Debot, Lambot & Toussaint, 1951).

These results, in addition to providing further information on the oxidizing properties of haemoglobin undergoing denaturation, indicate that a system containing oxyhaemoglobin should be saturated with carbon monoxide before attempting to assay for 5-hydroxyindoles.

SUMMARY

1. Oxyhaemoglobin, when it is undergoing denaturation, can oxidize serotonin and several related 5-hydroxyindoles. The reaction is completely inhibited by carbon monoxide and partially inhibited by ascorbic acid.

2. The oxidized product(s) is characterized by a pink colour, a change in the ultraviolet-absorption spectrum and a loss of fluorescence.

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The Isolation and Structure of Actinomycins II and III

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Since the discovery of actinomycin A by Waksman & Woodruff (1940) it has been recognized (Vining & Waksman, 1954) that the actinomycin complexes A, B and X consist of the same components, actinomycins I, II, III, IV and V, in different por-

tions and that actinomycin D is essentially pure actinomycin IV. The structure of actinomycin IV (I; A = B = L-proline) has been determined by Bullock & Johnson (1957). Recently, Katz, Pienta & Sivak (1958) have shown that the composition of

actinomycin A varies according to the conditions of growth of the organism, and Katz & Goss (1958, 1959) have studied the effect of adding various amino acids to the culture medium of *Streptomyces antibioticus*. In particular, they found that the addition of sarcosine stimulated the production of actinomycins II and III, which are normally trace components, and that with an optimum concentration (0.05 %) of sarcosine these two actinomycins accounted for about 25 and 35 % respectively of the complex.

This paper describes the properties of actinomycins II and III isolated from this sarcosine-stimulated actinomycin complex.

EXPERIMENTAL

A sample of the actinomycin complex produced by the sarcosine-stimulated organism was provided through the courtesy of Professor Waksman and Dr Katz of the Institute of Microbiology, Rutgers State University.

All melting-point determinations were carried out on the Kofler hot-plate. 'Acid-washed alumina' refers to alumina (type H; P. Spence and Sons, Ltd., Widnes, Lancs) which has been washed for 2 hr. with 10 % (v/v) acetic acid at 70°, with hot water to pH 4-5 and then with methanol, and dried in air.

Paper chromatography of the actinomycin complex produced by the sarcosine-stimulated organism. Circular paper chromatograms were run on disks (24 cm. diam.) of Whatman no. 1 paper in the solvent system di-*n*-butyl ether-tetrachloroethane-10 % (w/v) aq. sodium *o*-cresotinate (2:1:3, by vol.) (Roussos & Vining, 1956), the paper being first impregnated with the aqueous phase. Actinomycins B and D were run on the same paper chromatograms to provide reference components. The crude complex was thus shown to consist of five principal components: I (R_{IV} 0.17), II (R_{IV} 0.45), III (R_{IV} 0.68), IV (R_{IV} 1.00) and V (R_{IV} 1.57) and another actinomycin (R_{IV} 1.28). (R_{IV} indicates the R_F relative to that of actinomycin IV.)

Adsorption chromatography of actinomycin complex. A solution of the crude complex (884 mg.) in benzene-chloroform (3:1, v/v) (50 ml.) was filtered and then brought on to a column (32 cm. $\times$ 4.8 cm.) of acid-washed alumina. Elution was commenced with the same solvent mixture, the proportion of chloroform being gradually raised to benzene-chloroform (1:3, v/v). The main orange band was collected in three fractions and elution was then continued with 1 % methanol in chloroform to collect a slower-running minor band in a fourth fraction. Each fraction was concentrated and examined by paper chromatography by the method described above. The first 600 ml. of eluate contained no actinomycins. Fraction (i) (250 ml.) consisted mainly of fast-running trace components which were more conveniently examined by paper chromatography in the solvent system di-*n*-butyl ether-tetrachloroethane-10 % (w/v) aq. sodium *o*-cresotinate (4:1:5, by vol.). Comparison with the actinomycin C complex (R_{IV} values: C₁, 1.00; C₂, 1.83; C₃, 3.17) on the same circular chromatogram indicated that one of these trace components corresponded with C₂ (Brockmann & Pfennig, 1952). Fraction (ii) (700 ml.) consisted principally of actinomycins III, IV and

V, and fraction (iii) (700 ml.) consisted almost entirely of actinomycins II and III. Fraction (iv) (800 ml.) consisted mainly of actinomycin I. The solvent was removed from fraction (ii) and the residue (280 mg.) was subjected to chromatography on a column (25 cm. $\times$ 1.9 cm.) of acid-washed alumina, the elution being commenced with benzene-chloroform (3:1, v/v) and gradually proceeding to benzene-chloroform (2:3, v/v), at which stage the main orange band had been collected. Five fractions were collected. Fraction (a) (300 ml.) contained actinomycin IV plus higher trace-components. Fraction (b) (100 ml.) was similar to fraction (a). Fraction (c) (75 ml.) contained actinomycin IV plus a trace of actinomycin III. Fraction (d) (75 ml.) contained actinomycins III and IV. Fraction (e) (300 ml.) contained actinomycins II, III, IV and the component of R_{IV} 1.28. Fractions (c), (d) and (e) were combined with fraction (iii) from the first column and the solvent was removed, leaving a red, semi-crystalline residue (377 mg.).

Preparative paper chromatography of the concentrated actinomycin complex. The mixture of actinomycins (377 mg.) obtained by adsorption chromatography was dissolved in acetone (25 ml.) and this solution (15 mg. of solid per sheet) was applied in horizontal streaks to square sheets (12 in. $\times$ 12 in.) of Whatman 3MM paper which had been previously impregnated with the aqueous phase of the solvent system di-*n*-butyl ether-tetrachloroethane-10 % aq. sodium *o*-cresotinate (2:1:3, by vol.). The mixture was separated on each sheet by the ascending chromatographic technique. The papers were dried in air and the coloured zones were cut out. Corresponding zones were combined and extracted in a Soxhlet apparatus with acetone (250 ml.).

Actinomycin III. The acetone extracts were evaporated and the residue, which was contaminated with salts, was shaken with water (100 ml.) and chloroform (100 ml.) and the layers were separated. The aqueous phase was washed with chloroform (3 $\times$ 25 ml., then 6 $\times$ 10 ml.); the chloroform extracts were combined, washed with water (3 $\times$ 100 ml.) and dried and the solvent was removed. The residue was dissolved in benzene-chloroform (2 ml.; 1:1, v/v) and chromatographed on a column (10 cm. $\times$ 1.0 cm.) of acid-washed alumina, with benzene-chloroform (1:1, v/v) as eluting solvent. The single orange band was collected and the solvent removed, leaving a red semi-crystalline residue (102 mg.) which crystallized as prisms, m.p. 237-238°, $[\alpha]_D^{20} - 205^\circ$ in chloroform (c, 0.22), from ethanol containing a little chloroform (Found, in a sample dried *in vacuo* at 78°: C, 58.4; H, 7.2; N, 13.1. C₆₆H₈₄O₁₆N₁₂ requires C, 58.6; H, 6.9; N, 13.7 %). Light-absorption in chloroform-ethanol (1:10): max. at 240 (log ϵ , 4.54), 430 (log ϵ , 4.38) and 450 m μ (log ϵ , 4.41). The infrared absorption (KBr disk) showed maxima at 781, 953, 1080, 1100, 1131, 1202, 1230, 1272, 1303, 1322, 1362, 1380, 1410, 1489, 1515, 1588, 1642, 1664, 1758, 2965 and 3430 cm.⁻¹. Circular paper chromatography of actinomycin III indicated the presence of only one component, R_{IV} 0.68.

Actinomycin II. Actinomycin II was obtained by the same method as actinomycin III, from the appropriate extracts of the preparative paper chromatograms. It crystallized from acetone-carbon disulphide as plates, m.p. 215-216°, $[\alpha]_D^{20} - 157^\circ$ in chloroform (c, 0.24) (Found, in a sample dried *in vacuo* at 100°: C, 58.0; H, 6.83; N, 13.8. C₆₈H₈₈O₁₆N₁₂ requires C, 57.9; H, 6.9; N, 14.0 %). Light-absorption in chloroform-ethanol (1:10): max. at

237 (log ϵ , 4.55), 429 (log ϵ , 4.33) and 447 m μ (log ϵ , 4.37). The infrared spectrum (KBr disk) showed maxima at 1079, 1131, 1200, 1230, 1319, 1401, 1489, 1517, 1587, 1660, 1750, 2330, 2351, 2930, 2960 and 3390 cm⁻¹. Circular paper chromatography of actinomycin II indicated the presence of one component only, R_{IV} 0.45.

Amino acid content of actinomycin III. Actinomycin III (10.19 mg.) was dissolved in 6*N*-HCl (2 ml.) in a sealed tube and the solution was heated at 100° for 46 hr., then taken to dryness *in vacuo* and twice dissolved in water (1 ml.), evaporated to dryness, re-dissolved in water and transferred quantitatively through a filter plug into a standard flask and diluted to 5 ml. For comparison, actinomycin D (12.39 mg.) was treated in the same way. The hydrolysates were run on two-dimensional paper chromatograms in (i) phenol-water (500 g. of phenol to 112 ml. of water) and (ii) butan-1-ol-acetic acid-water (4:1:5, by vol.). Actinomycin III gave the following spots on treatment with ninhydrin (R_F values in the butanol solvent first; colour purple unless otherwise stated): sarcosine (0.17, 0.73); threonine (0.19, 0.44); proline (0.25, 0.85, yellow); valine (0.39, 0.72) and *N*-methylvaline (0.42, 0.93). Descending paper chromatograms of the actinomycin III and IV hydrolysates were run in butan-2-ol-aq. 3% NH₃. The chromatograms were run for 4 days, the solvent being allowed to drip from the serrated edge of the paper. The papers were dried in air and treated first with isatin (0.2% in acetone) and heated at 100°, and then with ninhydrin (0.2% in acetone) and again heated at 100°. The various spots in the two hydrolysates corresponded exactly, the distances run being as follows (purple unless otherwise stated): threonine (7.7 cm.); sarcosine (9.6 cm.); proline (11.7 cm., blue); valine (17.5 cm.) and *N*-methylvaline (24.0 cm.).

The proline content of actinomycin III was determined as follows: paper chromatograms to which measured quantities (microsyringe) of actinomycins IV and III hydrolysates had been applied were run in (i) butan-2-ol-aq. 3% NH₃ and (ii) butanol-acetic acid-water (4:1:5, by vol.). The papers were dried, dipped in a solution of isatin (0.2% in butanol containing 4% acetic acid) and kept in the dark at room temperature for 24 hr. The blue spots were scanned at intervals of 0.1 in. along the mid-line with a reflexion densitometer, and curves of densitometer reading against distance were drawn. The values obtained for the proline content of actinomycin III, calculated by comparison of the areas under the curves with those obtained for actinomycin IV, were (i) 1.0 mole and (ii) 1.1 moles.

The method of Krishnamurthy & Swaminathan (1955) was applied to circular paper chromatograms of actinomycins IV and III hydrolysates run in the solvent system butanol-phenol-acetic acid-water (30:10:8:40, by vol.) (R_F values: threonine, 0.23; sarcosine, 0.29; proline, 0.41; valine, 0.49; *N*-methylvaline, 0.59). Comparison of the two hydrolysates gave the following molar ratios for actinomycin III: valine:*N*-methylvaline:sarcosine, 2.0:2.1:2.9 (molecular weights: actinomycin IV, 1255; actinomycin

III, 1229; actinomycin II, 1203). Threonine analyses were unsatisfactory because of decomposition of this amino acid during hydrolysis.

Amino acid content of actinomycin II. Actinomycin II (6.46 mg.) was dissolved in 6*N*-HCl (1 ml.) and the solution was heated in a sealed tube at 100° for 46 hr., and then evaporated and made up to 5 ml. by the procedure used for actinomycins III and IV. A two-dimensional paper chromatogram of the hydrolysate in the same solvent systems as those used previously gave the following spots (all purple) after ninhydrin treatment (R_F in the butanol solvent first): sarcosine (0.24, 0.73); threonine (0.23, 0.43); valine (0.41, 0.72); *N*-methylvaline (0.49, 0.91). Comparison of the hydrolysate with that of actinomycin III by descending paper chromatography in butan-2-ol-aq. 3% NH₃ showed the two hydrolysates to be identical, except that the proline spot was absent from the actinomycin II hydrolysate. The method of Krishnamurthy & Swaminathan (1955) was applied as before to actinomycin II hydrolysate. Comparison with actinomycin IV hydrolysate gave the following molar ratios for actinomycin II: valine:*N*-methylvaline:sarcosine, 2.0:1.9:3.7.

Oxidation of actinomycin III. Actinomycin III (15 mg.) was dissolved in methanol (1.5 ml.) containing chloroform (0.1 ml.), and aq. 30% H₂O₂ (0.2 ml.) was added, followed by aq. 2% NaOH (1 ml.) and water (0.5 ml.). After 1 hr. at room temperature aq. 30% H₂O₂ (0.2 ml.) was again added. After a further hour at room temperature the solution was adjusted to pH 3-4 with HCl, diluted with water (35 ml.) and extracted with butan-1-ol (3 × 10 ml.). The combined butanol extracts were washed with water (10 ml.) and evaporated to dryness, leaving a pale-yellow amorphous solid. Paper chromatography of the latter in propan-2-ol-aq. NH₃ (sp.gr. 0.880)-water (7:1:2, by vol.) gave two spots; one, fluorescent in u.v. light, had R_F 0.51. The oxidation product was streaked on a sheet (18 in. × 5 in.) of Whatman 3MM paper and run by the descending method in the same solvent system. The relevant areas of the paper were cut out and extracted with ethanol, the ethanol was removed *in vacuo* and the two residues were hydrolysed in sealed tubes in 6*N*-HCl at 117° for 7 hr. Paper-chromatographic examination of the hydrolysates indicated that both fractions contained the same five amino acids as did actinomycin III itself.

DISCUSSION

Chromatography of the complex on columns of acid-washed alumina gave only a partial separation of the various components; a final separation was achieved by preparative paper chromatography with the solvent system (Roussos & Vining, 1956) di-*n*-butyl ether-tetrachloroethane-10% sodium *o*-cresotinate.

Some of the physical properties of the pure crystalline actinomycins are given in Table 1.

Table 1. *Physical properties of actinomycins II, III and IV*

Actinomycin	Crystalline form	M.p.	$[\alpha]_D^{19}$ (CHCl ₃)	$\lambda_{\max.}$ (ethanol)
II	Plates	215-216°	-157°	237, 447
III	Prisms	237-238°	-205°	240, 450
IV	Prisms	235.5-236.5°	-337°	242, 444

Paper-chromatographic examination of the amino acids released after acid hydrolysis of the actinomycins showed that actinomycin III contains the same five amino acids (threonine, valine, proline, sarcosine and *N*-methylvaline) as does actinomycin IV, but that actinomycin II lacked proline. The relative proportions of the various amino acids present in the two hydrolysates was determined by paper-chromatographic methods, by the method of Krishnamurthy & Swaminathan (1955) and by taking densitometer readings on paper chromatograms carrying also an actinomycin IV hydrolysate as a standard, after development of the amino acid spots with ninhydrin. The results showed that actinomycin III contains three sarcosine residues and one proline residue and that actinomycin II contains four sarcosine residues and no proline, whereas actinomycin IV contains two sarcosine residues and two proline residues. Actinomycins IV, III and II thus constitute a series in which proline and sarcosine are interchangeable. Brockmann & Manegold (1958) have shown that actinomycins I ('X₀₈') and V ('X₂') differ from IV ('X₁') in that the proline has been replaced partially by 4-hydroxyproline and 4-oxoproline respectively; otherwise the sequence of amino acids in the peptide chains was the same as in those of the actinomycins of the C series (where the *D*-valine of actinomycin D is replaced by *D*-alloisoleucine). A tentative structure for the actinomycins II and III is therefore shown in (I)

Actinomycin II: A = B = Sarcosine

Actinomycin III: A = Sarcosine; B = *L*-proline
and/or A = *L*-proline;
B = Sarcosine

Actinomycin IV: A = B = *L*-Proline

Bossi, Hütter, Keller-Schierlein, Neipp & Zahner (1958) have shown that 'actinomycin Z' contains *N*-methylalanine and no proline, but the crude product contained six components and information is not yet available on the detailed structures.

Actinomycin III may be a mixture of two isomers formed by random oxidative coupling (Johnson, 1956) of the two 3-hydroxy-4-methylanthraniloyl peptides, i.e. II-type and IV-type peptides. These isomers would be difficult to separate by chromatography, but a possible means of detecting their presence would be by oxidative fission and subsequent hydrolysis (Bullock & Johnson, 1957). Paper-chromatographic separation and subsequent hydrolysis of the two pentapeptides obtained by a small-scale oxidation of actinomycin III indicated that all five amino acids were present in both peptides; had only one possible form of actinomycin III been present then one of the peptides would have lacked proline.

SUMMARY

1. A sarcosine-stimulated actinomycin complex has been resolved into its components by preparative paper chromatography. Actinomycins II and III have been isolated and their physical properties are described.

2. The amino acid contents of these two actinomycins have been determined and tentative structures are proposed.

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