478. *Isolation and Properties of Pure Actinomycins.*

By G. G. Roussos and L. C. Vining.

Seven pure components have been separated by means of partition chromatography on powdered-cellulose columns from the mixtures of actinomycins produced by three different *Streptomyces* species. These seven components were shown to represent only three different actinomycins, suggesting that the natural complexes of the X, B, A, and D type are constituted from the same component actinomycins and that differences may be attributed to the relative amount of each component produced by the respective *Streptomyces* species.

It has been shown [1,2] that actinomycins A, B, C, and X are not homogeneous substances but are mixtures of closely related polypeptides linked to a chromophore which may be identical [3] for all members of the group. There have recently been reported [4,5] two

[1] Brockmann and Grone, *Chem. Ber.*, 1954, **87**, 1036.
[2] Vining and Waksman, *Science*, 1954, **120**, 389.
[3] Brockmann and Vohwinkel, *Naturwiss.*, 1954, **41**, 257.
[4] Brockmann and Bohne, *ibid.*, p. 65.
[5] Manaker, Gregory, Vining, and Waksman, "Antibiotics Annual 1954—1955," Medical Encyclopedia, Inc., New York, 1955, p. 853.

additional types, I and D, which, although substantially closer to homogeneity, nevertheless, contain subsidiary compounds in significant amounts. In spite of the investigation of products elaborated by a large number of actinomycin-producing *Streptomyces* species, no culture has yet been found which produces a single actinomycin. A complete knowledge of the chemistry and biological properties of this group depends, therefore, upon a satisfactory method for separating the natural complex into its components.

Paper chromatography of the products from twelve different cultures has shown [6] that the actinomycin complexes can be conveniently grouped into six different types according to the proportion of components at specific R_F values. They were represented by actinomycins A, B, C, D, and those from cultures * 3436 and 3491. Subsequent examination of the actinomycins from four other cultures has revealed no new types of complex. Brockmann and his co-workers,[1,7] after an examination of the products from twenty-one actinomycin-producing cultures, were able to classify them into three different types, represented by actinomycins C, I, and X. It was concluded [6] that actinomycin X is identical with the actinomycin produced by culture 3491. The actinomycin from culture 3436 was very similar to a sample of actinomycin A produced [8] from culture 3435 in 1940, but was classified separately because of differences in the amounts of minor components.[2] However, since such variations have now been found to be easily effected by changes in the method used for producing and isolating the antibiotic it is considered unnecessary to maintain this distinction and actinomycins of the type obtained from culture 3436 will henceforth be referred to as actinomycin A.

The actinomycin A, B, D, and X complexes contained many components which appeared identical by paper chromatography and it was tentatively suggested [9] that they might differ from each other only through quantitative differences in the amount of each component present. Paper-chromatographic comparison of the complexes in additional solvent systems has failed to show any differences between these components. We have found that circular paper chromatography with a mixture of aqueous sodium *o*-cresotinate, di-*n*-butyl ether, and *s*-tetrachloroethane, butan-1-ol, or ethyl acetate afforded the most satisfactory resolution of the actinomycin complexes. *s*-Tetrachloroethane was slightly the best and the R_F values in this solvent system are reported in Tables 1 and 2. In

TABLE 1. *Percentage composition of actinomycins from circular paper chromatography with di-*n*-butyl ether–*s*-tetrachloroethane–10% aqueous sodium* o*-cresotinate (2 : 1 : 3).*

	$R_{D,IV}$					
Actinomycin complex from culture no.	I 0·27	II 0·40	III 0·56	IV 1·00	V 1·35	VI * 1·55—1·97
3724 (X type)	4·9	Trace	Trace	11·5	84·6	Trace
3723 (B type)	9·5	Trace	Trace	28·1	59·3	3·1
3436 (A type)	6·6	2·9	Trace	66·7	23·8	Trace
3677 (D type)	Trace	Trace	Trace	100	Trace	—

	$R_{D,IV}$		
	1·00 (C_1)	1·43 (C_2)	1·99 (C_3)
3657 (C type)	10·3	48·3	41·4

* Actinomycin B_{VI} separated indistinctly into three components at $R_{D,IV}$ 1·55, 1·69, and 1·97 in this system, but in other actinomycins no distinct zones were discernible.

naming the actinomycin components a letter has been retained to designate the complex type produced by the micro-organism, and a subscript Roman numeral used for the component appearing at a specific R_F value in this solvent mixture. Actinomycins with $R_{D,IV}$ values (*i.e.*, the value relative to D_{IV}) greater than 1·35 (Table 1) have been referred

* Culture numbers refer to the culture collection of the Institute of Microbiology, Rutgers University.

[6] Gregory, Vining, and Waksman, *Antibiotics and Chemotherapy*, 1955, **5**, 409.
[7] Brockmann, *Angew. Chem.*, 1954, **66**, 1.
[8] Waksman and Woodruff, *Proc. Soc. Exp. Biol. Med.*, 1940, **45**, 609.
[9] Vining, Gregory, and Waksman, *Antibiotics and Chemotherapy*, 1955, **5**, 417

to collectively as component VI, and where they have been separated, as in the case of actinomycin B, as B_{VIa}, B_{VIb}, etc.

Further evidence that the components with similar behaviour on paper chromatography are identical, was obtained by the isolation of actinomycins B_I, B_{IV}, B_V, A_I, A_{IV}, A_V, and D_{IV} in quantities sufficient for examination of their chemical properties. This was achieved by partition chromatography on powdered-cellulose columns, which was found to be more

TABLE 2. *$R_{D,IV}$ values of actinomycin components from circular chromatography with di-*n*-butyl ether–*s*-tetrachloroethane–10% aqueous sodium o-cresotinate* (5 : 1 : 6).

Actinomycin complex	$R_{D,IV}$									
	I	II	III	IV	V	VI*a*	VI*b*	VI*c*	VI*d*	VI*e*
	0·20	0·39	0·63	1·00	1·20	1·77	2·20	2·66 *	2·90	3·27
B	×	×	×	×	×	←		Traces		→
V_{VI}					×	×	×	×	×	×
				(C_1)		(C_2)		(C_3)		
C				×		×		×		

× denotes presence in substantial amount.

* Although actinomycins B_{VIc} and C_3 had identical R_F values in this system they were separated in a system containing di-*n*-butyl ether, ethyl acetate, and 10% aqueous sodium *o*-cresotinate (2 : 1 : 3) ($R_{D,IV}$ of actinomycin B_{VIc} 2·13, and of C_3 2·90). Actinomycins B_{VIa} and C_2 were not separated in either system.

TABLE 3. *Properties of pure actinomycins.*

	B_I	B_{IV}	B_V	A_I	A_{IV}	A_V	D_{IV}
Crystalline form (from acetone–CS_2)	Plates	Prisms	Fine needles	Plates	Prisms	Fine needles	Prisms
M. p. (decomp.) (Kofler hot plate)	237·5—238°	235·5—236·5°	246—246·5°	237—238°	235—236°	245—246·5°	235·5—236·5°
$[\alpha]_D^{23}$ (*c*, 0·25 in 95% EtOH)	−235°	−268°	−320°	—	−261°	−323°	−262°
$\lambda_{max.}$ (Å) in 95% EtOH	2425, 4410	2420, 4430	2400, 4450	2420, 4410	2420, 4440	2400, 4450	2420, 4440
$\lambda_{min.}$ (Å) in 95% EtOH	2300, 3310—3320	2270, 3300	2270—2280, 3300	2300, 3310	2270, 3300	2280, 3300	2270, 3300
ε_{4440}	17·8	18·8	19·2	18·0	19·2	19·6	19·6
$R_{D,IV}$ in the 2 : 1 : 3 system	0·27	1·00	1·35	0·27	1·00	1·35	1·00

TABLE 4. *Amino-acid analyses of actinomycins (expressed as moles of amino-acid per mole of actinomycin of mol. wt.* 1200).

	Threonine	Sarcosine	Proline	Valine	*N*-Methylvaline
B_I	1·3	1·8	1·1	1·9	1·9
B_{IV}	1·2	1·9	2·2	1·9	2·0
B_V	1·0	2·0	1·1	2·0	2·1
A_I	1·3	1·9	1·0	2·0	2·0
A_{IV}	1·2	2·0	2·1	2·0	2·0
A_V	1·1	1·9	1·00	1·9	1·9
D_{IV}	1·2	2·0	2·1	2·1	2·1

satisfactory on a preparative scale than the adsorption-chromatographic method previously used.[9] The properties of the pure actinomycins are summarized in Table 3.

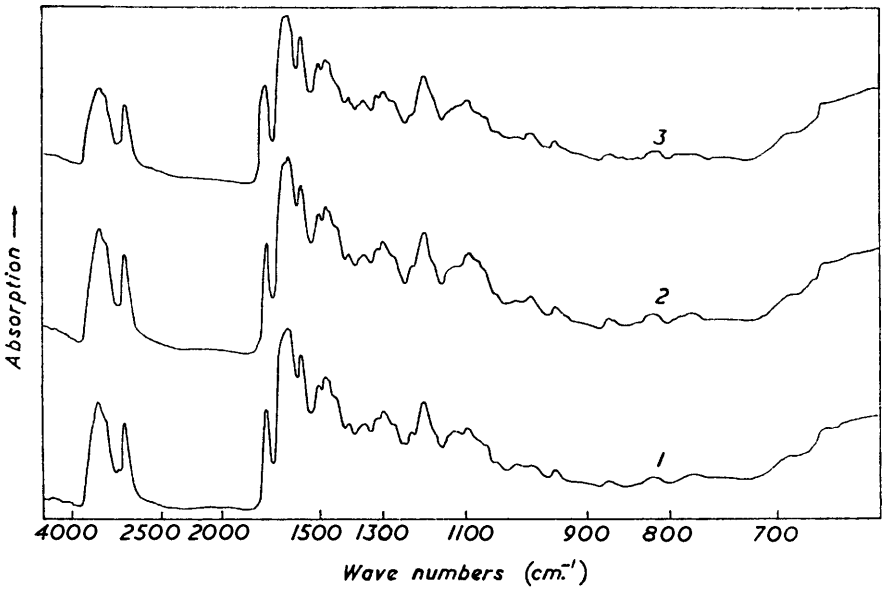
The absorption spectra (ultraviolet and visible) were very similar, differing only slightly in wavelength and intensity. In the infrared region actinomycins A_I and B_I were identical; and no differences were discovered between actinomycins A_{IV}, B_{IV}, and D_{IV}, or between actinomycins A_V and B_V. The differences in the spectra which allowed a separation into the three groups were small, however, in comparison with the overall similarities (see Figure).

Determination of the amino-acid contents (Table 4) revealed that actinomycins A_I (B_I) and A_V (B_V) each possessed one mol. of proline compared with the 2 mols. present in A_{IV} (B_{IV}, D_{IV}). There were only small differences between the samples in the values

obtained for threonine, but Brockmann and his co-workers [10] have recently shown that the use of hydrochloric acid and vigorous conditions for hydrolysis of the actinomycins causes considerable destruction of this amino-acid. It cannot be said with certainty, therefore, that these actinomycins do not differ in their total threonine content. In absence of such a difference, actinomycins A_I (B_I) and A_V (B_V) possess an identical amino-acid composition and variations in the order of amino-acids in the polypeptide chain or the linkage to the chromophore may account for the observed differences in their properties. In this respect, it is interesting that the infrared spectra of B_V and I_V contain an additional band at 1760 cm.$^{-1}$ suggestive of a type of ester or lactone grouping not found in the other samples.

A comparison of the properties of the isolated components indicated that actinomycins A_I and B_I were identical; similarly actinomycins A_{IV} and B_{IV} were identical with actinomycin D_{IV}, and actinomycin A_V with actinomycin B_V. It appeared, therefore, that

Infrared spectra of actinomycins.



1, *Actinomycin* B_{IV}. 2, *Actinomycin* B_I. 3, *Actinomycin* B_V. (*All in* KCl *discs.*)

actinomycins A, B, and D belonged to a group of complexes which were composed of the same components, but in different proportions. To the group can also be added actinomycin X, shown by Brockmann [7] to differ from actinomycin B only in the proportion of the components present in each sample. The four types of complex within the group are characterized by a steady increase in the proportion of component IV, and a corresponding decrease in component V in the order X, B, A, D (Table 1). It is probable that in this group the remaining minor components with corresponding R_F values will also prove to be identical, irrespective of the complex from which they were separated. The difficulty of isolating substances present in such small amounts makes their study difficult, however, unless a means can be found of influencing the producing organism to increase substantially the proportion of these components in the complex. The relation between the members of the X, B, A, D group of actinomycins is emphasised by the fact that the cultures producing both actinomycins A and X have been shown [2,7] by a suitable change in the conditions of growth to be capable of producing an actinomycin complex chromatographically indistinguishable form actinomycin B.

[10] Brockmann, Grone, and Timm, *Naturwiss.*, 1955, **42**, 125.

Brockmann and Bohne [4] made a direct comparison of actinomycins A and I, and reported that they were very similar, if not identical. The properties we have found for the components of actinomycin A are not in agreement, however, with those described by Brockmann and his co-workers [1,7] for the actinomycin I complex. The differences reside principally in the fact that, whereas the corresponding components from actinomycins A and B were identical, the German workers have reported differences between actinomycins I and X (B) in their behaviour to paper chromatography in certain solvent systems. Moreover, none of the pure components isolated from actinomycin I was identical with those from actinomycin X. A closer relation appeared to exist between actinomycins I and C, and, in fact, Brockmann and Grone have suggested [1] that the corresponding components in these two complexes may be identical.

Experimental

Circular Paper Chromatography.—A filter-paper disc (Whatman No. 2; 15 cm. diam.) was impregnated by dipping it in the aqueous phase of the solvent system di-*n*-butyl ether–*s*-tetrachloroethane–10% aqueous sodium *o*-cresotinate (2 : 1 : 3), and excess of moisture was removed between fresh filter paper. The actinomycin samples were applied in acetone solution (5 mg./ml.) from a capillary pipette to segments of a small circle drawn around the centre of the filter paper where a thin cotton wick was attached. The paper was placed upon an open Petri dish (14 cm. diam.) containing the organic phase of the solvent system, and covered with a large watch glass. The chromatogram was allowed to develop until the outer actinomycin zone was close to the glass rim. Best resolution was obtained with low R_F values when, after the front had reached the outer edge of the paper, the solvent flow was restriced to the rate at which it evaporated from the narrow strip extending outside the walls of the Petri dish. R_F values varied greatly with the amount of aqueous phase left in the paper after blotting but could be conveniently adjusted to the blotting technique employed by alterations in the proportion of *s*-tetrachloroethane in the solvent system.

The following samples were examined: actinomycins from cultures **3436** (A type), **3723** (B type), **3724** (X type), **3677** (D type), and **3677** (C type). The actinomycin components could best be seen by viewing the developed chromatograms under ultraviolet light of 2570 Å; zones due to actinomycin appeared as dark absorbing areas against a fluorescent background.

Separation of Components by Partition Chromatography on a Cellulose Column.—A column ($1\frac{3}{4}$ × 30 in.) was packed with dry cellulose powder (Whatman's Ashless Standard Grade) to a height of 20 in. The aqueous phase (500 ml.) of di-*n*-butyl ether–*s*-tetrachloroethane–10% aqueous sodium *o*-cresotinate (4 : 1 : 5) was passed through the column, followed by the organic phase until an equilibrium had been reached and no further aqueous phase appeared in the eluate. The actinomycin (300 mg.), dissolved under reflux in water-saturated di-*n*-butyl ether–*s*-tetrachloroethane (6 : 1), was applied to the column and washed in with a little fresh solvent. With this solvent mixture its movement on the column was extremely slow, and development did not occur until the proportion of *s*-tetrachloroethane was increased to 5 : 1. It was continued until the strong zone due to component V was close to the bottom. Air was then forced through the column to remove excess of solvent. Minor components running ahead of component V were collected in the eluate. The remaining zones were separated mechanically and the actinomycins extracted with benzene. Alternatively, when the component was present in small amounts only, a more complete recovery was obtained by eluting the actinomycin from the impregnated cellulose with water, then extracting it in benzene.

Two procedures were used for obtaining crystalline products: (*a*) The solution was extracted once with dilute sodium carbonate solution, then washed with water, and the solvent removed *in vacuo*. The residual actinomycin was crystallized to constant m. p. from the appropriate solvent. (*b*) The solution was passed through a column of silicic acid (Mallinkrodt 2847), the actinomycin being strongly adsorbed. The column was washed thoroughly with benzene and developed with pure ethyl acetate. The actinomycin was recovered from the column by extrusion and elution of the appropriate section with methanol. Concentration of the methanolic solution and, where necessary, addition of an equal volume of di-*n*-butyl ether yielded crystalline actinomycin which required no further purification.

Whenever possible, these operations were carried out in the dark and under nitrogen to avoid photocatalytic oxidation of the actinomycin. A solvent system consisting of di-*n*-butyl

ether–ethyl acetate–10% aqueous sodium *o*-cresotinate (3 : 1 : 4) also gave good separation of actinomycin components. It was less satisfactory than that described above because of the large amounts of a non-volatile liquid contaminant originating from the cresotinate which remained with the actinomycin after evaporation of the solvent *in vacuo* when procedure (*a*) was used. Separation of the actinomycin from this contaminant could only be achieved by chromatography on silicic acid, as in procedure (*b*).

The actinomycins crystallized well from acetone– or ethyl acetate–carbon disulphide, except components A_V and B_V, for which either methanol or methanol–di-*n*-butyl ether proved more satisfactory. The actinomycin complexes investigated in this way each separated on the column into zones which corresponded to those found by circular paper chromatography. An additional yellow zone at the upper surface of the column was due to degraded material. From the actinomycins produced by cultures (i) **3436**, (ii) **3723**, and (iii) **3677**, components (i) A_I, A_{IV}, and A_V, (ii) B_I, B_{IV}, and B_V, and (iii) D_{IV}, were isolated. In addition the fraction B_{VI} from the actinomycin produced by culture **3723** was concentrated and crystallized. Circular paper chromatography of each fraction demonstrated its purity, except in the case of B_{VI} which on resolution in di-*n*-butyl ether–*s*-tetrachloroethane–10% aqueous sodium *o*-cresotinate (5 : 1 : 6) was found to consist of six components (Table 2).

Amino-acid Analysis.—Samples (5 mg.) of actinomycins B_I, B_{IV}, B_V, A_{IV}, A_V, and D_{IV} were hydrolysed in sealed tubes at 100° for 48 hr. with 20% hydrochloric acid (1 ml.), then the solutions were evaporated repeatedly to dryness in a vacuum-desiccator over sodium hydroxide. The residues were each redissolved in distilled water (0·25 mg.), filtered, and applied in measured amounts from a micropipette to filter-paper strips (Whatman No. 1). The strips, together with a series containing standard amounts of L-threonine, sarcosine, L-proline, D-valine, and *N*-methylvaline were transferred to a large tank and subjected to descending chromatography in the solvent system butan-2-ol–3% aqueous ammonia (7 : 2) for three days.[11] The solvent was allowed to drip from the serrated lower edge of the paper strips. The chromatograms were dried in a current of air and dipped in a freshly prepared solution of ninhydrin (0·2%) in acetone containing acetic acid (1%). After drying for 30 min. in air, the papers were placed for a further 20 min. at 90° over a tray of water.[12] They were then stored in a desiccator in the dark until the colour intensity of the zones could be measured with a densitometer. Good resolution was obtained of the five amino-acids present in each of the actinomycins. The concentrations were calculated from a comparison with the colour intensity produced by known concentrations of the corresponding standards. Duplicate analyses showed good agreement.

In a similar experiment, the chromatograms were sprayed with a solution of isatin (0·2%) in butan-1-ol containing acetic acid (4%), then kept for 24 hr. in the dark to allow development of the colour.[13] The proline content determined by this method agreed well with the values obtained above.

The authors are indebted to Drs. G. W. Downing of Merck and Co., Inc. Rahway, N.J., J. A. Aeschlimann of Hoffmann La-Roche Inc., Nutley, N.J., and to the Baker Chemical Co., Easton, Pa., for generous gifts of samples, and to Mr. A. D. Mebane of Ortho Research Foundation, Raritan, N.J., for the infrared spectra. One of them (G. G. R.) acknowledges the award of a fellowship from the Institute of Microbiology, Rutgers University.

INSTITUTE OF MICROBIOLOGY, RUTGERS UNIVERSITY, THE STATE UNIVERSITY OF NEW JERSEY, NEW BRUNSWICK, N.J., U.S.A.
PRAIRIE REGIONAL LABORATORY, NATIONAL RESEARCH COUNCIL OF CANADA, SASKATOON, SASKATCHEWAN, CANADA.
[Present address (L. C. V.): SASKATOON.]
[*Received, January 10th,* 1956.]

[11] Roland and Gross, *Analyt. Chem.*, 1954, **26**, 502.
[12] Redfield and Barron, *Arch. Biochem. Biophys.*, 1952, **35**, 443.
[13] Acher, Fromageot, and Jutisz, *Biochim. Biophys. Acta*, 1950, **5**, 81