127. *An Antibiotic Similar to Xanthomycin A.*

By D. K. Dougall.

A species of *Streptomyces* (N.C.I.B. 8697), obtained from a sample of Malayan soil, has been shown to produce an antibiotic that is either identical with, or very similar to, xanthomycin A. Some of the properties of the antibiotic isolated from the culture fluid of the Malayan Streptomyces are different from those described by Rao and Peterson [1] for xanthomycin A, but an explanation of these discrepancies is presented. The titration curve of the antibiotic is discussed together with the results of estimation of the simple bases liberated by acid hydrolysis.

Xanthomycin A, an antibiotic produced by an unidentified *Streptomyces*, was first described by Thorne and Peterson [2] in 1948. Mold and Bartz [3] isolated an antibiotic which was either identical with, or very similar to, xanthomycin A. Rao and Peterson [1] subsequently described the behaviour of the antibiotic and interpreted it as indicating the presence of a benzoquinone system in the molecule. Rao, Peterson, and van Tamelen [4] described some degradations of the antibiotic. They showed that 2-aminoethanol, methylamine, and ammonia were present in acid hydrolysates.

In a preliminary communication, Dougall and Abraham [5] reported the isolation of an antibiotic from the culture fluid of a *Streptomyces* isolated in Malaya (N.C.I.B. 8697). Some of its properties were so similar to those reported for xanthomycin A that it seemed possible for the two substances to be identical. In other properties, however, there

[1] Rao and Peterson, *J. Amer. Chem. Soc.*, 1954, **76**, 1335, 1338.
[2] Thorne and Peterson, *J. Biol. Chem.*, 1948, **176**, 413.
[3] Mold and Bartz, *J. Amer. Chem. Soc.*, 1950, **72**, 1847.
[4] Rao, Peterson, and van Tamelen, *ibid.*, 1955, **77**, 4327.
[5] Dougall and Abraham, *Nature*, 1955, **176**, 256.

appeared to be surprising differences. The work described in the present paper suggests an explanation of this apparent inconsistency.

The antibiotic produced by the Malayan *Streptomyces* was isolated from the culture fluid by extraction into trichloroethylene. It was present in the culture fluid in at least two forms, one of which was extracted rapidly into the solvent while the other required 8—12 hours' stirring with solvent for maximum extraction. The relative proportions of the two forms varied with the conditions of growth of the organism and in the later cultures the rapidly extracted form predominated. Further purification of the antibiotic was effected by transferring the active material into and out of water by varying the pH of the aqueous phase. The final aqueous extract was freeze-dried and yielded the antibiotic dihydrochloride as an orange amorphous powder. The most potent preparations obtained had a specific antibacterial activity of 1500 units/mg. Assay of the antibiotic was carried out by the hole plate method [6] with *Staphylococcus aureus* (N.C.T.C. 6571) as test organism. The unit of activity was defined in terms of the weight of a standard preparation, so that one unit/ml. gave a zone of inhibition of approx. 25 mm. diam.

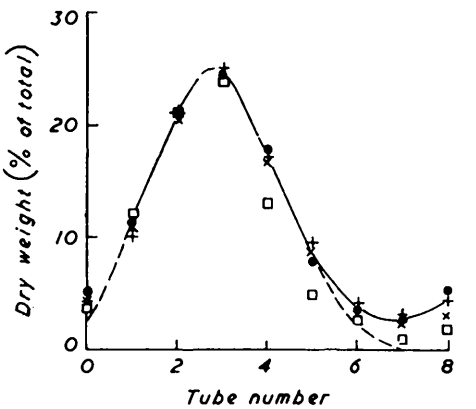

FIG. 1. *Eight-transfer countercurrent distribution of the antibiotic between equal volumes of trichloroethylene and 0·5N-sodium citrate buffer at* pH 6·0 *(upper phase mobile).*

+, *% of total dry weight.*
□, *relative antibacterial activity.*
×, *relative ninhydrin colour density before hydrolysis.*
●, *relative ninhydrin colour density after hydrolysis.*
The broken line is the calculated curve due to a component (90%) *with a partition coefficient* $K = 0{\cdot}6$.

Material of specific activity 1500 units/mg. was shown, by an eight-transfer countercurrent distribution, to contain one component which accounted for 90% of the total material (see Fig. 1). This component could be crystallized as a dihydrochloride and it was subsequently shown that samples of the amorphous dihydrochloride of activity 1500 units/mg. could also be crystallized. The antibiotic dihydrochloride which had been crystallized twice appeared to contain only one component when subjected to ionophoresis on paper at pH 7·0. It could be located by its antibacterial activity and by its reaction with ninhydrin. The twice crystallized hydrochloride from different batches of material had similar antibacterial activity and exhibited similar physical and chemical properties.

The dihydrochloride of the antibiotic was shown to be very hygroscopic and the amount of water it absorbed varied rapidly with the time of exposure to the air. Elementary analyses indicated an atomic N : Cl ratio of 3 : 2. If the presence of water was allowed for, the analytical values were in approximate agreement with the empirical formula $C_{23}H_{29\text{-}31}O_7N_3,2HCl$ proposed by Rao and Peterson [1] for xanthomycin A. However, the divergence of different analyses leaves this formula provisional.

In Table 1 the more important biological, physical, and chemical properties of the antibiotic isolated from the Malayan *Streptomyces* are listed and compared with the properties of xanthomycin A reported by Rao and Peterson.[1] The clear difference in the midpoints of the titration curves (the pK values) of the ionizable groups of the two antibiotics (7·3 and 3·0 for the antibiotic from the Malayan *Streptomyces*, and 4·5 and 2·0 for xanthomycin A) seemed to indicate that the substances were different. However, the

[6] Brownlie, Delves, Dorman, Breen, Grenfell, Johnson, and Smith, *J. Gen. Microbiol.*, 1948, **2**, 40.

infrared spectra (see Fig. 2) showed only minor differences in the region of 9·0 μ (thought to be due to difference in the resolution obtained with different spectrometers [7]) and it was concluded that the two specimens used were very similar and were probably identical. This could not be confirmed by direct comparison because the samples of xanthomycin A in Professor Peterson's possession had decomposed.

The major points of difference between the two sets of properties shown in Table 1 are the differences in the p*K* values of the ionizable groups, the uptake of hydrogen in the

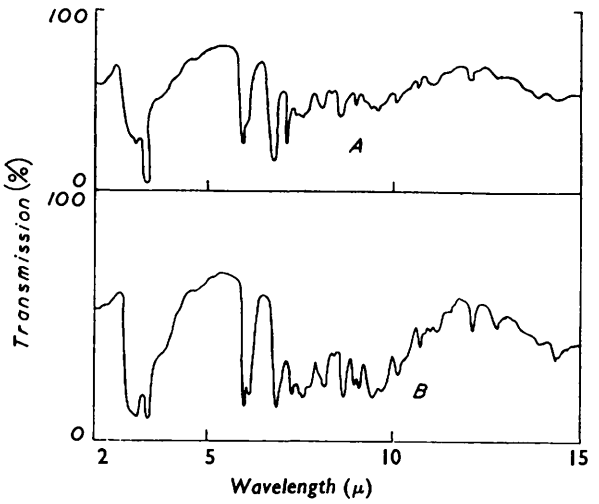

FIG. 2. *Comparison of the infrared spectrum of xanthomycin A (A, redrawn from Rao and Peterson [1]) with the antibiotic dihydrochloride (B). Samples in paraffin paste.*

TABLE 1. *Comparison of some reported [1] properties of xanthomycin A with those of the antibiotic isolated from the culture fluid of the Malayan* Streptomyces.

Property	Antibiotic from Malayan *Streptomyces*	Xanthomycin A
Antibacterial activity	Highly active against Gram-positive bacteria	Highly active against Gram-positive bacteria
Toxicity	30 μg. killed a mouse (20 g.)	M.L.D.$_{50}$ to mice (20 g.) 3·2 μg.
p*K* values	7·3, 3·0	4·5, 2·0
$[\alpha]_D$ of the hydrochloride	+ 98° (water)	+ 115° (water)
Ultraviolet absorption spectra :		
(*a*) in 0·01N-HCl	$\lambda_{max.}$ 263 mμ ($E^{1\%}_{1\,cm.}$ 179) 350 mμ ($E^{1\%}_{1\,cm.}$ 16·4)	$\lambda_{max.}$ 265 mμ ($E^{1\%}_{1\,cm.}$ 196) 345 mμ ($E^{1\%}_{1\,cm.}$ 19·8)
(*b*) in EtOH	$\lambda_{max.}$ 267 mμ ($E^{1\%}_{1\,cm.}$ 182) (for a sample whose pH in water was 7·0)	$\lambda_{max.}$ 288 mμ ($E^{1\%}_{1\,cm.}$ 148) 460 mμ ($E^{1\%}_{1\,cm.}$ 118) (free base)
Degree of unsaturation	Absorbed 3 mols. H_2/540 g. (Pd–C)	Absorbed 2 mols. H_2/mole (PtO_2)
Stability :		
(*a*) in alkali	12% of the activity lost in 15 min. at pH 11·0	Decomposed rapidly above pH 6·0
(*b*) in acid	Activity not changed after 15 days in N-HCl	14% activity lost at 100° in N-acid. No loss at 20° in N-acid in 60 min.
Bases detected after acid hydrolysis	NH_2Me, 2-aminoethanol	NH_2Me, 2-aminoethanol, NH_3

presence of palladium–charcoal, and the ultraviolet absorption spectrum of the free-base form of the antibiotic. When measured in aqueous solution at various pH values the ultraviolet absorption of the antibiotic from the Malayan *Streptomyces* did not resemble that described for the free-base xanthomycin A. However, when an attempt was made to

[7] Dr. F. B. Strauss, personal communication.

convert the antibiotic dihydrochloride into the free base by the method which was reported by Rao and Peterson [1] to yield xanthomycin A from its salts, the ultraviolet absorption spectrum of the product was very similar to that described for xanthomycin A. The properties of this product (subsequently referred to as the " free base ") are compared with the properties described for xanthomycin A in Table 2. The two groups of properties are sufficiently alike to suggest that the two products are closely related and to confirm that the antibiotic from the Malayan *Streptomyces* is very similar to, or identical with, xanthomycin A.

TABLE 2. *Comparison of the properties of the " free base " from the antibiotic produced by the Malayan* Streptomyces *with those reported for free-base xanthomycin A.*

Property	" Free base "	Xanthomycin A
pK values	4·8	4·5, 2·0
Stability	Decomp. above pH 8·0	Decomp. above pH 8·0
Degree of unsaturation	absorbed 2 mols. (H_2/460 g. (Pd–C)	absorbed 2 mols. H_2/460 g. (PtO_2)
Ultraviolet absorption in EtOH	$\lambda_{max.}$ 287 mμ ($E^{1\%}_{1\,cm.}$ 124)	$\lambda_{max.}$ 288 mμ ($E^{1\%}_{1\,cm.}$ 148)
	450 mμ ($E^{1\%}_{1\,cm.}$ 63)	460 mμ ($E^{1\%}_{1\,cm.}$ 118)
	$\lambda_{infl.}$ 315 mμ ($E^{1\%}_{1\,cm.}$ 90)	$\lambda_{infl.}$ 315 mμ ($E^{1\%}_{1\,cm.}$ 116)

Further examination of the material termed the " free base " revealed that it contained only 20% of the activity of the starting material, and was not homogeneous when subjected to ionophoresis on paper at pH 7·0. Ninhydrin revealed the presence of three components, the largest of which was neutral and possessed no antibacterial activity. The antibacterial activity of the preparation was associated with a basic component which migrated to the same position as the antibiotic itself. The smallest component behaved in the same way as 2-aminoethanol. Further, after treatment of the " free base " with hydrochloric acid, neither the ultraviolet absorption spectrum nor the antibacterial activity returned to that of the antibiotic dihydrochloride.

Thus it seems that, although the " free base " prepared from xanthomycin A and from the antibiotic produced by the Malayan *Streptomyces* are similar, yet during the preparation of the " free base " from the antibiotic dihydrochloride by the method of Rao and Peterson [1] the material had undergone an irreversible transformation.

The titration curve reported by Rao and Peterson [1] for xanthomycin A seems to be that of the " free base " and is different from that of the antibiotic dihydrochloride isolated from the Malayan *Streptomyces*. The titration curve of the antibiotic dihydrochloride (Fig. 3) is reversible up to pH 10·5 and together with the effect of pH on the partition coefficient of the antibiotic shows the presence of two basic groups in the molecule. The amount of alkali required to discharge each group in the molecule indicated that the equivalent weight of the antibiotic dihydrochloride is approx. 540. If a sample of the antibiotic is maintained at pH 11·5—12·0 for two hours and then titrated, a second curve (see Fig. 3) is obtained. The product can be titrated reversibly and has groups with pK values of 9·5, 4·5, and 3·0. This material is found almost exclusively in the aqueous phase when it is distributed between chloroform and water at pH 6·0, which suggests that the group with pK 9·5 is basic and that one of the remaining groups is acidic. The pK values of 9·5 and 4·5 might well be assigned to an amino- and a carboxylic acid group respectively. However, the result of alkali treatment of the antibiotic cannot be described merely in terms of the hydrolysis of an amide linkage because the appearance of the basic group with pK 9·5 was accompanied by the disappearance of a basic group with pK 7·3.

When the antibiotic from the Malayan *Streptomyces* was hydrolysed in hydrochloric acid, methylamine and 2-aminoethanol were liberated.[5] Estimation of the methylamine present in hydrolysates of the antibiotic by Conway's method,[8] or by distillation and

[8] Conway, *Biochem. J.*, 1935, **29**, 2755.

chromatography on Dowex 50-X4, indicated that 0·4—0·6 mole of this base was liberated from 540 g. of the dihydrochloride. Estimation of 2-hydroxyalkylamines in the hydrolysates by Gordon, Martin, and Synge's method [9] indicated that 1·8—1·9 moles of compounds similar to 2-aminoethanol had been liberated by the hydrolysis in addition to 0·4—0·6 mole of methylamine. When the 2-aminoethanol was recovered from hydrolysates of the antibiotic by azeotropic distillation with toluene [10] and chromatography on Dowex 50-X4, only 0·5 mole of 2-aminoethanol/540 g. of the dihydrochloride was obtained.

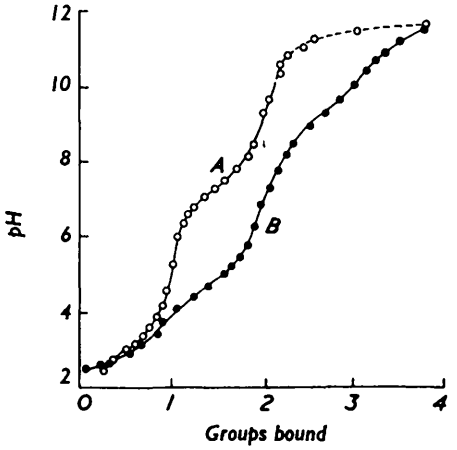

FIG. 3. *Titration curve of the antibiotic dihydrochloride in water at room temperature.*
A, titration with alkali.
B, titration with acid after 2 hr. at pH 11·7.
The broken line shows the region of breakdown of the material.

This could indicate that only one mole of base obtained in the estimation of 2-hydroxyalkylamines arose from 2-aminoethanol and the remainder from another 2-hydroxyalkylamine which was not volatile in toluene.

EXPERIMENTAL

Isolation of the Antibiotic.—Cultivation of the *Streptomyces* (N.C.I.B. 8697) was carried out at the Medical Research Council's Antibiotics Research Station, Clevedon, and the resulting culture fluid (250 l.) was adjusted to pH 4·0 and filtered to remove the mycelium. The clear filtrate was stirred with trichloroethylene (0·1 vol.) for 15 min. and the pH maintained at 9·0—9·5 by the occasional addition of sodium hydroxide. The solvent was removed by centrifugation and the extraction repeated twice. The combined trichloroethylene extracts were concentrated to approximately 2 l. in a climbing film evaporator [11] at approx. 26°.

This solution was extracted with water (2 × 0·1 vol.) maintained at pH 3·0—3·5 with hydrochloric acid The active material was extracted from the aqueous phase (maintained at pH 6·0—6·5) into chloroform (2 × 0·1 vol.). Finally, the active material was removed from the chloroform by two extractions with a convenient volume of water, the pH of which was maintained at 2·0—2·5 by addition of hydrochloric acid, and the aqueous solution was freeze-dried. The yield was 0·4—1·4 g. This material was crystallized from 10% water–propan-1-ol by the addition of acetone and yielded the antibiotic dihydrochloride. This salt ($[\alpha]_D$ +100°; *c* 0·4 in H_2O) decomposed without melting at approx. 145°. Samples for analysis were dried *in vacuo* at room temperature but no special precautions were taken to avoid subsequent hydration (Found: C, 51·0, 49·5, 50·3; H, 6·4, 7·1, 6·8; N, 6·6, 7·5, 7·1; Cl, 12·6, 14·3, 12·3. Calc. for $C_{23}H_{29-31}O_7N_3,2HCl$: C, 51·7; H, 5·8—6·2; N, 7·9; Cl, 13·2%).

Countercurrent Distribution.—The system used consisted of equal volumes (4 ml.) of trichloroethylene and 0·5M-sodium citrate in equilibrium at pH 6·0. The countercurrent distribution was carried out in the fundamental manner [12] with the upper aqueous phase moving. After

[9] Gordon, Martin, and Synge, *Biochem. J.*, 1943, **37**, 538.
[10] Synge, *ibid.*, 1945, **39**, 355.
[11] van Heyningen, *Brit. J. Exp. Path.*, 1949, **30**, 302.
[12] Craig and Craig, "Techniques of Organic Chemistry," Interscience Publ., Inc., London, 1950, Vol. III, p. 171.

eight transfers, the aqueous phase of each tube was adjusted to pH 9·0—9·5 at equilibrium with sodium hydroxide and discarded. The trichloroethylene in each tube was washed with water (1 ml.) (pH 9·0—9·5), and the washings were discarded. The trichloroethylene in each tube was extracted with 0·25N-acetic acid (4·0 ml.) and yielded an aqueous solution of the antibiotic acetate. The results of determinations of the antibacterial activity and the ninhydrin colour densities before and after hydrolysis were related to the determinations of the dry weight of material in each tube, and are shown in Fig. 1.

The material in tubes 1—4 was converted into the dihydrochloride by extraction into chloroform and back into dilute hydrochloric acid.

Examination of Hydrolysates of the Antibiotic.—The antibiotic was hydrolysed by hot N- or 6N-hydrochloric acid in nitrogen-filled sealed tubes at 105° for 10—16 hr. The excess of acid was evaporated in a vacuum-desiccator, and the residues dissolved in water. To isolate the bases, a sample of the antibiotic dihydrochloride (50 mg.) was hydrolysed in 6N-hydrochloric acid (1 ml.) as above for 10 hr. The hydrolysate was dried *in vacuo* in the outer compartment of a standard Conway cell, and the volatile bases were distilled from saturated potassium carbonate (2 ml.) into N-hydrochloric acid (1 ml.) in the centre compartment. The solution from the centre compartment was dried *in vacuo*. The residue from the outer compartment of the Conway cell was azeotropically distilled with toluene, and the aqueous distillate acidified and evaporated to dryness.

The bases volatile from potassium carbonate at 37° were chromatographed on a column of Dowex 50-X4 (105 × 0·9 cm.) and eluted with 0·97N-hydrochloric acid.[13] Chromatography of authentic specimens under these conditions disclosed peaks for ammonia at 146 ml., 2-aminoethanol at 155 ml., and methylamine at 176 ml. (When authentic samples were chromatographed on Dowex 50-X8 in the manner described by Rao, Peterson, and van Tamelen [4] the order of elution was 2-aminoethanol–ammonia–methylamine.) The peak at 176 ml. from the bases volatile from potassium carbonate at 37° accounted for 89% of the ninhydrin colour density of the material applied to the column and the material in this band behaved in the same way as methylamine when subjected to ionophoresis on paper at pH 7·0.

The elution curve of the bases recovered by azeotropic distillation showed peaks at 158 ml. and 177 ml. The first was shown to be due to 2-aminoethanol and the second to methylamine, by ionophoresis on paper at pH 7·0. 2-Aminoethanol accounted for 87%, and the methylamine 16% of the ninhydrin colour density of the material applied to the column.

The recovery of bases by this method (*a*) is compared with the recovery by the methods of Conway [8] and of Gordon, Martin, and Synge [9] (*b*) in the annexed Table.

	Moles of base recovered from 540 g. of the antibiotic.	
Method	Methylamine	2-Aminoethanol
(*a*) Chromatography	0·39	0·53
(*b*) Distilln. and titration	0·55	1·8 *

* Estimated as NH_3 liberated from 2-hydroxyalkylamines by periodate oxidation.

I am indebted to Dr. E. P. Abraham for advice and for much helpful discussion, to Messrs. Hale, Kelly, and Miller of the Medical Research Council's Antibiotics Research Station at Clevedon, Somerset, for the preparation of culture fluids and concentrates of the antibiotic, and to the Australian National University for a scholarship.

Sir William Dunn School of Pathology, Oxford. [*Received, August 3rd,* 1956.]

[13] Hirs, Moore, and Stein, *J. Amer. Chem. Soc.*, 1954, **76**, 6063.