[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, UNIVERSITY OF WISCONSIN]

Xanthomycin A. Production, Isolation and Properties[1]

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Xanthomycin has been produced on a pilot plant scale (50 gal.) and conditions modified so that maximal yields were obtained in 24 hr. The fermented medium contains at least two active components designated A and B. Xanthomycin A accounts for most of the antibiotic activity and has been separated from B by countercurrent distribution. A crystalline hydrochloride of A has been obtained and from this the free base has been prepared. Elementary analyses of the salt and free base and a molecular weight determination made on the free base lead to the molecular formula, $C_{23}H_{29-31}N_3O_7$. Xanthomycin A contains two basic, one methoxyl, one N-methyl and two terminal methyl groups.

The isolation of xanthomycins A and B, two new antibiotics elaborated by an unidentified species of *Streptomyces*, designated S-94, was first reported by Thorne and Peterson.[2] Subsequently Mold and Bartz[3] described an antibiotic which closely resembles xanthomycin A. In addition, several private communications have been received concerning the isolation of xanthomycin-like antibiotics in several pharmaceutical laboratories engaged in the search for new antibiotics.

The xanthomycins are characterized by the bright orange yellow color of their solutions, high activity toward a wide variety of gram-positive and gram-negative organisms and their extraordinary toxicity. Earlier work on these antibiotics dealt with their production in submerged culture fermentation, and separation of the components by Craig countercurrent distribution. Xanthomycin A, the major component of the antibiotic mixture was converted into the crystalline reineckate salt and some of its general properties were described. This paper is a continuation of the work on xanthomycin A and includes further studies on the production and properties of the antibiotic as a first step toward gaining insight into its chemical nature.

Production and Isolation of Xanthomycin A.—The antibiotic mixture was produced following, in general, the procedure of Thorne and Peterson with some modifications. The organism was grown in submerged culture in both 30-liter and 50-gallon fermentors. When the temperature of the fermentation was raised from 25 to 30°, the time required for the maximum yield was considerably shortened and the amount of the antibiotic produced was somewhat higher. For instance, the antibiotic production reached a maximum of approximately 25,000 to 30,000 units (38 to 45 γ) per ml. in about 24 hours as contrasted with a yield of 20,000 units per ml. in 45 to 50 hours at 25°. However, the destruction of the antibiotic at 30° also proceeds at a more rapid rate, thus necessitating rapid isolation after the maximum yield has been reached.

Separation of the antibiotic mixture into the components A, B and the minor component was carried out by Craig counter-current distribution. The xanthomycin A fractions were purified by a repetition of the same procedure and were obtained either as the free base or hydrochloride.

Homogeneity and Stability of Xanthomycin A.—Thorne and Peterson reported that the xanthomycin complex could be resolved into three components by using a 24-transfer countercurrent distribution method. Mold and Bartz, who employed a 48-transfer distribution, found four components in their mixture. They also reported that when seemingly pure major fractions were recycled in the Craig apparatus there were indications of the reappearance of the other fractions. They concluded that the antibiotic could not be purified by the technique employed.

It also has been observed repeatedly in the present work that xanthomycin A continually undergoes transformation into other active components which differ markedly from the starting material in solubility behavior. For instance, when an aqueous solution of xanthomycin A is extracted with chloroform at *p*H 6.0 to 7.0, it passes almost completely into the solvent phase. When the chloroform extract is washed with dilute hydrochloric acid the antibiotic goes into the water layer, but when this acid solution is again neutralized to *p*H 6.0 to 7.0 and extracted with chloroform only part of the color and activity can be obtained in the solvent phase. The fraction left in the aqueous layer behaves like xanthomycin B in a countercurrent distribution analysis. This type of transformation takes place constantly in solution so that all samples obtained by the Craig countercurrent distribution procedure contain varying amounts of the other component or components which behave like xanthomycin B.

A study of this transformation was made by means of paper chromatography. The results of this study are shown in Fig. 1. All preparations of xanthomycin A show two active zones, one of which is close behind the solvent and the other near the origin. The slower moving component corresponds to xanthomycin B while the faster moving one is xanthomycin A (strip A in Fig. 1). When a sample of xanthomycin A was extracted from aqueous solution at *p*H 6.0 to 7.0 into chloroform, the chloroform extract contained all the xanthomycin A and a small amount of xanthomycin B (strip B) and the aqueous layer contained only xanthomycin B (strip C). In order to observe the transformation of xanthomycin A into the other components, a sample was chromatographed in the usual manner. After the development the strip was cut in half lengthwise. One half of this was plated to locate the zones. The portions on the

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(2) C. B. Thorne and W. H. Peterson, *J. Biol. Chem.*, **176**, 413 (1948).

(3) J. D. Mold and Q. R. Bartz, THIS JOURNAL, **72**, 1847 (1950).

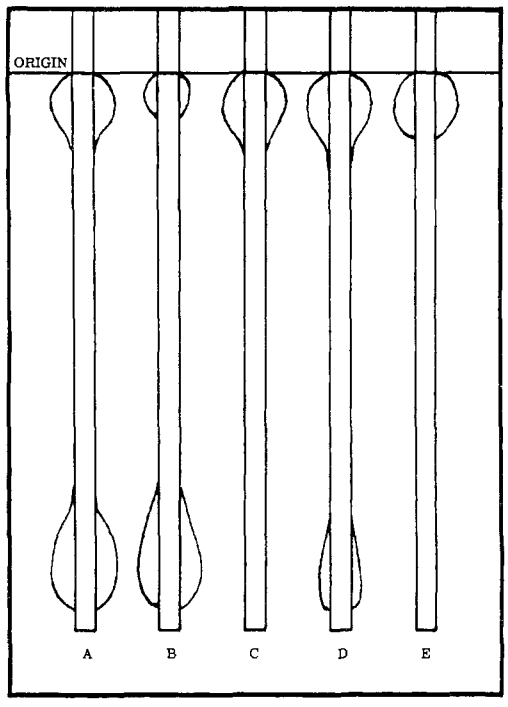

Fig. 1.—Paper chromatograms on xanthomycin A preparations. The samples were applied at the marks near the top and the ends of the trips mark the solvent front.

other half of the strip corresponding to zones of activity were cut out and eluted separately with 80% aqueous methanol containing some hydrochloric acid. These eluates were respotted on two different strips which were developed and plated. It was found that the slower moving component gave rise to two spots, a small amount of the faster moving component corresponding to xanthomycin A and a slower moving spot accounting for the major portion of the activity (strip D). The faster moving component (xanthomycin A) was completely converted into the slower moving material (strip E). These results add further support to the ready transformation of xanthomycin A into other active materials even under mild acid conditions. The reverse change evidently proceeds only to a limited extent under normal conditions. All samples of xanthomycins purified by the Craig countercurrent distribution showed this behavior and it is not possible to prevent this facile change. For the chemical work described in this and subsequent papers the samples of xanthomycin A used were those obtained by the Craig countercurrent distribution procedure repeated twice, even though these showed the presence of a minor component resembling xanthomycin B.

Elementary Composition of Xanthomycin A.—Elementary analysis of the crystalline reineckate of xanthomycin A previously has been reported by Thorne and Peterson as well as by Mold and Bartz. These analyses agreed in general except in the case of sulfur. This led to the assignment of two different empirical formulas for the reineckate salt by the two groups of workers: $C_{34}H_{50}N_6O_{13}\cdot H[Cr(CNS)_4(NH_3)_2]$ and $C_{25}H_{35}N_3O_7\cdot H[Cr(CNS)_4(NH_3)_2]$. Based on these formulas the equivalent weight of the free base would be 742 and 489, respectively.

The above discrepancy may be due in part to the choice of the reineckate for analytical purposes. The tendency of the reineckate ion to decompose during crystallization and the high proportion of the salt contributed by the reineckate ion, make the analytical values obtained uncertain.

In order to resolve this discrepancy xanthomycin A was converted into the crystalline hydrochloride and analyzed. Several batches of the hydrochloride gave consistent values in agreement with the formula $C_{23}H_{29-31}N_3O_7\cdot 2HCl$. This formula was further confirmed by a complete elementary analysis and molecular weight determination of the free base of xanthomycin A.

In addition xanthomycin A has been found to contain one methoxyl, one methylimide and two terminal methyl groups. Zerewitinoff determination showed the presence of one active hydrogen atom per molecule of the free base. A Van Slyke determination of primary amino groups in xanthomycin A hydrochloride showed the absence of such groups in 5- or 20-minute reaction times. When the reaction was prolonged for several hours, a slow evolution of nitrogen took place, due probably to the decomposition of the substance.

Properties of Xanthomycin A.—Xanthomycin A is a deep orange-red basic substance with a marked bitter taste. Its solutions in dilute acid are bright yellow in color and in dilute alkali are deep reddish pink. This color change is essentially reversible although the activity is lost during this treatment. When left in contact with air, the alkaline solution changes irreversibly to pale yellow. With alcoholic sodium ethoxide xanthomycin A gives an evanescent purple color which changes to yellow.

Xanthomycin A exhibits a characteristic absorption spectrum in the ultraviolet region. There has been some discrepancy between the results of Thorne and Peterson and of Mold and Bartz regarding a part of the ultraviolet absorption spectrum. The spectrum reported by the former group for the hydrochloride showed two maxima, one at 265 mμ ($E^{1\%}_{1\,cm.}$ 185) and another at 326 mμ ($E^{1\%}_{1\,cm.}$ 76.5). The spectrum described by Mold and Bartz for the same salt exhibited the major maximum at 264.5 mμ ($E^{1\%}_{1\,cm.}$ 179.4) but the intensity of the minor maximum was much less ($E^{1\%}_{1\,cm.}$ 18.8 at 335 mμ).

The absorption spectra of xanthomycin A free base and of the hydrochloride are shown in Fig. 2. The hydrochloride has a sharp maximum at 265 mμ and a low flat rise between 340 and 380 mμ with a maximum at 345 mμ. This maximum depends on the solvent employed and in the case of water on the *p*H of the solution. The sharp peak agrees well with that previously reported by Thorne and Peterson but the second is in better agreement with that given by Mold and Bartz. In solutions above *p*H 7.0 xanthomycin A readily undergoes decomposition and the spectrum changes rapidly with time. The absorption spectrum of the free base of xanthomycin A is strikingly different from that

of the hydrochloride and has not been described before. It shows two maxima at 288 and 460 mμ and two points of inflection at 225 and 315 mμ.

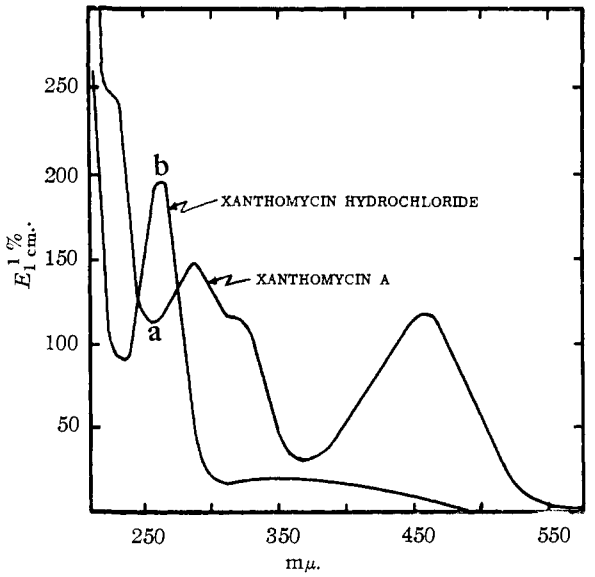

Fig. 2.—Absorption spectra of xanthomycin A: a, free base in absolute ethanol; b, hydrochloride in 0.1 *N* hydrochloric acid.

The infrared absorption spectra of xanthomycin A and its hydrochloride are shown in Figs. 3 and 4. The spectra seem to indicate the absence of hydroxyl, amino and carboxyl groups and the presence of an amide or highly conjugated carbonyl group in the molecule.

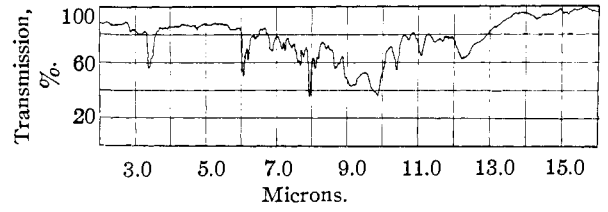

Fig. 3.—Infrared absorption spectrum of xanthomycin A in carbon tetrachloride.

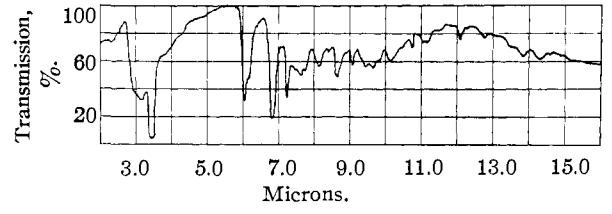

Fig. 4.—Infrared absorption spectrum of xanthomycin A hydrochloride in a Nujol mull.

In order to determine the nature of the basic groups in xanthomycin A, a study was made of its titration behavior. The titration curves of xanthomycin A and its hydrochloride as measured with a glass electrode are shown in Fig. 5. Xanthomycin A possesses two weakly basic groups with approximate mid-point titration values of 2.0 and 4.5. The titration curve of the hydrochloride also shows similar behavior. When the titration of the hydrochloride with alkali was continued beyond *p*H 7.0, at least one more equivalent of alkali was consumed with a mid-point of titration at *p*H 9.5. This is due to the rapid and irreversible decomposition of xanthomycin A above *p*H 7.0.

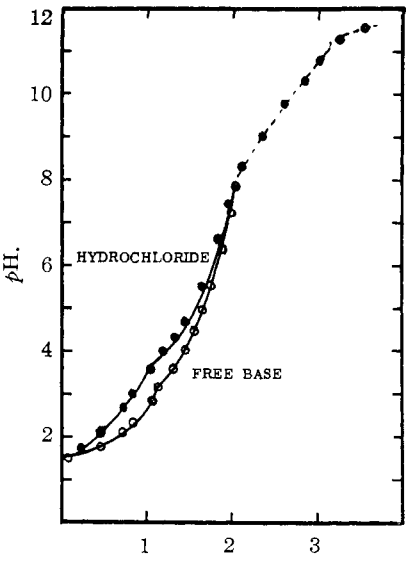

Fig. 5.—Titration curves of xanthomycin A and of the hydrochloride.

Experimental

Fermentation.—A 50-gallon glass-lined Pfaudler fermentor equipped with agitation, sterile air supply and automatic temperature and foam control described by Buelow and Johnson[4] was employed for the fermentation. The medium slightly modified from that of Thorne and Peterson consisted of the following components, g. per liter: corn steep liquor, 20; soybean meal, 10; sodium chloride, 10; calcium carbonate, 1; lactose, 10; glucose, 5.

The mixture was adjusted to *p*H 7.0 and sterilized in the tank. A solution of dibasic ammonium phosphate sufficient to give 1% concentration in the medium was adjusted to *p*H 7.0, sterilized separately and added to the rest of the medium in the tank. Five per cent. by volume of a 48-hour vegetative inoculum was used. The temperature was maintained at 30°, the agitation at 150–180 r.p.m. and aeration at 1.2 cu. ft. per minute. A solution of 6% Alkaterge C in lard oil was employed for foam control. The broth was harvested after 24–28 hours when the activity reached about 25,000 to 30,000 units per ml. After the maximum is reached the activity starts to decrease rapidly. As much as 50–60% of the maximum activity was found to be destroyed in 2–5 hours. The activity could be preserved until the broth is processed by adjusting it to *p*H 2.0 and chilling.

The antibiotic was assayed either by the turbidimetric procedure of Thorne and Peterson[2] or by the Oxford cup-plate method[5] with *Micrococcus pyogenes* var. *aureus* strain H as the assay organism. Crystalline xanthomycin was used as the standard.

The method of isolation of the crude antibiotic mixture from the broth was in general similar to that employed by Thorne and Peterson. Separation of the components by countercurrent distribution between chloroform and 0.5 *M* citrate buffer at *p*H 4.2 was carried out in 16 to 20 separatory funnels for handling conveniently large enough batches of material. After the countercurrent distribution, the fractions containing xanthomycin A were collected, brought into chloroform and again subjected to countercurrent distribution. The fractions containing xanthomycin A were repeatedly extracted with chloroform, the solution was

(4) G. H. Buelow and M. J. Johnson, *Ind. Eng. Chem.*, **44**, 2945 (1952).

(5) W. H. Schmidt and A. J. Moyer, *J. Bact.*, **47**, 199 (1944).

dried over sodium sulfate, filtered and evaporated to dryness, to give xanthomycin A as a deep orange-red amorphous solid.

Crystalline Hydrochloride of Xanthomycin A.—Xanthomycin A was dissolved in absolute ethanol and treated with a solution of dry hydrogen chloride in the same solvent. The deep orange-red solution turned bright yellow. The solution was evaporated to dryness *in vacuo* and the orange-yellow crystalline residue recrystallized from a mixture of absolute ethanol and ethyl acetate (3:1). It separated out in the form of bright orange-yellow rectangular plates.

Anal. Calcd. for $C_{23}H_{29-31}N_3O_7 \cdot 2HCl$: C, 51.76; H, 6.08; N, 7.88; Cl, 13.32; $-OCH_3$ for 1, 8.44; $-NCH_3$ for 1, 2.81; $-CCH_3$ for 2, 5.62. Found: C, 51.76, 51.94; H, 6.25, 6.71; N, 7.94, 7.82; Cl, 13.71, 12.57; $-OCH_3$, 9.15; $-NCH_3$, 2.62; $-CCH_3$, 5.26. An aqueous solution of the hydrochloride showed a specific rotation, $\alpha^{25}D$ +115° (*c* 0.4 in water). The absorption spectrum in 0.1 *N* hydrochloric acid (Fig. 2) showed maxima at 265 mμ ($E^{1\%}_{1\,cm.}$ 196) and at 345 mμ ($E^{1\%}_{1\,cm.}$ 19.8).

The free base of xanthomycin A was purified by precipitating twice from a chloroform solution with ether. The product was a deep orange-red amorphous solid.

Anal. Calcd. for $C_{23}H_{29-31}N_3O_7$: C, 60.00; H, 6.52; N, 9.01; O, 24.35; $-NCH_3$ for 1, 3.26; mol. wt., 460. Found: C, 60.15, 60.40; H, 6.32, 6.56; N, 8.75, 9.00; O by direct analysis, 24.73; $-NCH_3$, 3.16; mol. wt. by ebullioscopic method in methanol, 455, 512, 520. The sum of the averages for the elementary composition, 100.32%, shows that xanthomycin A contains no other elements than C, H, N and O. The absorption spectrum in absolute ethanol (Fig. 2) has two maxima, one at 288 mμ ($E^{1\%}_{1\,cm.}$ 148) and the other at 460 mμ ($E^{1\%}_{1\,cm.}$ 118), and points of inflection at 225 mμ ($E^{1\%}_{1\,cm.}$ 253) and 315 mμ ($E^{1\%}_{1\,cm.}$ 116).

Paper Chromatography.—One to two micrograms of xanthomycin A hydrochloride was spotted on filter paper strips (0.5 × 60 inches, Eaton Dikeman 613) and developed with 1-butanol containing 1% by volume of 1 *N* hydrochloric acid. After the downflow development at 37° the strips were dried and analyzed by plating them on agar seeded with spores of *Bacillus subtilis* according to the method of Karnovsky and Johnson.[6]

(6) M. L. Karnovsky and M. J. Johnson, *Anal. Chem.*, **21**, 1125 (1949).

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Xanthomycin A. Quinonoid Behavior[1]

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Xanthomycin A ($C_{23}H_{29-31}N_3O_7$) has quinonoid properties. Sulfur dioxide, stannous chloride and titanous chloride reduced it to dihydroxanthomycin which was isolated as the sulfate $C_{23}H_{31-33}N_3O_7 \cdot H_2SO_4$. In the presence of Adams catalyst xanthomycin A takes up two moles of hydrogen to form tetrahydroxanthomycin A which is readily reoxidized by ceric sulfate to isodihydroxanthomycin A. In this oxidation only one mole of oxygen is consumed. Because of this behavior it appears that xanthomycin A has two centers of unsaturation. One of these is irreversibly reduced by catalytic hydrogenation. The other is a quinonoid system which may be reversibly reduced and oxidized. The acetyl derivative of tetrahydroxanthomycin A forms an unstable crystalline chloroplatinate having the composition $C_{23}H_{33-35}N_3O_7(CH_3CO)_4 \cdot H_2PtCl_6$. The corresponding methyltetrahydroxanthomycin A has been prepared and isolated as the stable crystalline chloroplatinate, $C_{25}H_{37-39}N_3O_7 \cdot H_2PtCl_6$. Oxidation–reduction potentials of xanthomycin A and isodihydroxanthomycin A indicate that a benzoquinone type of structure exists in xanthomycin A.

Xanthomycin A is a basic antibiotic with a molecular formula $C_{23}H_{29-31}N_3O_7$ and contains two salt-forming groups, one methoxyl, one methylimide and two terminal methyl groups.[2]

Xanthomycin A exhibits several properties typical of quinonoid substances. It is deep orange-red in color, dissolves in aqueous alkali to give a bright reddish pink solution. In alcoholic sodium methoxide it forms an intense purple color. It releases iodine from acidified potassium iodide solutions and is reduced by sulfur dioxide.

In their preliminary investigation on xanthomycin A, Mold and Bartz[3] showed that the antibiotic could be reduced catalytically to a colorless, microbiologically inactive product which regained its color but not its activity on shaking with air. This reoxidized material could again be reduced to a colorless compound but less hydrogen was consumed. Thiele acetylation or chemical reductive acetylation of xanthomycin A gave no loss of color. However, no quantitative studies were reported on the reduction of xanthomycin A in their work. Our paper deals with studies on the reduction of xanthomycin A, made with a view to obtaining information regarding the nature of the quinonoid system in the molecule.

Stannous chloride, titanous chloride and sulfur dioxide reduce xanthomycin A to a light yellow, unstable dihydro derivative. In the presence of air this product readily undergoes reoxidation. Attempts to stabilize dihydroxanthomycin A by acetylation under various conditions resulted invariably in the formation of dark-colored resinous products.

Xanthomycin A can be reduced in the presence of Adams catalyst to the tetrahydro derivative. If tetrahydroxanthomycin A is exposed to air while in solution, it rapidly undergoes reoxidation to the corresponding quinone, isodihydroxanthomycin A. Conventional methods of acetylation of tetrahydroxanthomycin led to considerable decomposition. However, when hydrogenation and acetylation were carried out simultaneously, a colorless, stable derivative was obtained. The acetyltetrahydroxanthomycin A was purified as the crystalline chloroplatinate and analyzed. It contained four acetyl and two basic nitrogen groups. Although stable to atmospheric oxidation, it was unsuitable for degradation work because it was readily deacetylated in acid or alkaline solutions and then gave rise to tarry decomposition products.

(1) Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. This investigation was supported in part by research grants from the National Institutes of Health, Public Health Service, and Chas. Pfizer and Co., Inc. Presented at the 121st meeting, American Chemical Society, Milwaukee, 1952.

(2) K. V. Rao and W. H. Peterson, THIS JOURNAL, **76**, 1335 (1954).

(3) J. D. Mold and Q. R. Bartz, *ibid.*, **72**, 1846 (1950).