An Antibiotic Related to the Xanthomycins

BY JAMES D. MOLD[1] AND QUENTIN R. BARTZ

An antibiotic, which possesses many properties similar to those reported for the xanthomycins,[2a,b] has been obtained from culture filtrates of several unidentified isolates of *Streptomyces*. A brief description of this work is presented in this paper.

A typical culture filtrate required the following dilutions to produce a 50% inhibition of growth when assayed turbidimetrically[3] against the following organisms: *S. aureus*, 1:12,000; *K. pneumoniae*, 1:500; *S. paradysenteriae* (Sonne), 1:400; *S. schottmuelleri*, 1:250; *E. coli*, 1:150. A dilution of 1:810 gave the same zone of inhibition as 1 γ of streptomycin base when assayed by the disc-plate agar diffusion method.[4] The microbiological activity in these filtrates, although relatively stable at *p*H 2, was rapidly lost at neutral or alkaline *p*H (Table I). The basic

TABLE I

STABILITY OF THE ANTIBIOTIC IN CULTURE FILTRATES UNDER VARIOUS CONDITIONS OF TEMPERATURE AND *p*H

*p*H	% of activity *vs. B. subtilis* remaining 5°, 24 hr.	25°, 24 hr.	100°, 15 min.
2	100	100	48
7	100	26	0
10	56	0	0

character of the antibiotic was indicated by its solubility in many organic solvents at basic or neutral *p*H and its insolubility at acid *p*H. When a crude culture filtrate was extracted with an equal volume of organic solvent at *p*H 7, the following per cent. of the total activity was found in the solvent phase: benzene, 61%; ethylene dichloride, 88%; ether, 32%; *n*-butyl alcohol, 85%; carbon tetrachloride, 42%; chloroform, 92%; petroleum ether, 0%; *n*-amyl acetate, 9%; and ethyl acetate, 47%. At *p*H 2 the antibiotic was not extracted by these solvents.

The procedure for concentration of the antibiotic from the crude beers was: (1) filtration at *p*H 2; (2) extraction at *p*H 7 with ethylene dichloride; (3) extraction of the ethylene dichloride solution with 0.1 volume of 0.01 *M* hydrochloric acid; (4) repetition of steps 2 and 3 twice more with this acid extract; (5) the final acid extract was dried from the frozen state. By this procedure good yields of yellow-orange glassy concentrates with activities of 30,000–40,000 units/mg. *vs. B. subtilis*[4,5] have been obtained. With material purified by this procedure, experiments were carried out to test the homogeneity of the preparation and to identify it chemically. The Craig technique of counter-current distribution was applied using the method of alternate withdrawal[6] and the active material was resolved into four biologically active components (Fig. 1), one being concentrated in the "aqueous-soluble" tubes (0–11), a trace of another in the "ethyl acetate-soluble" tubes (33–48), and the other two closely associated in the central tubes (12–32).

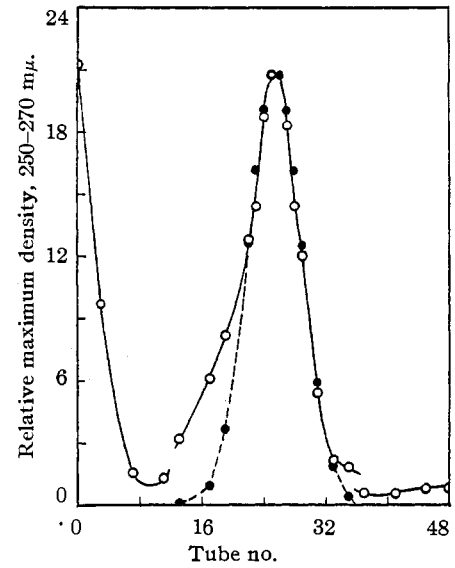

Fig. 1.—Craig distribution: -O- -O-, antibiotic concentrate; -●- -●-, theoretical for a single component. (The biological activities were found to conform to a similar plot.)

The latter two seemed to have identical properties except for their partition coefficients. When seemingly pure central fractions were recycled in the Craig apparatus, indications of the reappearance of the other fractions made it evident that the antibiotic could not be purified by this technique. The most active fraction obtained had an $E^{1\%}_{1\,cm.} = 179.4$, $\lambda = 264.5$ mμ and $E^{1\%}_{1\,cm.} = 18.8$, $\lambda = 335$ mμ (aq., *p*H 2) (*cf.* Fig. 2). The value for the major maximum corresponds very closely to that reported for xanthomycin A while the minor maximum is approximately one-fourth the value reported for xanthomycin A and appears at somewhat longer wave length.[2a] When the ultraviolet absorption was determined in 0.1 *N* sodium hydroxide, an altered spectrum was observed. This was essentially reversible upon reacidification. It was found necessary to conduct experiments with this antibiotic in the absence of ultraviolet light since a rapid loss of microbiological activity was noted upon exposure even at acid *p*H. After irradiation for three hours with a 100 w. Westinghouse Mazda AH4 mercury vapor lamp at a distance of 20 cm., the activity *vs. B. subtilis* had dropped to

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(2) (a) Thorne and Peterson, *J. Biol. Chem.*, **176**, 413 (1948). (b) The major portion of the work reported in this note was carried out prior to the publication of Thorne and Peterson.

(3) Joslyn and Galbraith, *J. Bact.*, **54**, 26 (1947).

(4) Loo, Skell, Thornberry, Ehrlich, McGuire, Savage and Sylvester, *ibid.*, **50**, 701 (1945).

(5) Assayed by the disc-plate agar diffusion method and expressed as equivalent streptomycin units.

(6) Craig, Hogeboom, Carpenter and du Vigneaud, *J. Biol. Chem.*, **168**, 665 (1947).

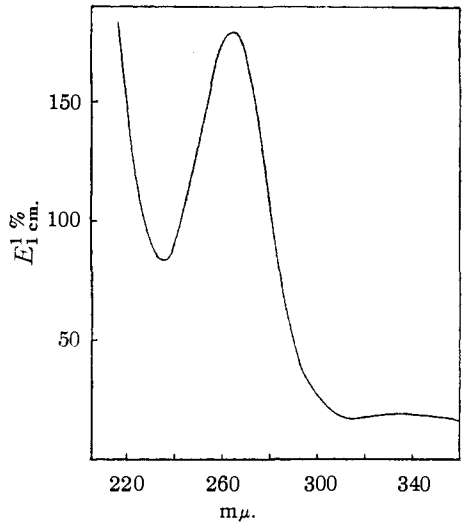

Fig. 2.—Ult raviolet absorption spectrum (aq. *p*H 2).

about one-third its original value and two maxima were observed in the ultraviolet absorption spectrum ($E^{1\%}_{1\,cm.}$ = 86.0, λ = 274 mμ; $E^{1\%}_{1\,cm.}$ = 80.0, λ = 290 mμ (aq., *p*H 2)).

An antibiotic concentrate with $E^{1\%}_{1\,cm.}$ = 176, λ = 267 mμ (aq., *p*H 2) was evaluated *vs.* a number of organisms. The following amounts per ml. were required to give 50% inhibition of growth by the turbidimetric method of assay[3]: *E. coli*, 0.18 γ; *K. pneumoniae*, 0.049 γ; *S. schottmuelleri*, 0.12 γ; *S. paradysenteriae* (Sonne), 0.074 γ; and *S. aureus*, 0.0029 γ.[7] Assay by the streptomycin disc-plate method gave 32,300 units/mg. *vs. B. subtilis* and 190 units/mg. *vs. B. mycoides*.[4,5] Acute intravenous toxicity in mice gave a M. T. D. of 1.2 γ/20 g. mouse and a LD_{50} of 2.0 γ/20 g. mouse.

Elementary analysis indicated that carbon, hydrogen, nitrogen, and ionic chlorine are present and sulfur is absent. Positive reactions were obtained with the following reagents: Benedict; bromine in carbon tetrachloride; potassium permanganate; silver nitrate ($Ag^+ \rightarrow Ag^0$); acidified potassium iodide ($2I^- \rightarrow I_2$); sodium hydrosulfite; and periodic acid. A slight precipitate was obtained with 2,4-dinitrophenylhydrazine and with dimedone and a deep red solution was formed with hydroxylamine. Negative reactions were obtained with acidified stannous chloride, fuchsin reagent and cysteine. The latter produced a stabilization of the activity. Acid solutions of the antibiotic are yellow and alkaline solutions deep red. Reduction with Adams platinic oxide catalyst gave a loss of color and activity. The color returned upon shaking with air but the product was inactive microbiologically. The same sample could then be reduced to a colorless compound again but consumed less hydrogen. Thiele acetylation gave no loss of color nor did chemical reductive acetylation. Catalytic reduction in acetic acid–acetic anhydride (1:4) gave a colorless crystalline product which was recrystallized from acetic acid by the addition of acetic anhydride ($E^{1\%}_{1\,cm.}$ = 86.2, λ = 295 mμ (aq., *p*H 2); $E^{1\%}_{1\,cm.}$ = 114.4, λ = 284 mμ (aq., *p*H 11). Irradiation of this material at *p*H 4.6 with ultraviolet light produced a relatively slight change in spectrum with a broadening of the band.

In order to compare these antibiotic concentrates with xanthomycin, the conditions for Craig counter-current distribution used by Thorne and Peterson[2a] were applied. A distribution pattern was obtained which closely duplicated that reported for xanthomycin giving evidence for the close similarity of the two preparations. A reineckate was also prepared. The analyses of this salt are in fair agreement with those reported for xanthomycin reineckate[2a] with the exception of the value for sulfur (15.62 compared with 12.10 found by Thorne and Peterson). The ash value obtained, also, is not in agreement with that calculated for the formula $C_{38}H_{57}N_{12}O_{13}S_4Cr$, proposed by these workers (calcd. ash, Cr_2O_3, 7.10; found, 10.77). Although the ash determination is subject to a rather larger error than the other analyses, the value obtained is in better agreement with the formula $C_{29}H_{42}N_9O_7S_4Cr$.

Many of the properties of this antibiotic, including microbiological activity, toxicity, ultraviolet absorption, and chemical reactivity, are consistent with a *p*-quinone system which is highly substituted.[8] Further work will be necessary to substantiate this.

Experimental

Preparation of Concentrates of the Antibiotic from Culture Beers.—A typical culture beer of the antibiotic (52 l.) was filtered through 1800 g. of Hyflo Super-Cel in a filter press and the cake rinsed with 7 l. of water. The combined filtrates and washings (46 l. containing 44.2×10^6 units *vs. B. subtilis*) were adjusted to *p*H 7.0 with aqueous sodium hydroxide and extracted with 46, 23 and 23 l. of ethylene dichloride. The combined ethylene dichloride solutions were extracted with 9 l. of 0.01 *M* hydrochloric acid (40.5×10^6 units). A combined lot of comparable acid extracts (10 l. containing 30.4×10^6 units) was adjusted to *p*H 7.8 and extracted with 10 l., 10 l., 5 l., and 5 l. of ethylene dichloride and the resulting ethylene dichloride solutions combined and extracted with 2 l. of 0.01 *N* hydrochloric acid (43×10^6 units). This extract was adjusted to *p*H 7.8 and extracted with four 2 l. portions of ethylene dichloride. The combined ethylene dichloride solutions were extracted with 400 ml. of 0.01 *M* hydrochloric acid (32.4×10^6 units) and dried from the frozen state to give 792 mg. of yellow-orange glassy solid which assayed 34,000 units/mg. *vs. B. subtilis*.

Counter-current Distribution with the Antibiotic Concentrate.—Concentrates of the antibiotic which had been prepared as described above were distributed by the Craig technique between ethyl acetate and an aqueous buffer solution 1.0 *M* in phosphate at a *p*H of 6.2–6.3. At the conclusion of the experiment each tube was acidified to *p*H 2 with concentrated hydrochloric acid and the active material extracted into the aqueous phase from which it was obtained by the procedure described above for crude concentrates.

(7) For comparison with Chloromycetin see Ehrlich, Bartz, Smith, Joslyn and Burkholder, *Science*, **106**, 417 (1947).

(8) Oxford, *J. Soc. Chem. Ind.*, **61**, 189 (1942); Gulland, *Biochem. J.*, **26**, 32 (1932); Braude, *J. Chem. Soc.*, 490 (1945).

Preparation of the Crystalline Reineckate of the Antibiotic.—This salt was precipitated by addition of a saturated aqueous solution of freshly recrystallized ammonium reineckate to a neutral aqueous solution of the antibiotic. After crystallizations from 95% ethanol and 50% aqueous acetone, red needles were obtained which gave no characteristic melting point. Assay: 15,000 units/mg. *vs. B. subtilis*.[4,5] *Anal.*[9] Calcd. for $C_{29}H_{42}N_9O_7S_4Cr$: C, 43.05; H, 5.24; N, 15.58; S, 15.85; ash (Cr_2O_3), 9.40. Found: C, 43.17, 43.27; H, 5.24, 5.33; N, 15.36; S, 15.62; ash, 10.77.

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(9) Microanalyses were performed by C. W. Beazley.

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The Decarboxylation of Simple Fatty Acids

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Although textbooks almost universally state that fusion of the fatty acid salts with sodium hydroxide yields hydrocarbons according to the equation: $RCO_2Na + NaOH \rightarrow RH + Na_2CO_3$, it is difficult to support this statement with evidence. Berthelot[1] reported in 1866 that this reaction was not general.

We have heated the sodium salts of acetic, propionic, butyric and caproic acids with equimolar quantities of sodium hydroxide, and separated the gaseous products. An examination of the data in Table I shows that the decomposition of sodium acetate alone gives products in accord with the above equation.

TABLE I

Sodium salt (0.05 mole)	Acetate	Propionate	Butyrate	Caproate
Reaction temp., °C.	371–376	370–380	360–365	355–360
Gas liberated, mole	0.045	0.044	0.07	0.056
	Composition of gas, %			
H_2	0.5	33	31	38
CH_4	98.9	20	39	37.6
C_2H_6	..	44	7	1.4
C_3H_8	..	..	17	1.3
C_4H_{10}	..	..	..	3.8
C_5H_{12}	..	..	..	12.1
Unsaturates	..	0.3	5.7	2.5

It is noted that these decompositions all occur in the same temperature range. The reaction is exothermic and the major portion of the gas is liberated in a few minutes. Analysis of the water-soluble residues showed that in all cases the amount of sodium carbonate formed was above 90% of the theoretical.

(1) Berthelot, *Ann. chim. phys.*, [4] **9**, 444 (1866).

Berthelot considered that the formation of methane, hydrogen, etc., from sodium propionate was caused by the thermal decomposition of the ethane first formed. Although this explanation may, in part, account for these products, the thermal stability of ethane and propane at such temperatures[2] and the rapidity of the reaction suggest that the primary pyrolytic products are complex. In any case the method is not suitable for the preparation of the simple paraffin hydrocarbons.

Experimental

The sodium salts, excepting the acetate, were prepared from aqueous sodium hydroxide and an excess of the acid, followed by evaporation to dryness. After washing with ether, the salts were recrystallized from water and dried *in vacuo* to constant weight over phosphorus pentoxide.

By calculation from the analyses for sodium, all the salts were better than 98% pure.

The apparatus for the fusion consisted of a side-arm test-tube (35 mm. o.d.) placed inside a jacket wound with a heating element, and insulated. The test-tube was closed with a rubber stopper carrying a thermocouple well and connected through a condenser to a gas collecting bottle.

A mixture of the dried sodium salt (0.05 mole) and sodium hydroxide (0.05 mole), powdered in a "dry box," was placed in the test-tube and the system flushed with dry nitrogen. The mixture was heated to the decomposition temperature, when the internal temperature increased and gas was rapidly evolved; most of the gas was evolved in about five minutes.

The gases were separated by a cryostat, using isothermal distillations at successive temperatures.[3,4] The gas fractions were identified by combustion analyses. The data are shown in Table I.

The carbonate in the residues was determined by standard procedures.

(2) Egloff, "Reactions of Pure Hydrocarbons," Reinhold Publishing Corp., New York, N. Y., 1937, p. 99 *et seq.*, p. 119 *et seq.*

(3) Ailman, Ph.D. Thesis, The Pennsylvania State College, 1938.

(4) We are indebted to Dr. H. D. Zook and Mr. W. J. McAleer for these analyses.

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Mass Spectrometric Evidence for a New Boron Hydride

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In examining with the mass spectrometer the residues from pure B_5H_{11} which had been stored at −78° for a long time, there was detected a small amount of $B_{10}H_{14}$, B_6H_{10} and B_5H_9 with the B_5H_{11}. In addition, a group of hitherto unobserved peaks, dominant peak mass 105, was found. It is believed they represent a new boron hydride, B_9H_{13}.

The spectrum of pure $B_{10}H_{14}$ is given in Fig. 1 (A), from mass 85 to 124, the parent peak. This was obtained with mass spectrometer operating conditions already described.[1] Peaks of double ionization for $B_{10}H_{14}$ were observed in the region 55–59.

(1) F. J. Norton, THIS JOURNAL, **71**, 3488 (1949)