g., with vasodepressor activity equivalent to 0.48 mcg. of histamine per mg. A small aliquot was treated with one-half its weight of Decalso without changing the histamine assay. This residual vasodepressor activity, a small fraction of that present in the crude material, appears to be inherent in streptothricin.

The material was then finally dissolved in water, treated with 30% (w./w.) of carbon to ensure freedom from pyrogens, filtered aseptically, and measured into sterile vials of 0.5 g. each. After freeze-drying, vials were plugged aseptically, capped and identified as Streptothricin Sulfate, Upjohn Research No. 8804. The product passed routine F.D.A. streptomycin control tests for sterility, toxicity and pyrogens, but not for vasodepressor activity. The bioassay against streptomycin on a *B. subtilis* plate[17] was about 400 mcg. of streptomycin free-base per mg. This material has been distributed widely to investigators since its preparation in 1948 and has served for numerous pharmacological, biochemical and chemical studies.

(17) Y. H. Loo, P. S. Skell, H. H. Thornberry, John Ehrlich, J. M. McGuire, G. M. Savage and J. C. Sylvester, *J. Bact.*, **50**, 701 (1945).

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[CONTRIBUTION FROM THE LILLY RESEARCH LABORATORIES, ELI LILLY AND COMPANY]

The Isolation of a Second Crystalline Antibiotic from *Streptomyces erythreus*

BY C. W. PETTINGA, W. M. STARK AND F. R. VAN ABEELE

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S. erythreus grown on a variety of media has been shown to produce two antibiotic substances, erythromycin and erythromycin B. Procedures are described for the separation of these antibiotics. Isolation of erythromycin B was accomplished by use of chromatography on powdered cellulose and countercurrent distribution. Properties and partial characterization of the new antibiotic are described.

The isolation of erythromycin, a clinically useful antibiotic, from *Streptomyces erythreus* has been reported.[1] Paper chromatographic methods,[2] developed primarily to aid in isolation and fractionation of erythromycin, gave evidence that more than one antibiotic substance can be produced by certain strains of *Streptomyces erythreus*. Details of the isolation and partial characterization of a second antibiotic substance, erythromycin B, are presented in this paper.

When *S. erythreus* is grown in a fermentation medium and the resultant broth is analyzed by paper chromatography using a solvent system composed of methanol, acetone and water (19:6:75), two zones of antibiotic activity can be detected. The more mobile zone having an R_f of approximately 0.7 has been identified and associated with erythromycin. The less mobile component, erythromycin B, has an R_f of 0.6. If the chromatograms are run two dimensionally, no further separation of the two components can be demonstrated. Furthermore, when pure erythromycin is added to a sample of erythromycin B obtained by elution of a paper chromatogram, two zones of antibiotic activity can be demonstrated by subsequent analysis of the mixture by paper chromatography.

The behavior on paper chromatograms was used as the basis for development of a method of isolation of erythromycin B. A crude concentrate containing erythromycin and erythromycin B was first obtained by extraction of the fermentation broth with chloroform or amyl acetate at *p*H 9.5. After concentration to a small volume, powdered cellulose[3] was added. The cellulose with adsorbed erythromycin was thoroughly dried and was packed into the top portion of a column that had been previously packed with dry cellulose powder. The column was developed with a solvent mixture composed of 0.01 *N* ammonium hydroxide saturated with methyl isobutyl ketone. In a typical experiment 15-ml. fractions were collected on an automatic fraction collector. Antibiotic activities in the various fractions were determined (*cf.* Fig. 1). Paper strip chromatography demonstrated that the first peak, fractions 9 to 18, contained only erythromycin, whereas the second peak, fractions 28 to 65, contained only erythromycin B. The latter fractions were pooled and further purified to yield the crystalline antibiotic.

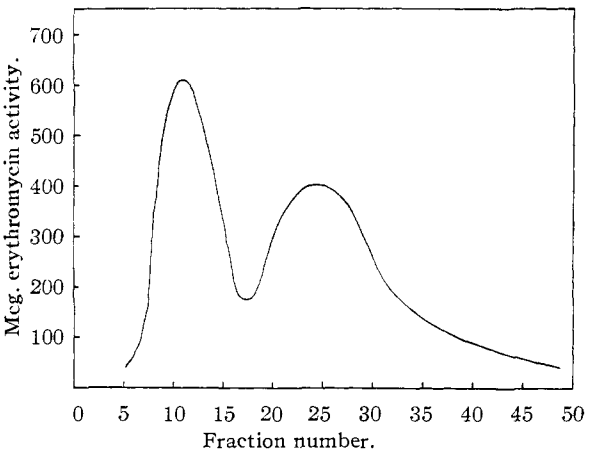

Fig. 1.—The separation of a mixture of erythromycin (fractions 6-14) and erythromycin B (fractions 21-48) on a cellulose column.

The solubility and distribution properties of erythromycin B prepared in this manner were sufficiently different from those of erythromycin to suggest the possibility of separation by countercurrent distribution. For distribution studies an all-glass 100-tube Craig apparatus was used.[4]

(1) J. M. McGuire, *et al.*, *Antibiotics and Chemotherapy*, **2**, 281 (1952).

(2) Details of the paper chromatographic methods will be published by H. L. Bird and C. T. Pugh.

(3) Solka-Floc, a purified wood cellulose was used in these experiments.

(4) The apparatus is described and illustrated in a chapter by L. C. Craig and D. Craig in "Technique of Organic Chemistry," Vol. III, Arnold Weissberger, Ed., Interscience Publishers, Inc., New York, N. Y., 1950, pp. 285–287. It was purchased from the H. O. Post Scientific Instrument Co., New York.

TABLE I

MEDIA USED FOR GROWING STREPTOMYCES ERYTHREUS

Sporulation medium	g./l.	Vegetative medium	g./l.	Fermentation medium	g./l.
Dextrin	15.0	N Z Amine A (Sheffield Farms)	10.0	Sucrose	68.4
Tryptone	5.0			Glycine	7.5
Betaine	0.5	Blackstrap molasses (USI)	20.0	DL-Alanine	0.9
Agar	20.0			K_2HPO_4	5.0
K_2HPO_4	0.2	K_2HPO_4	1.5	NaCl	5.0
NaCl	.2	*p*H 7.0–7.2		$MgSO_4$	0.5
$MgSO_4$	.2			$FeSO_4 \cdot 7H_2O$	.02
$CaCl_2$	.08			$ZnSO_4 \cdot 7H_2O$	.01
Trace elements ($FeSO_4$, $ZnSO_4$, $CuSO_4$, $MnCl_2$ and $CoCl_2$)				$MnCl_2 \cdot 4H_2O$	.008
*p*H 7.2				$CoCl_2 \cdot 6H_2O$	.001
				$CaCO_3$	3.0
				*p*H 7.5	

The solvent system was composed of 20 parts of methyl isobutyl ketone, 1 part of acetone and 20 parts of 0.1 *N* phosphate buffer at *p*H 6.5. A typical activity distribution curve is shown in Fig. 2.

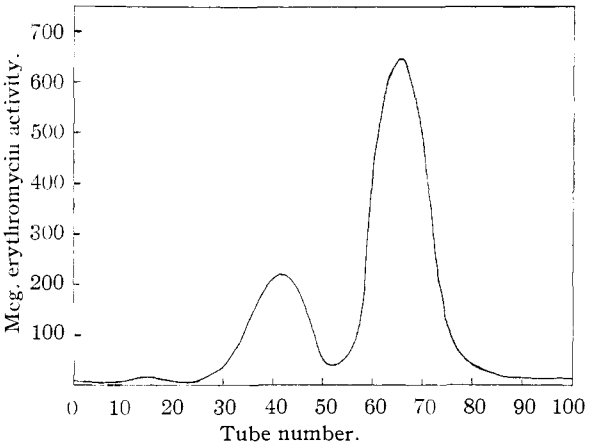

Fig. 2.—Countercurrent distribution of a crude antibiotic preparation containing erythromycin (tubes 30–50) and erythromycin B (tubes 60–80).

Final purification and crystallization of erythromycin B was accomplished in the following manner. The appropriate fractions from the column or the countercurrent separation were concentrated *in vacuo* to about one-tenth volume, and the antibiotic was recovered from the aqueous solution at *p*H 9.5 with chloroform. After removing the chloroform by evaporation, the antibiotic was crystallized from acetone.

Properties of Erythromycin B

Erythromycin B is similar to erythromycin in most of its physical and chemical properties. Both are basic compounds, relatively insoluble in water and extremely soluble in ether, acetone, chloroform and ethyl acetate. Acid salts of both compounds are water soluble.

Electrometric titration in 66% dimethylformamide–water showed a titratable group with a pK'_a of 8.5. The molecular weight determined from titration data is about 736 ± 36. The specific rotation at 25° (2% in ethanol) was −78°, and the melting point was 191–195° (capillary tube).

Anal. C, 61.54; H, 9.45; N, 2.00; O (by difference), 27.01.

The ultraviolet absorption spectrum in methanol showed a single broad peak of weak intensity having a maximum at 286 mμ and a molar extinction coefficient of about 59. For comparative purposes, the infrared absorption spectrum of erythromycin B is shown on the same plot as that of erythromycin, Fig. 3. Although it is obvious the two compounds are closely related, discrete differences do exist.

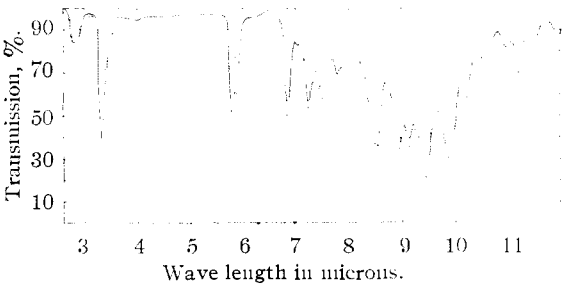

Fig. 3.—Infrared absorption spectra of erythromycin (----), and erythromycin B (——), 5.5% in chloroform, on Beckman IR-2T spectrophotometer.

The microbiological spectrum of erythromycin B is similar to that of erythromycin; however, erythromycin B is only about 75–85% as active.

Experimental

Organism, Conditions for Growth, and Assay.—The culture was grown for 12 days at 33° on a sporulation medium (Table I). A spore suspension was used to inoculate 75 ml. of vegetative medium (Table I) in a 250-ml. erlenmeyer flask. After incubation at 28° on a rotary shaker (240 r.p.m.) for 72 hours, the vegetative growth was centrifuged and washed, and was used in 2.5-ml. portions to inoculate 75 ml. of fermentation medium in a 250-ml. flask.

Several fermentation media were found suitable; the synthetic medium shown in Table I was utilized in most of these studies. The sucrose was sterilized separately and was added aseptically to the remainder of the sterile medium; the initial *p*H was 7.5. The fermentation flasks were incubated at 28° for 7 days on a rotary shaker (240 r.p.m.).

Antibiotic potency was determined with *Micrococcus Pyogenes* var. *aureus* using a 4-hour turbidimetric test. The relative concentration of the two antibiotics was determined by bioautographs of paper chromatograms using either *Micrococcus pyogenes* var. *aureus* or *Bacillus subtilis*.

Extraction.—Three liters of broth containing activity equivalent to 540 mg. of erythromycin was adjusted to *p*H

9.6 with 1.0 *N* sodium hydroxide and was extracted with three 300-ml. portions of chloroform. After drying over anhydrous sodium sulfate, the chloroform solution was concentrated under reduced pressure to about 50 ml. The antibiotic activity was transferred to aqueous solution by extracting the chloroform with four 20-ml. portions of 0.1 *M* phosphate buffer at *p*H 5.2. The combined aqueous extracts were adjusted to *p*H 9.5, and again were extracted with chloroform. Evaporation of the chloroform yielded a light brown residue that weighed 0.89 g. and contained an activity equivalent to 460 mg. of erythromycin. Further purification of such preparations was achieved by chromatography or countercurrent distribution.

Chromatography.—Three grams of cellulose powder was added to 10 ml. of an acetone solution that contained an antibiotic preparation equivalent to 176 mg. of erythromycin. The mixture was evaporated to dryness under vacuum.

The activity adsorbed on cellulose powder was added to a column previously packed with dry cellulose, and the column was developed by gravity with 0.01 *N* ammonium hydroxide saturated with methyl isobutyl ketone. Fractions (15-ml.) were collected by an automatic fraction collector. Fractions 1 to 5 contained no activity. Fractions 6–14 contained erythromycin, and fractions 15–20 contained a mixture of erythromycin and erythromycin B. The remainder of the active fractions (21–48) contained only erythromycin B. The recovery of activity from the column was almost quantitative. The erythromycin B fractions were combined, adjusted to *p*H 10.5, and extracted with two 100-ml. portions of chloroform. After drying over anhydrous sodium sulfate, the chloroform was removed under reduced pressure. The residue was extracted with three 25-ml. portions of ether which were combined, filtered, and dried. After evaporation of the ether, the residue was dissolved in 5 ml. of warm acetone. Crystalline erythromycin B was deposited as the solution cooled.

Craig Countercurrent Distribution.—The solvent system was prepared by mixing 10 l. of 0.1 *M* phosphate buffer at *p*H 6.5, 10 l. of methyl isobutyl ketone and 0.5 l. of acetone. Ten ml. of the upper phase was used to dissolve 590 mg. of the crude antibiotic. A 100-tube countercurrent distribution was performed in a Craig machine having a capacity of 10 ml. per phase in each tube. Upon completion of the distribution, samples from every fifth tube were withdrawn for assay. The results are plotted in Fig. 1. Paper chromatograms showed that tubes number 30 through 50 contained only erythromycin while tubes number 60 through 80 contained only erythromycin B. The latter fractions were combined and concentrated to about 40 ml. under reduced pressure. The *p*H was adjusted to 9.6 and the antibiotic activity was extracted into chloroform. The residue remaining after removal of the chloroform by evaporation was crystallized from 6 ml. of acetone. The first crop of rectangular plates weighed 95 mg.

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[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY AND NUTRITION, DUKE UNIVERSITY SCHOOL OF MEDICINE]

Mechanism of Thiamine-catalyzed Reactions[1]

BY SHUNZI MIZUHARA[2] AND PHILIP HANDLER

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High concentrations of thiamine in alkaline solution catalyze several reactions of carbonyl compounds known to occur in biological systems, among them the formation of acetoin and acetate from acetaldehyde and diacetyl. Acetaldehyde-C^{14} has been employed to demonstrate the mechanism of this reaction.

It is well established that in biological systems thiamine functions in the oxidative and nonoxidative decarboxylation of pyruvic and other α-keto acids which arise in metabolism.[3] Under some circumstances, however, thiamine pyrophosphate together with a specific protein such as the "carboxylase" of pig heart has been observed to catalyze the formation of CO_2 and acetoin, rather than acetaldehyde or acetate, when incubated with pyruvate. This reaction is favored by the presence of acetaldehyde.[4] Presumably related is the "diacetylmutase" reaction in which diacetyl yields acetate and acetoin with preparations from pigeon breast muscle.[5] The formation of furoin from furfural also has been observed in protein-free, model systems in the presence of various quaternary thiazole compounds including thiamine[6,7] which also catalyzes acetoin formation from pyruvate[8] and the diacetylmutase reaction.[9] Significantly these reactions proceed only if 2 equivalents of alkali are added per mole of thiamine hydrochloride,[8,9] since under these conditions there exist appreciable quantities of thiamine pseudobase.[10]

A mechanism for the mode of action of thiamine in these systems has been suggested[8,9] which considers that the lone pair of electrons on the tertiary thiazole nitrogen of thiamine pseudobase coordinates with the carbo-cation of pyruvate or diacetyl. The resulting intermediate decarboxylates (or loses a ketone-like group) leaving a complex which acetylates acetaldehyde to form acetoin. To strengthen the arguments on which this mechanism rests, the catalytic action of thiamine in alkaline solution was verified and, with the use of

(1) This work was done under contract AT-(40-1)-289 between Duke University and the Atomic Energy Commission.

(2) Department of Biochemistry, Okayama University Medical School, Okayama, Japan.

(3) S. Ochoa, *Physiol. Rev.*, **31**, 56 (1951).

(4) D. E. Green, W. W. Westerfeld, B. Vennesland and W. E. Knox, *J. Biol. Chem.*, **145**, 69 (1942).

(5) D. E. Green, P. N. Stumpf and K. T. Zarundnaya, *ibid.*, **167**, 811 (1941).

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(8) S. Mizuhara, R. Tamura and H. Arata, *Proc. Japan Academy* **27**, 302 (1951).

(9) S. Mizuhara and K. Cono, *ibid.*, **27**, 705 (1951).

(10) S. Mizuhara and H. Arata, *ibid.*, **27**, 700 (1951).