not possible to establish any immunological differences between the two dextran fractions 1 and 9 which differed in molecular weight. Despite these limitations, however, and in view of the high purity of the C^{14}-dextran used,[2] the data clearly establish that the precipitin produced by human beings in response to the injection of small amounts of dextran is indeed antibody to dextran.

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[CONTRIBUTION FROM THE NOYES CHEMICAL LABORATORY, UNIVERSITY OF ILLINOIS, AND THE UPJOHN COMPANY]

Streptothricin. I. Preparation, Properties and Hydrolysis Products[1]

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Streptothricin is a basic antibiotic with an empirical formula of about $C_{20}H_{34}N_8O_9$. On hydrolysis, it yields ammonia, carbon dioxide and three ninhydrin-positive components, none of which give significant ninhydrin–carbon dioxide values. One of these hydrolysis products has been identified as β,ϵ-diaminocaproic acid. A large-scale carbon chromatogram is described which conveniently affords pure sulfates or hydrochlorides of streptothricin or streptomycin.

Streptothricin is a basic antibiotic produced by *Streptomyces lavendulae*, and first isolated by Waksman and Woodruff[3] in 1942. Although of little clinical importance because of its nephrotoxicity,[4] streptothricin is of considerable interest due to its high antibacterial activity. A study of its structure and properties has been undertaken, therefore, and the present paper records initial data on the properties and degradation products of streptothricin. Fried and Wintersteiner[5] reported preliminary data on streptothricin, and later Peck, *et al.*,[6] prepared the crystalline helianthate and reineckate, reporting analytical data and properties for these salts.

The streptothricin used in these studies was prepared in the research laboratories of The Upjohn Company. Essentially pure sulfates of either streptothricin or streptomycin were obtained by chromatography of neutral aqueous solutions on columns of active carbon. Very dilute acetone (1%, v./v.) in water was found to be a suitably selective eluting agent for obtaining the pure antibiotic sulfates.

The crystalline helianthate and the amorphous hydrochloride were prepared and analyzed. The data obtained agree reasonably well with those for salts of a tribasic substance of empirical formula $C_{20}H_{34}N_8O_9$. The helianthate analyses approximate those of Peck, *et al.*, as do the rotation and the analyses of the hydrochloride. The tribasic nature of the antibiotic is also demonstrated by the titration data which indicate the presence of three groups with pK_a' values of 7.1, 8.2 and 10.1.[7] These data are not consistent with the presence of a free carboxyl group, and the pK_a' of 10.1 suggests the possible presence of a guanido group. Analyses disclosed the absence of O-methyl C-methyl and N-methyl groups.

The Van Slyke nitrous acid determination liberated 25% of the total nitrogen, whereas the ninhydrin–carbon dioxide value was essentially zero. Streptothricin showed only end-absorption in the ultraviolet and took up no hydrogen in the presence of platinum catalyst.

A study was made of the behavior of streptothricin toward a variety of color reagents and the results (Table I) are in agreement with the more limited data of Fried and Wintersteiner.

TABLE I

RESULTS OF COLOR TESTS WITH STREPTOTHRICIN

Test	Result	Test	Result
Benedict	+	Ninhydrin	+
Fehling	+	Sakaguchi	−
Tollens	+	Molisch	−
Neutral permanganate	+	Anthrone	+(weak)
Schiff	−	Elson–Morgan	+
Ferric chloride	−	Hopkins–Cole	−
Biuret	+	Millon	−
Pauly	+		

The negative Sakaguchi test is not consistent with the presence of a monosubstituted or asymmetrically disubstituted guanidine group. Thus, if the basic group of pK_a' 10.1 is a guanido group, it is not of the type present in streptomycin. The Pauly test did not give the typical color of an imidazole, and may be due to an active methylene group.

Because streptothricin gave positive Pauly, biuret and ninhydrin tests, and amino groups were liberated during hydrolysis, a peptide structure for this antibiotic seemed probable. The products of acid and alkaline hydrolysis were, therefore, investigated.

(1) The authors wish to express their thanks to the Abbott Laboratories, Eli Lilly and Company, and The Upjohn Company for a generous grant in support of this work.

(2) Part of the material in this paper is taken from theses submitted to the Graduate College of the University of Illinois in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Chemistry.

(3) S. A. Waksman and H. B. Woodruff, *Proc. Soc. Exp. Biol. Med.*, **49**, 207 (1942).

(4) G. Rake, D. Hamre, F. Kavanagh, W. L. Koerber and R. Donovick, *Am. J. Med. Sci.*, **210**, 61 (1945).

(5) J. Fried and O. Wintersteiner, *Science*, **101**, 613 (1945).

(6) R. L. Peck, A. Walti, R. P. Graber, E. Flynn, C. E. Hoffbine, Jr., V. Allfrey and K. Folkers, THIS JOURNAL, **68**, 772 (1946).

(7) Private communication from W. W. Davis, The Lilly Research Laboratories.

In the course of the hydrolysis, the Van Slyke amino nitrogen value increased to about 75% of the total nitrogen, but, significantly, the ninhydrin–carbon dioxide value increased but slightly and never accounted for more than a small fraction of a mole per mole of streptothricin. This unexpected result was the first clear-cut indication that streptothricin does not belong in the group of so-called peptide-antibiotics, since no α-amino acids are liberated during hydrolysis.

Acid or alkaline hydrolysis produced three ninhydrin-positive components, which could be separated on paper chromatograms in the systems 75% phenol:water or *n*-propyl alcohol:acetic acid:water in the volume ratio 2:1:1. Subsequent spraying with ninhydrin solution and heating led to the formation of three dark-purple spots, representing compounds designated A, B and C, respectively. Acid hydrolysis is accompanied by less darkening than that obtained with alkali, and most of the work on this problem has been done on hydrolysates prepared with hydrochloric acid. It has been discovered recently that very mild acid or alkaline hydrolysis liberates one mole of carbon dioxide forming a single new product in quantitative yield. This new substance is ninhydrin-positive but microbiologically inactive by an agar-plate assay utilizing *Mycobacterium* 607. Details of its preparation and properties will be reported as a future paper. Stronger conditions result in the further loss of one mole of ammonia, which begins only after the carbon dioxide evolution is complete, and it seems probable that the elimination of carbon dioxide affects the lability of the group yielding the ammonia.

The separation of the three components of streptothricin hydrolysates proved troublesome, and whereas preliminary work with carbon columns provided compound A in relatively pure form, the method was unsatisfactory for compounds B and C. The sulfate of compound C is relatively insoluble in methanol and may be precipitated in a crude form from streptothricin hydrolysates. Powdered cellulose chromatography[8a,b] was found to be satisfactory for the separation of streptothricin hydrolysates. The solvent system used was *t*-butyl alcohol:acetic acid:water in the ratio 2:1:1. This afforded preparations of each component uncontaminated with the others. Results obtained from chromatography of 8.8 g. of streptothricin hydrolysate in this manner are recorded in Table II.

TABLE II

CHROMATOGRAPHY OF 8.8 G. OF STREPTOTHRICIN HYDROLYSATE ON 1 KG. OF POWDERED CELLULOSE

Fraction eluate volume, ml.	Weight, g.	Papergram assay
0– 760	1.145	NH_3
760– 1309	0.946	NH_3 + compound A
1309– 2986	2.910	Compound A
2986– 3695	0.951	Compounds A + B
3695–11396	2.118	Compound B
11396–11991	0.037	Blank
11991–13729	2.159	Compound C

(8) (a) L. Hough, J. K. N. Jones and W. H. Wadman, *Nature*, **162**, 448 (1948), and *J. Chem. Soc.*, 2511 (1949); (b) D. H. Peterson and L. M. Reineke, *J. Biol. Chem.*, **181**, 95 (1949).

Compound A has been identified as (+)-β,ϵ-diamino-*n*-caproic acid and given the trivial name of β-lysine.[9] The characterization and synthesis of this compound, which also has been isolated from streptolin[10] and viomycin[11] hydrolysates, will be reported later. Each of these antibiotics is a highly active antibacterial agent[8,12,13] and the presence of this same unusual amino acid in each renders this compound of more than usual biochemical interest. Preliminary data indicate that β-lysine possesses anti-lysine activity. Work on the characterization of compound B, the guanido-like compound (for which an amino-imidazoline-type structure is postulated), compound C, the reducing compound, and the compound obtained from streptothricin by the loss of one mole of carbon dioxide is now in progress.

Experimental

Preparation of Streptothricin Helianthate.—Crude streptothricin hydrochloride (10.0 g.) was dissolved in 100 ml. of methanol and treated with an aqueous solution containing 8.0 g. of methyl orange. The solution was allowed to stand for 1 hour at room temperature with occasional shaking, and refrigerated overnight. The precipitate was recrystallized three times from aqueous methanol to afford 7.26 g., 32% yield, bio-assay 72% of theory.

Anal. Calcd. for $C_{20}H_{34}N_8O_9\cdot(C_{14}H_{15}O_3N_3S)_3$: C, 51.5; H, 5.52; N, 16.40; S, 6.6. Found: C, 52.15; H, 5.88; N, 16.50; S, 7.08.

Preparation of Streptothricin Hydrochloride.—Streptothricin helianthate (5.0 g.) was suspended in 30 ml. of methanol, 1 ml. of concentrated hydrochloric acid was added dropwise, and the solution became violet. The solution was filtered through carbon and the carbon cake was washed thoroughly with methanol. The filtrate was treated with 300 ml. of acetone and the precipitate centrifuged, washed with acetone, and dried *in vacuo* over phosphorus pentoxide. The total yield was 1.21 g., 55% by weight, of white amorphous material, $[\alpha]^{25}D$ −51.9° (water).

Anal. Calcd. for $C_{20}H_{34}N_8O_9\cdot 3HCl$: C, 37.5; H, 5.79; N, 17.50; Cl, 16.70. Found: C, 38.22; H, 5.79; N, 17.79; Cl, 16.34.

Acid Hydrolysis of Streptothricin.—Nine and one-half grams of streptothricin sulfate (Upjohn, Research Lot 8804) was dissolved in water and diluted to 145 ml. The solution was placed in a 1-liter flask and attached to an aeration train. Ascarite was used to protect the system and nitrogen was employed as the flushing agent. The system was flushed with nitrogen, 145 ml. of concentrated hydrochloric acid was introduced, and the mixture was brought to boiling. The carbon dioxide was trapped in 5 *N* carbonate-free sodium hydroxide solution. Within 10 minutes the mixture had turned cherry-red, darkening to ruby-red in 40 minutes, and to amber in 100 minutes. The mixture was heated under reflux for 8 hours with continuous nitrogen flushing.

The cooled reaction mixture was evaporated to dryness *in vacuo*, taken up in water and freeze-dried. The dried powder was again dissolved in water and neutralized to *p*H 6.5 with Amberlite IR-45 resin in the hydroxyl cycle. The neutral solution was freeze-dried to afford 8.8 g. of amber powder.

The alkali trap, which originally contained 238 ml. of 5 *N* sodium hydroxide, was transferred quantitatively, without undue exposure to air, into an aqueous solution containing 64 g. of ammonium chloride. To this solution was added 238 ml. of a solution containing 87.5 g. of barium

(9) H. E. Carter, W. R. Hearn, E. M. Lansford, Jr., A. C. Page, Jr., N. P. Salzman, D. Shapiro and W. R. Taylor, THIS JOURNAL, **74**, 3704 (1952).

(10) E. E. Smissman, R. W. Sharpe, B. F. Aycock, E. E. van Tamelen and W. H. Peterson, *ibid.*, **75**, 2029 (1953).

(11) T. H. Haskell, S. A. Fusari, R. P. Frohardt and Q. R. Bartz, *ibid.*, **74**, 599 (1952).

(12) R. W. Rivett and W. H. Peterson, *ibid.*, **69**, 3006 (1947).

(13) John Ehrlich, R. M. Smith, Mildred A. Penner, Lucia E. Anderson and A. C. Bratton, Jr., *Am. Rev. Tuberc.*, **63**, 7 (1951).

chloride. The flask was allowed to stand overnight. The resultant precipitate was filtered into a tared, sintered-glass funnel and washed with 10, then 100 ml. of water. The precipitate was dried *in vacuo* at 108° for 24 hours to afford 3.00 g. of barium carbonate, equivalent to 15.2 millimoles of carbon dioxide. Thus, 1.08 moles of carbon dioxide was produced per mole of streptothricin, based on a molecular weight of 530 for streptothricin free-base.

Preparation of the Cellulose Chromatographic Column.—Twenty-five hundred grams of Solka-Floc cellulose powder (Brown Company, New York, 18) was extracted three times with 11-liter portions of 1 *N* hydrochloric acid, filtered, washed with water and dried. A 1232-g. sample of acid-washed dried powder was stirred with 6250 ml. of 0.5 *N* hydrochloric acid for 90 minutes and allowed to stand overnight. The powder was filtered, washed to neutrality with water, and air-dried for 15 hours. The powder was extracted twice with 3.6 liters of solvent mixture *t*-butyl alcohol:acetic acid:water in the ratio 2:1:1, then washed with 16 liters of water, filtered, and dried in the hood for 72 hours.

One kilogram of the dried powder was packed and tamped in 20-g. portions into a column of 3-in. diameter (45.6 cm.^{2} cross-section). The packed height was 63 cm.

Chromatography.—The packed cellulose column was aligned with a fraction cutter and an initial 25 ml. of the solvent mixture was added to the top of the column and allowed to flow through under gravity. As the level of the solvent mixture dropped to the top of the adsorbent, 20 ml. of water containing 8.8 g. of streptothricin hydrolysate was added. This was followed in succession by 5 and 10 ml. of water, and 10, 10 and 400 ml. of solvent mixture. A pressure reservoir was then attached for continuous feed. When the solvent front had proceeded half-way down the dry column, 2.5 pounds pressure was applied, to afford an elution rate of 0.9 ml. per minute throughout most of the run. Progress of the column was followed by papergrams, chloride and ninhydrin tests. Fractions were pooled on the basis of their papergram behavior, concentrated *in vacuo*, and freeze-dried. Dried residues were freed of traces of cellulose by extraction with three 20-ml. portions of water and filtration. Freeze-drying of these aqueous extracts afforded the amounts of products recorded in Table II.

Extraction and Purification of Streptothricin Sulfate; Isolation of the Crude Product.—This was done essentially as described by VanderBrook, *et al.*, of the Upjohn Research Laboratories.[14] The crude sulfate thus prepared, which served as starting material for the following purification steps, assayed 35% of ultimate purity. It showed excessive vasodepressor activity[15] equivalent to 20 mcg. of histamine per mg.

Decalso Treatment to Reduce Vasodepressor Activity.—Fifteen liters of aqueous solution containing 4.42 kg. of crude product was divided into portions of 10 and 5 liters, respectively, and treated countercurrently with two 3.3-kg. portions of Decalso (Permutit according to Folin) each suspended in 4–5 liters of water. The combined supernatants and numerous washes of the adsorbent were filtered and pooled to afford 37 liters of clear aqueous solution containing 3.5 kg. of streptothricin sulfate assaying 50% of ultimate purity and with optical activity of $[\alpha]_D$ −22.3° (water). It showed vasodepressor activity equivalent to 0.8 mcg. of histamine per mg. The yield on this operation was 81%; the loss substantiates previous data on the adsorption of streptothricin and similar antibiotics on Permutit-type adsorbents.

Carbon Chromatography.—An intimate dry mixture of 41 kg. of Darco G-60 and 13.6 kg. of Celite-545 was gradually made into a smooth aqueous slurry by the addition of water to the churning dough mixer in which the dry mixing had been accomplished. The slurry was poured into a 16-inch pipe, 5 ft. in height, equipped on the bottom with a hinged, grooved and tapped cover which supported a piece of canvas upon which rested the column charge. The top cover had a sparger, through which solutions could be pumped to the column without release of pressure, two sight glasses, compressed air line from pressure regulator, and a 0.25-inch hole through which passed a rod, containing on its lower end a 16-inch disk of perforated stainless steel. After the excess water had been drained from the slurry, and it had been further compressed by passage of water under about 20 p.s.i.g., the perforated plate was lowered to the surface of the carbon to protect it from disruption. The "liquid hold-up" (determined at this point by subtracting all water put through from the sum of that pumped on plus that used in making the slurry) was 86 liters. It was found essential to guard against accidental lowering of the liquid or supernatant level below the surface of the packed section of adsorbent. Other precautions found necessary to prevent failure due to cracking in this size of operation were: thorough removal by "puddling" of all air bubbles trapped when the slurry was poured into the column, and limiting of pressure fluctuations to gradual changes, when necessary, no greater than about 1 pound per minute. By observing these precautions, nearly uniform success was obtained in chromatography of either streptothricin or streptomycin hydrochlorides or sulfates in pipes up to 24 inches in diameter, containing up to 440 kg. of adsorbent.[16]

To the column thus prepared was added the 37 liters of neutral aqueous solution containing 3.5 kg. of streptothricin sulfate. The starting material was put on at a rate of about 5 liters per hour (4 pounds pressure) and was followed by 15 liters of de-ionized water at a rate of 5 liters per hour. One per cent. (v./v.) of acetone in de-ionized water was then put through at 10–12 liters per hour (9–11 pounds pressure) during the remainder of the chromatography.

The eluate was tested frequently with barium chloride spot reagent for total sulfate, and by polarimeter readings for streptothricin. A recorder and a conductivity cell of cell-constant 20 afforded a continuous record of electrical conductance of the eluate. This allowed appropriate pools to be made for bioassay and other analyses. The results are shown in Table III.

TABLE III

CHROMATOGRAPHY OF 3.5 KG. OF STREPTOTHRICIN SULFATE ON 41 KG. OF CARBON

Fraction[a]	l.	g./l.	$[\alpha]_D$
0– 55	55	0	...
55–140	85	23.0	0°
140–501	361	1.9	0
501–505	4	1.0	−36
505–521	16	4.0	−48
521–532	11	9.5	−52
532–548	16	12.5	−52
548–564	16	9.5	−53
564–584	20	6.6	−54
584–600	16	4.8	−55
600–620	20	3.6	−54
620–682	62	1.2	−55
682–721	39	1.0	−20

[a] Fraction number represents cumulative volume in liters eluted, starting with first penetration of the adsorbent by the starting material. Thus with a liquid hold-up of 86 liters, and starting material volume of 37 liters, completely unadsorbed material appeared in fraction 86–123. The active material beginning at 501 liters was slowed about 4.8 liquid hold-ups by the adsorption–elution process.

Isolation of Chromatographed Product.—Fraction 521–600 was chosen as essentially pure streptothricin sulfate, on the basis of optical rotation, bioassay, conductivity measurements and semi-quantitative Pauly and sulfate tests. The solution was concentrated under reduced pressure, treated with carbon to remove a trace of color and freeze-dried. The product was a white amorphous powder weighing 607

(14) M. J. VanderBrook, A. N. Wick, W. H. DeVries, Roberta Harris and G. F. Cartland, *J. Biol. Chem.*, **165**, 463 (1946). We are indebted to Dr. E. C. Saudek for the fermentation, and to Mr. W. H. DeVries for the extraction of crude streptothricin.

(15) Determined by Dr. K. J. Olson in cats according to a method subsequently described in "U. S. Pharmacopeia," Vol. XIV, p. 698. It seems significant that the curve for blood-pressure drop and recovery *vs.* time has the knife-edge shape of histamine itself, rather than the slow response found with streptomycin. This is interesting in view of the amino-imidazoline structure proposed for component B of streptothricin.

(16) Acknowledgment is due Mr. Bruce S. Lane for equipment design and to Messrs. R. W. Barr and W. J. Kowal for technical assistance in running chromatograms.

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.

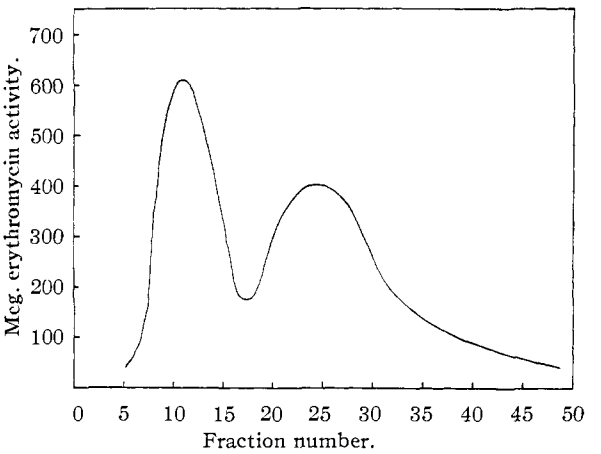

Fig. 1.—The separation of a mixture of erythromycin (fractions 6-14) and erythromycin B (fractions 21-48) on a cellulose column.

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.