tained from the mother liquors. The combined product was dissolved in dry chloroform and chromatographed on alumina. After washing two narrow brown bands down the column with additional chloroform, a third dark-red band was separated mechanically and eluted with methanol. Evaporation of the methanol *in vacuo* to a small volume yielded 17 mg. of yellow crystals, m. p. 228–230°. One recrystallization from a mixture of ethyl acetate and high-boiling petroleum ether yielded 11 mg. of fine yellow needles, m. p. 230–231°.

Anal. Calcd. for $C_{27}H_{30}O_9N_5$ (DNP of tetrahydrocolchiceine): C, 57.03; H, 5.32; N, 12.32. Found: C, 56.42; H, 5.36; N, 11.78.

Summary

Periodate oxidation studies on hexahydrocolchiceine and several of its derivatives indicate that it has a 1,2-diol structure. This furnishes strong support for the Dewar formula for ring C, and is incompatible with the Windaus structure.

ROCHESTER, N. Y. RECEIVED DECEMBER 30, 1948

[CONTRIBUTION FROM THE DIVISION OF CHEMICAL DEVELOPMENT, E. R. SQUIBB & SONS]

Separation of the Streptomycins[1]

BY ANDREW E. O'KEEFFE, MORRIS A. DOLLIVER AND ERIC T. STILLER

Early work in the streptomycin field was hampered by the limitations and vagaries inherent in biological assay methods. Gradually, as more highly purified material became available, many workers turned their attention to the development of chemical assay methods to supplement the basic bioassay. Of the several methods proposed, probably the most popular is that outlined by Schenck and Spielman,[2] involving the alkaline degradation of streptomycin to yield maltol, which could be readily determined either through its characteristic absorption spectrum or by the formation of colored complexes, as with iron.

During the standardization of the maltol method as adopted in these laboratories, it was noted that, as between solid samples of varying degrees of purity, there was an apparent deviation from Beer's law. That this deviation was apparent rather than real was shown by the fact that it was not reproduced when a given solid sample was progressively diluted. These two facts led to the conclusion[3] that a maltol-producing substance other than streptomycin was present in varying degrees in the different samples.

Pursuit of the above hypothesis by Fried and Titus[4,5,6,7] led to the isolation, characterization and identification of "Streptomycin B," later[8] called mannosidostreptomycin.[9]

While Fried and Titus,[4,7] as well as Plaut and McCormack,[10] were able to perform analytical separations of streptomycin and mannosidostreptomycin by the application of the Craig[11,11a] technique of countercurrent extraction, these separations were not directly applicable to preparative work, both because of scale limitations inherent in the method and because their interests stopped short of the recovery of the antibiotics in usable form.

Concurrently and in collaboration with the work mentioned above, the present authors were engaged in parallel exploitation of the basic finding[12] that streptomycin, normally a strongly hydrophilic substance, could be rendered preferentially soluble in organic solvents by the introduction of a "carrier." A "carrier" is defined as an organic compound which is: (1) capable of reacting reversibly with the functional groups of the compound being treated (in the case of streptomycin, such a compound would be an organic acid, which can react with the guanidine groups of the antibiotic); and (2) of sufficient chain length to render its adduct with the compound being treated selectively soluble in organic rather than aqueous media. It will be noted that these criteria approach very closely the definition of a detergent; generally, it was found that any of the common anionic detergents can be used as carriers for streptomycin. Among some of those tested were: (a) fatty acids of varying chain lengths from C_5 to C_{18}; (b) alkyl sulfonic acids (*e. g.*, dodecyl); (c) alkyl sulfuric acids (*e. g.*, *n*-dodecyl, 2-ethylhexyl, 7-ethyl-2-methyl-undecyl-(4), and 3,9-diethyltridecyl-(6)); and (d) aryl or aralkyl sulfonic acids (*e. g.*, sulfonated cumene).

At the time when our attention became centered upon the problem of separating streptomycin from mannosidostreptomycin we used "Pentasol" (mixed synthetic amyl alcohols; Sharples Chemical Co.) as our solvent phase. This choice was dictated by several factors including availability, cost, minimal miscibility with water, minimal emulsification, etc. As our carrier we used a commercial grade of lauric acid, of a purity of about 85%, the remainder being largely myristic acid.

(1) Presented at the First Meeting in Miniature, North Jersey Section, American Chemical Society, January 10, 1949.

(2) J. R. Schenck and M. A. Spielman, THIS JOURNAL, **67**, 2276 (1945).

(3) J. A Shannon, private communication.

(4) J. Fried and E. Titus, *J. Biol. Chem.*, **168**, 391 (1947).

(5) E. Titus and J. Fried, *ibid.*, **168**, 393 (1947).

(6) J. Fried and H. Stavely, THIS JOURNAL, **69**, 1549 (1947).

(7) J. Fried and E. Titus, *ibid.*, **70**, 3615 (1948).

(8) E. Titus and J. Fried, *J. Biol. Chem.*, **174**, 57 (1948).

(9) S. A. Waksman, *Science*, **107**, 233 (1948).

(10) G. W. E. Plaut and R. B. McCormack, THIS JOURNAL, **71**, 2264 (1949).

(11) L. C. Craig, *J. Biol. Chem.*, **155**, 519 (1944).

(11a) L. C. Craig, G. H. Hogeboom, F. H. Carpenter and V. du Vigneaud, *ibid.*, **168**, 665 (1947).

(12) Lott, Braker and O'Keeffe, and Lott, Braker and Heuser, U. S. Patent Applications.

Upon stirring or shaking a solution of lauric acid in amyl alcohol with an aqueous solution of streptomycin hydrochloride, no detectable extraction into the solvent phase takes place. If, however, sodium hydroxide is gradually added to the agitated mixture, and samples of the mixture are removed at intervals for assay of the phases, a curve may be constructed wherein the logarithm of the distribution coefficient of streptomycin, D, is plotted against pH. This curve, shown in Fig. 1, exhibits a remarkably sharp rise in the pH range 6.25–6.5. At higher pH's, above about 8, the distribution coefficient falls off again, but much less abruptly. Determinations of the distribution coefficient at the higher pH's are less accurate, because of the formation of very stable emulsions, presumably due to the increasing presence of sodium laurate.

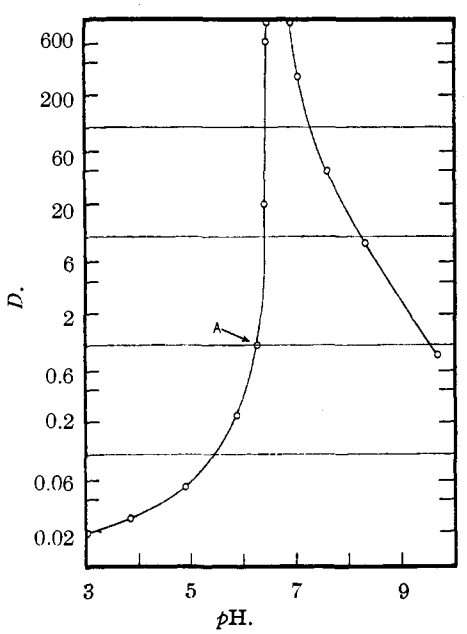

Fig. 1.—Effect of pH on streptomycin distribution coefficient in the system: water *vs.* 15% lauric acid in amyl alcohol.

In order to obtain separation by means of countercurrent extraction, it is necessary to adjust the system so that the distribution coefficient of one component is greater than unity, while that of the other component is less than unity (*i. e.*, $D_A > 1 \simeq D > D_B$). Therefore, assuming no effect of one component upon the other, the distribution coefficient of a binary mixture should be adjusted to approximately one (assays being in terms of the sum of the two components).

Accordingly, the point marked "A" on the curve of Fig. 1 was chosen. However, since the system is extremely sensitive to minute changes of pH, it was necessary to add a buffer in order to stabilize the system at this point.

The buffer chosen (from preliminary data kindly furnished by Dr. Elwood Titus) was 0.375 M borate and 0.125 M phosphate. The presence of this high ionic concentration had a pronounced depressant effect upon the distribution coefficient. Thus it became necessary to prepare a new curve, similar to that of Fig. 1, but for the system buffer lauric acid–amyl alcohol. This curve is shown in Fig. 2; it includes D_A and D_B as well as D.

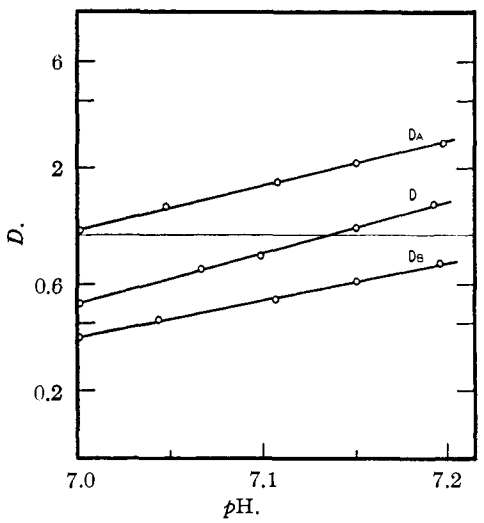

Fig. 2.—Effect of pH on streptomycin distribution coefficient in the system: 0.375 M borate and 0.125 M phosphate buffer *vs.* 15% lauric acid in amyl alcohol.

Prior to attempting actual separations, we felt it necessary to determine the effect upon distribution coefficient of several other known variables; *viz.*, (a) concentration of streptomycin; and (b) temperature. These were evaluated by means of single-stage extractions carried out in separatory funnels. Results obtained are shown in Figs. 3 and 4, respectively.

Before continuing with the description of the remainder of our preliminary work, it is necessary to give a brief description of the apparatus to which this information was to be applied. This consisted of a system designed for increment operation, such that it could be readily scaled up for continuous plant processing.[12a] An odd number of separatory funnels were mounted in a rack, with spaces number $-u \rightarrow 0 + u$, so that the contents of all could be mixed simultaneously by rotating the rack about an axis perpendicular to the long axes of the funnels. This process is shown in Fig. 5. The countercurrent pattern produced by repetition of the process is shown schematically in Fig. 6. An inspection of these figures will show that,

(12) (a) The process hereinafter described was suggested by the work of Craig and others. In fact, our process may be performed in the Craig machine by the "Alternate Withdrawal" process (ref. 11a, p. 677), if increments of solute are added to the appropriate tube at each cycle. Such operation might be advantageous for a special case involving a micro-separation of a binary mixture. Our process is frankly limited to binary separations, but has the compensating advantage of continuous operation.

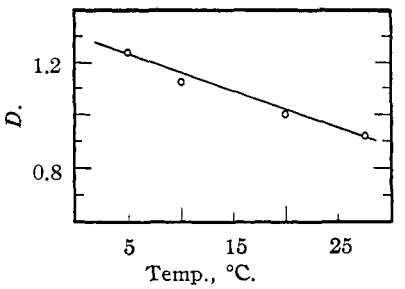

Fig. 3.—Effect of temperature on distribution coefficient.

for a mixture A + B, where $D_A > 1$ and $D_B < 1$ (*i. e.*, "A" is preferentially solvent-soluble, while "B" is preferentially buffer-soluble), continued operation as described above will lead to the removal of a portion of component A with the solvent leaving the train at each cycle, and a simultaneous removal of a portion of component B with buffer leaving the train.

Further study and mathematical expansion[13] of Fig. 6 will show the course of the distribution over a large number of cycles. This may be calculated by the equations furnished by Stene.[14] Such calculations, for a typical case, are shown graphically in Figs. 7 and 8. The striking point of these figures is the pronounced build-up in and about the feed stage. Since Σ, the asymptotic concentration in the system, will be determined by this build-up, it becomes necessary to know the magnitude of the effect, so that operation may be kept within the concentration range wherein D remains constant (the straight-line portion of Fig. 4). Stene[15] gives a simple formula for evaluating the build-up effect. His formula, converted into terms of the present problem, is

$$\Delta_A = \Sigma_A(1 - D_A)/(1 + D_A) \quad (1)$$

and

$$\Delta_B = \Sigma_B(1 - D_B)/(1 + D_B) \quad (2)$$

Another point brought out by Stene's treatise is that maximum separation can be obtained when the extraction factors of the two components are mutually reciprocal

$$E_A = 1/E_B \quad (3)$$

(13) It is not felt necessary in this paper to go into the mathematical details of the separation. Our co-workers Bartels and Kleiman (*Chem. Eng. Progress*, in press) have discussed that phase of the problem at length in a separate publication. The following notation, used hereinafter, is taken in part from their paper:

D = Distribution coefficient = $\frac{\text{concentration in solvent phase}}{\text{concentration in aqueous phase}}$

V = Volume (of either phase, as indicated by subscript)

R = Ratio of volumes of phases = V_S/V_H

E = Extraction factor = RD

Δ = Increment added to system at each cycle

Σ = Asymptotic concentration within system

Subscripts A, B refer to streptomycin and mannosidostreptomycin, respectively.

Subscripts S, H refer to solvent and aqueous phases, respectively.

Terms relating to solute, when used without subscripts, indicate constants determined on a mixture of 67% streptomycin and 33% mannosidostreptomycin.

(14) S. Stene, *Ark. Kem. Mineral. Geol.*, **18H**, No. 18 (1944) (121 pp.).

(15) Ref. 14, p. 75.

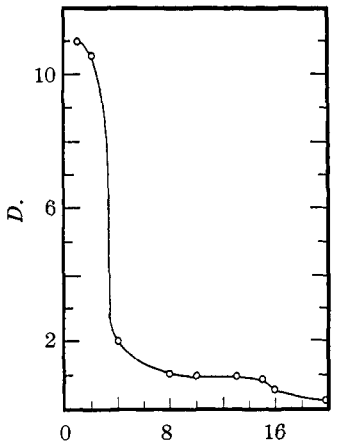

Fig. 4.—Effect of streptomycin concentration on distribution coefficient.

or, by definition of E

$$RD_A = 1/RD_B \quad (4)$$

Since it is simpler to adjust R than D, we solve this for R[16]

$$R_{opt} = (D_A D_B)^{-1/2} \quad (5)$$

Using the known values for D_A and D_B, this gives

$$R_{opt} = (2.20 \times 0.63)^{-1/2} = (1.385)^{-1/2} = 1.2 \quad (6)$$

If we substitute the terms E_A and E_B in equations (1) and (2) above, and add these two equations, it will simplify the calculation of the maximum permissible feed increment

$$\Delta_A + \Delta_B = \Sigma_A (1 - E_A)/(1 + E_A) + \Sigma_B (1 - E_B)/(1 + E_B) \quad (7)$$

But it can be shown that under the conditions which satisfy equation (3)

$$(1 - E_A)/(1 + E_A) = (1 - E_B)/(1 + E_B) \quad (8)$$

whence equation (7) becomes

$$\Delta_A + \Delta_B = (\Sigma_A + \Sigma_B)(1 - E_A)/(1 + E_A) \quad (9)$$

in which the sum $(\Delta_A + \Delta_B)$ is the maximum feed increment of mixed streptomycins, and the sum $(\Sigma_A + \Sigma_B)$ is the maximum concentration of mixed streptomycins which is without effect upon distribution coefficient (Fig. 4). We must, however, use E_A (or E_B) and not the extraction factor corresponding to the apparent distribution coefficient of Fig. 4. Numerically, we have

$$\Delta_A + \Delta_B = 15000 \frac{[1 - (1.2 \times 2.2)]}{[1 + (1.2 \times 2.2)]} = 6700 \text{ γ/ml.} \quad (10)$$

(The algebraic sign of this product is without significance.)

Laboratory-scale separations applying the data outlined above served to confirm the validity of the data. Starting with various mixtures, containing from 30 to 60% mannosidostreptomycin,

(16) Ref. 14, p. 87.

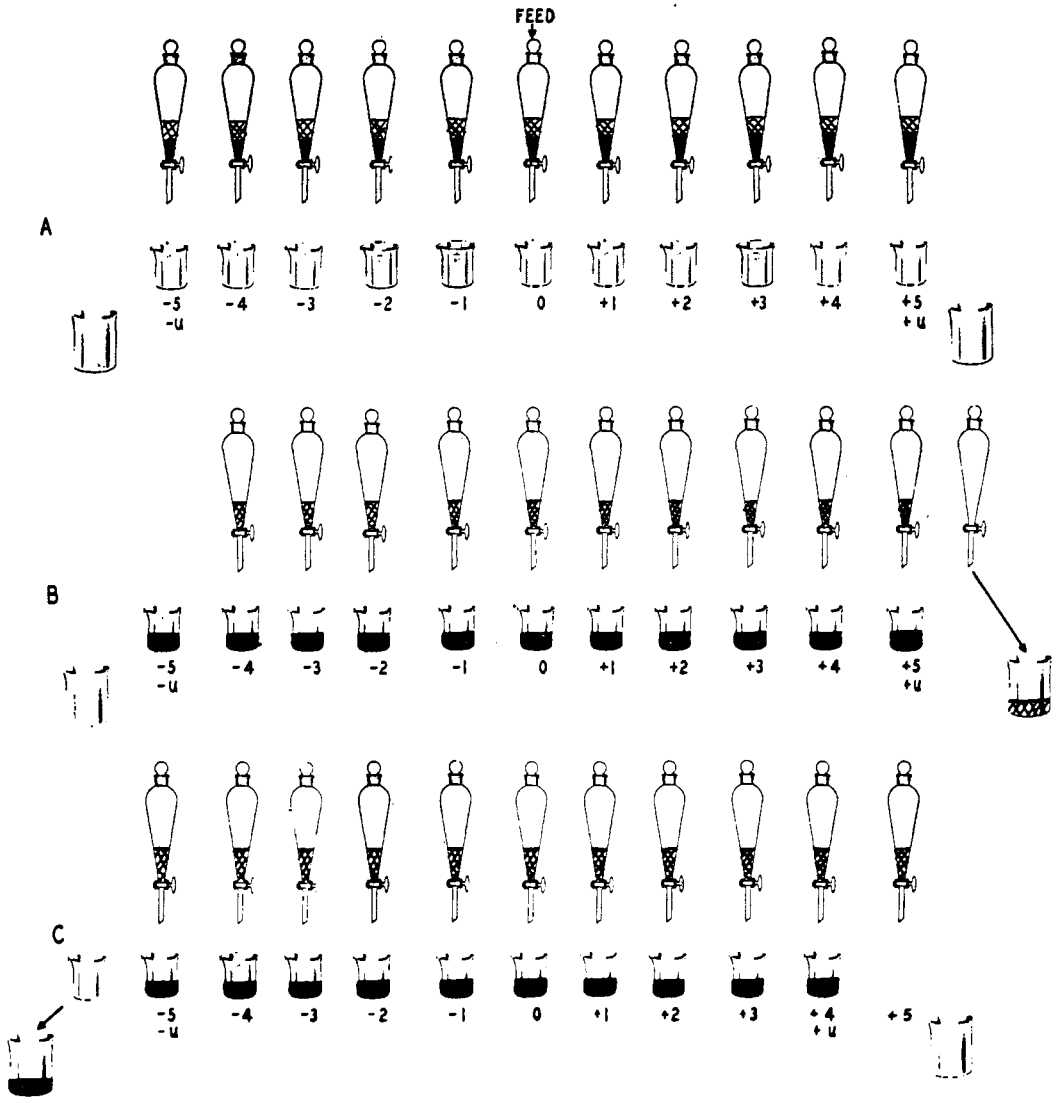

Fig. 5.—Apparatus for continuous countercurrent extraction with center feed: A, solvent and buffer have been introduced into each funnel, and an increment of the binary mixture to be separated has been introduced into the funnel in space "O" as a solid or as a concentrated aqueous solution. The phases have been mixed and settled; B, each funnel, still containing its solvent, has been moved one place to the right; the one on the extreme right has been emptied into a collection vessel; C, the empty funnel has been replaced at the extreme left and has been replenished with fresh solvent. Each beaker of buffer has been moved one place to the left; the one on the extreme left has been emptied into a collection vessel; A, the empty beaker has been replaced at the extreme right and has been replenished with fresh buffer. Each beaker has been emptied into the funnel directly over it. The second increment of the mixture has been added to the funnel now in space "O." The second cycle is now under way.

we were able to obtain solvent fractions containing less than 1% of the mannoside. Material balances ranged from about 95 to 105%.

Our principal problem was now one of recovering the streptomycin from the effluent solvent in usable form. (The recovery of mannosidostreptomycin from the buffer was considered largely an academic problem; its treatment will be included in a subsequent publication.) The solvent contains, besides streptomycin, the following substances: amyl alcohol, lauric acid, water and sodium ion (this last is also subject to carrier extraction, and therefore follows the streptomycin to some extent.) Of these, the first two are readily separated from the streptomycin by means of a reverse extraction into mineral acid at *p*H 5. This *p*H was chosen after an inspection of the curve of Fig. 1 and a number of trial extractions in which it was found that, at *p*H's between 5 and 6.25, intractable emulsions were formed. Water is, of course, removed in a later freeze-drying step. The removal of the sodium ion presented some dif-

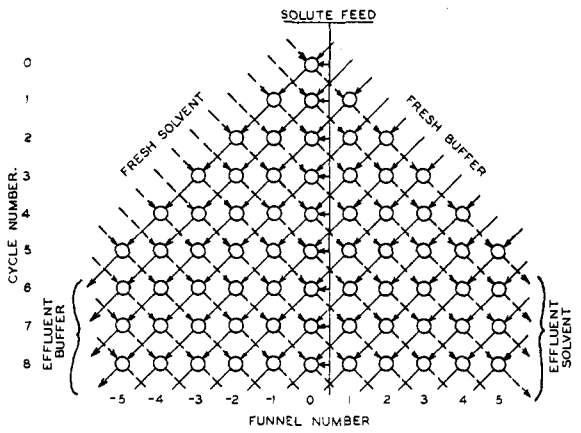

Fig. 6.—An eleven-funnel separation.

ficulty, but was finally solved by extracting the solvent countercurrently with distilled water. Mechanically, this may be accomplished in either of two ways: in batchwise operations, if the water is added to the well-stirred solvent, the two phases will separate with relatively little difficulty; if the solvent is added to the water, or if stirring is stopped and then started again, very stubborn emulsions are formed. The second method, found satisfactory for larger-scale operations, consisted in bubbling the solvent upward through a stream of water moving slowly downward in a glass column. If a sparger is used of such dimensions that bubbles of solvent about 1.5–2 mm. in diameter are formed, and generous sections of the column at the top and bottom are arranged as settling zones, the phases will separate by gravity, and a clear, ashless solvent can be obtained with little trouble. There is a slight loss of streptomycin activity in such washing; this is usually under 10%.

Experimental

1. **Preparation and Mutual Presaturation of Phases.**—348 grams of boric acid and 267 g. of disodium phosphate were dissolved in 1200 ml. of normal sodium hydroxide solution and the mixture made up to 15 liters. The *p*H of this mixture was 8.75.

1980 Grams of lauric acid (Eastman Kodak Co. Tech.) was dissolved in Pentasol (Sharples Chemical Co.) and made up to 15 liters with the same solvent.

The two solutions described above were stirred together and adjusted while stirring to *p*H 7.15 by the addition of 10% sodium hydroxide solution. The two phases were then separated and stored in separate containers for use in the operation described below.

2. **Apparatus.**—An apparatus was used consisting of eleven one-liter separatory funnels arranged in a rotatable rack with spaces numbered $-5 \rightarrow 0 \rightarrow +5$. Eleven beakers were provided to facilitate the transfers of the aqueous phase.

3. **Operation.**—Mechanically, the operation of the separation train was as described in Figs. 5 and 6.

The volume of solvent in each funnel was 495 ml.; the volume of buffer was 450 ml. Each feed increment consisted of 4.50 g. of solid streptomycin complex hydrochloride having a total streptomycin activity of 500 γ/mg. and consisting of 67% (wt.) streptomycin, 33% mannosidostreptomycin.

In order to approach the conditions of continuous operation of large-scale equipment, samples of the two effluent phases were collected from the sixteenth to the twenty-fifth cycles, inclusive.

Assays were run directly on an aliquot of the collected aqueous phase to give total streptomycin activity and the proportions of this activity due to streptomycin and to the mannoside, respectively. An aliquot of the solvent phase was extracted into aqueous hydrochloric acid (3 portions of one-third normal acid were used, to ensure quantitative extraction) and this extract was assayed in the same manner. Craig distribution curves for the original material and for both products are shown in Fig. 9.

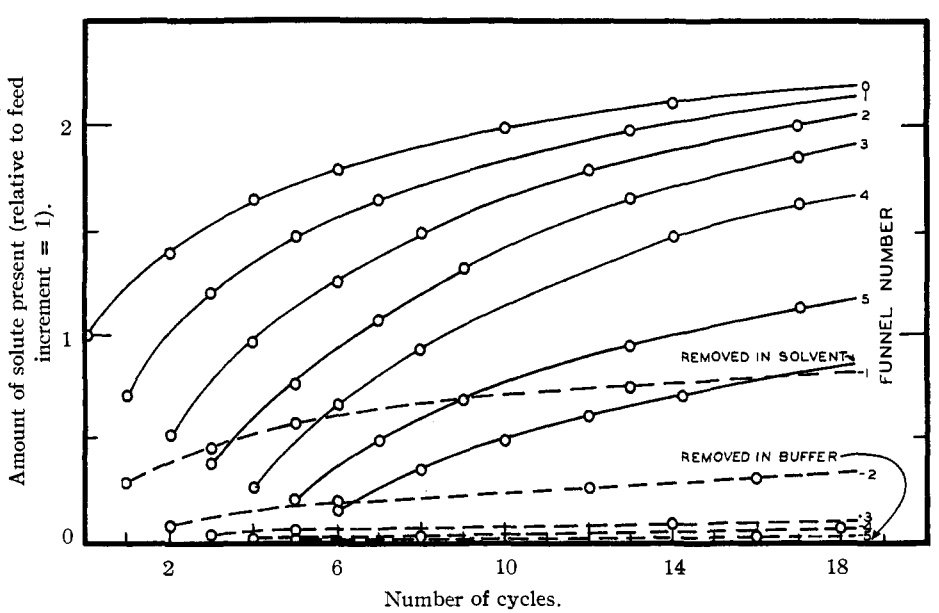

Fig. 7.—Approach toward steady state in eleven-funnel center feed countercurrent separation of components A and B where $D_A = 2.20$, $D_B = 0.63$ and $R = 1.2$.

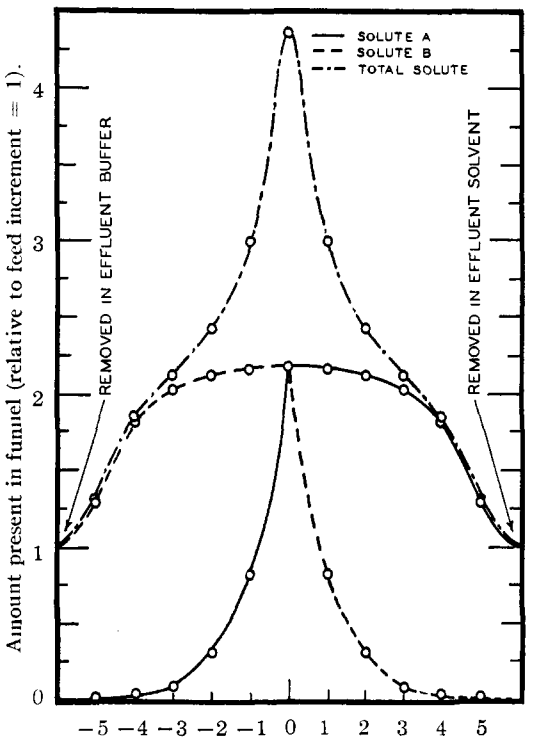

Fig. 8.—Distribution of solutes A and B over eleven-funnel center feed countercurrent train after reaching steady state where $D_A = 2.20$, $D_B = 0.63$ and $R = 1.2$.

Data for the entire run are tabulated below:

Activity of influent complex	500 γ/mg.
Activity of effluent solvent	2850 γ/ml.
Composition of effluent solvent (Craig distribution)	99% Streptomycin
(% of total activity)	1% Mannosidostreptomycin
Activity of effluent buffer	1850 γ/ml.
Composition of effluent buffer (Craig distribution)	12% Streptomycin
(% of total activity)	88% Mannosidostreptomycin

Material balance	Streptomycin γ × 10^{-6}	Mannoside γ × 10^{-6}	Total γ × 10^{-6}
Input: 10 feeds × 4500 mg./feed × 500 γ/mg. =	15.1	7.4	22.5
Effluent solvent: 10 feeds × 495 ml./feed × 2850 γ/ml. =			14.1
14.1 × 0.99	14.0		
14.1 × 0.001		0.1	
Effluent buffer: 10 feeds × 450 ml./feed × 1850 γ/ml. =			8.3
8.3 × 0.12	1.0		
8.3 × 0.88		7.3	

Recovery of streptomycin to solvent 14.0/15.1 = 93%
Total recovery of streptomycin (14.0 + 1.0)/15.1 = 99%
Recovery of mannosidostreptomycin to buffer 7.3/7.4 = 99%
Total recovery of mannosidostreptomycin (7.3 + 0.1)/7.4 = 100%

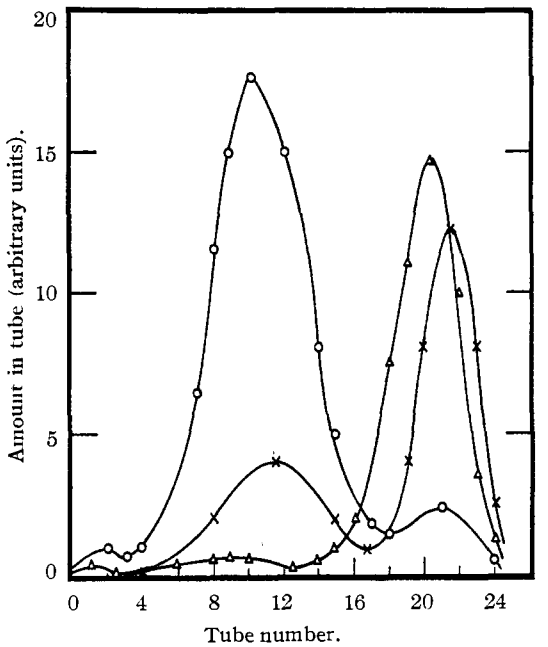

Fig. 9.—Craig distributions of: streptomycin complex, ×—× 67% streptomycin–33% mannosidostreptomycin; effluent solvent, △—△ 99% streptomycin–1% mannosidostreptomycin; effluent buffer, O—O 12% streptomycin–88% mannosidostreptomycin.

A second aliquot of the solvent phase was washed five times with equal volumes of water, always adding the water to the well-stirred solvent, so as to prevent emulsification. The washed solvent was then extracted twice with water plus sufficient hydrochloric acid to adjust the *p*H of the mixture to 5.0. This extract was neutralized to *p*H 5.5 by means of the anion-exchange resin "IR4B" (Resinous Products Co.) and freeze-dried; 1000 ml. of solvent yielded, after washing, reverse extracting and drying, 3.40 g. of streptomycin hydrochloride having an activity of 740 γ/mg.

Solvent: 1000 ml. × 2850 γ/ml. = 2.85 × 10^6γ
Final product: 3400 mg. × 740 γ/mg. = 2.5 × 10^6γ
Yield from solvent: 2.5/2.85 = 88%

Acknowledgment.—The authors wish to express their appreciation for the able assistance furnished by Mr. Martin Greshes, Mr. Frank Russo-Alesi, Mr. Lawrence Hedden and Mrs. Ethel Newman.

Summary

Streptomycin and mannosidostreptomycin have been separated by countercurrent distribution in a system composed of lauric acid in amyl alcohol and an aqueous phosphate–borate buffer.

The chemical and mechanical techniques described are believed to have wide application in the isolation and separation of other naturally occurring compounds.

NEW BRUNSWICK, N. J. RECEIVED JANUARY 28, 1949