The Identity of the Neomycin Complex, as Measured by Countercurrent Distribution and Microbiological Analyses[1,2]

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Forty-nine transfer countercurrent distributions of neomycin preparations, between borate buffer and stearic acid in pentasol, using the method of alternate withdrawals in the 25-tube Craig machine, has been adopted as the tentative criterion for determining the purity of neomycin preparations. Several preparations of neomycin have been studied in this manner, including neomycin A and neomycin B. Neomycin A was demonstrated to be a homogeneous material; it was characterized as that substance produced in cultures of *Streptomyces fradiae*, that occurs around tube 33. All of the other preparations gave distribution patterns characteristic of mixtures of closely related substances. Neomycin has been characterized as that material, produced in cultures of *S. fradiae*, which is essentially composed of at least three similar substances that occur around tubes 36, 40 and 46. "Neomycin B" has been shown to be no different from those substances commonly referred to as "neomycin" except that it lacks the neomycin A component. It is recommended, therefore, that the designation "neomycin B" be discarded. This study has demonstrated that the clinical activities reported for neomycin may be attributed to those components occurring between tubes 35 and 49 when the neomycin complex is partitioned as described above.

I. Introduction

Neomycin, one of the newer antibiotics, was first isolated from culture filtrates of a strain of *Streptomyces fradiae*, one of the soil actinomycetes.[3] It was soon recognized even in the study of crude preparations of this antibiotic that it was not a single substance but a mixture of closely related compounds.[4] It was suggested, therefore, that the proper designation would be "neomycin complex." Several laboratories, in addition to our own, have sought to isolate the individual components of this mixture, as evidenced by the reported isolations of neomycin A[5] and neomycin B.[6]

The isolation of neomycin received general interest because of its great chemotherapeutic potentialities as shown by the reports[7,8,9] of several institutions investigating the activity of some of the purified commercial products in experimental animals and in clinical medicine. It was not known whether these preparations were single components, or mixtures of components, of the neomycin complex, even though these materials were obtained by laborious procedures devised to yield a single substance. Five preparations have been studied in this work in an attempt to determine which components of the complex are responsible for the antimicrobial activities associated with neomycin.

II. Experimental

Assay Methods Used.—Two methods were used in the evaluation of the relative potency of the neomycin preparations and of the fractions obtained from them: (a) the cup method,[4] (b) the streak dilution method.[10] Four test organisms were used for characterizing the antibiotic spectrum: *Escherichia coli*, *Bacillus subtilis*, *Staphylococcus aureus* and *Mycobacterium* 607.

Neomycin Preparations.—Five neomycin preparations have been used in this study. These can be designated as: I, Neomycin hydrochloride—Lot 18-19 prepared in our own laboratories[11]; II, neomycin sulfate—submitted by Upjohn Company; III, neomycin sulfate—prepared by Commercial Solvents Corporation; IV, neomycin B sulfate—product of Chas. Pfizer and Company; V, neomycin A hydrochloride—product of Merck & Company, Inc.

Countercurrent Distribution Studies.—Twenty-four transfer distributions of the various neomycin preparations were carried out in the borate buffer-stearic acid-pentasol system at *p*H 7.6 as described before[12] except that: (1) equilibration of the two phases was effected by rotating the Craig machine for three minutes instead of two minutes; (2) the upper half of the machine was not rotated to the next position until the layers, viewed through the glass plates at the top and bottom of the machine, were seen to have separated; (3) chloroform was substituted for benzene, in the shaking at acid *p*H to displace the neomycin into the aqueous layer, because of the higher solubility of stearic acid in the chloroform.

Forty-nine transfer distributions in the same system at *p*H 7.6, using the method of alternate withdrawals,[13] were carried out on 300 to 500 mg. of samples as described before,[4] with the same exceptions noted above for 24 transfer distributions. In general, 80–90% of the original activity was recovered after distribution. Thus, 58700 units (87%) and 55700 units (89%) were, respectively, recovered from the distributions of 500 mg. (66500 units) of sample II and 430 mg. (62350 units) of sample IV.

III. Discussion

It was necessary, first of all, to adopt a criterion for establishing the purity of neomycin preparations; "purity" is used in this sense to signify the presence of only one antibiotically active substance in a given preparation. The countercurrent distribution technique of L. C. Craig was chosen for this purpose. Figure 1 summarizes the results obtained when 24 transfer distributions were carried out in the Craig machine on samples I, II and III. The peaks around tube 21 have been considered to be characteristic of the neomycin complex.[4] The presence of other, lesser components was also indicated around tubes 16, 12, 5 and 2. However, theoretical distribution curves, calculated according to the method of Williamson and Craig[14] did not show close fits to the corresponding experimental curves. Hence it became apparent that more than 24 transfers were required to separate these similar components. These substances were successfully

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(2) Presented before the Division of Biological Chemistry at the 118th Meeting of the American Chemical Society at Chicago, Ill., September 3 to 8, 1950.

(3) S. A. Waksman and H. A. Lechevalier, *Science*, **109**, 305 (1949).

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(8) O. Felsenfeld, I. F. Volini, S. J. Ishihara, M. C. Bachman and V. M. Young, *J. Lab. Clin. Med.*, **35**, 428 (1950).

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(10) S. A. Waksman and H. C. Reilly, *Ind. Eng. Chem., Anal. Ed.*, **17**, 556 (1945).

(11) This material was prepared by a process involving the use of ion exchange resins. Details of the process will be published shortly by Dr. W. L. Ruigh of this Laboratory to whom we are indebted for this sample.

(12) E. A. Swart, THIS JOURNAL, **71**, 2942 (1949).

(13) L. C. Craig, G. H. Hogeboom, F. H. Carpenter and V. du Vigneaud, *J. Biol. Chem.*, **168**, 665 (1947).

(14) B. Williamson and L. C. Craig, *J. Biol. Chem.*, **168**, 687 (1947).

resolved when 49 transfers were effected. Therefore, 49 transfer distributions in the borate buffer–stearic acid–pentasol system at *p*H 7.6, using the method of alternate withdrawals, was adopted as the present tentative criterion for determining the purity of neomycin preparations. It should be clearly understood, however, that the appearance of only one peak in the distribution curve of a particular sample cannot be considered as proof of the presence of only one antibiotic in that sample. Such proof of homogeneity would require the testing of the sample in other systems and/or by other methods.

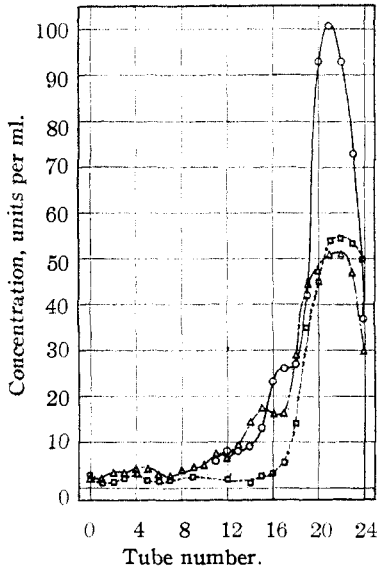

Fig. 1.—Distribution patterns for 24 transfer distributions, in borate buffer–stearic acid–pentasol at *p*H 7.6, of neomycin samples: I, -O-O-O-; II, -□-□-□- and III, -△-△-△-.

The results of studies of such 49 transfer distributions of the five solids are graphically presented in Figs. 2, 3, 4, 5 and 6. Apparently none of the samples except solid V was a single component of the neomycin complex. All of these materials, except neomycin A, showed three major components around tubes 36, 40 and 46. Neomycin A, on the other hand, was apparently a homogeneous material as shown by the presence of only one peak around tube 33. This major component of neomycin A appeared as a minor component of preparations I and III, as demonstrated by plateaus or slight peaks in the region of tube 33.

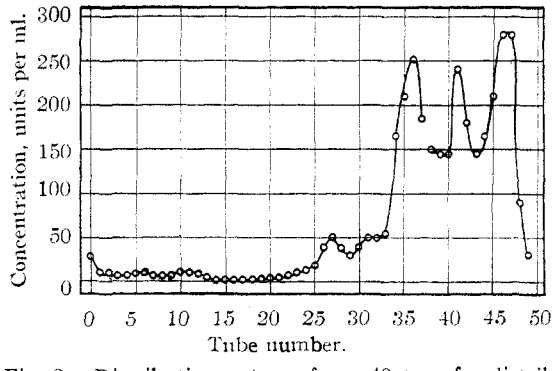

Fig. 2.—Distribution pattern for a 49 transfer distribution, in borate buffer–stearic acid–pentasol at *p*H 7.6, of neomycin sample I.

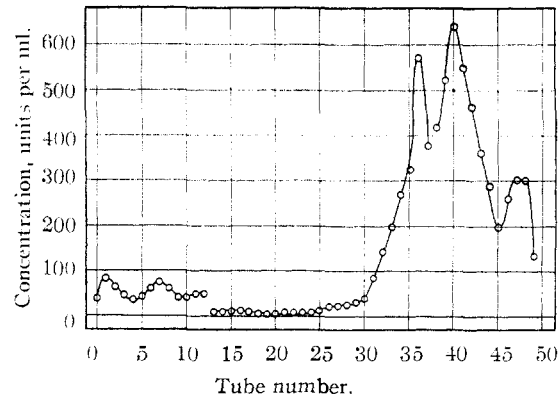

Fig. 3.—Distribution pattern for a 49 transfer distribution, in borate buffer–stearic acid–pentasol at *p*H 7.6, of neomycin sample II.

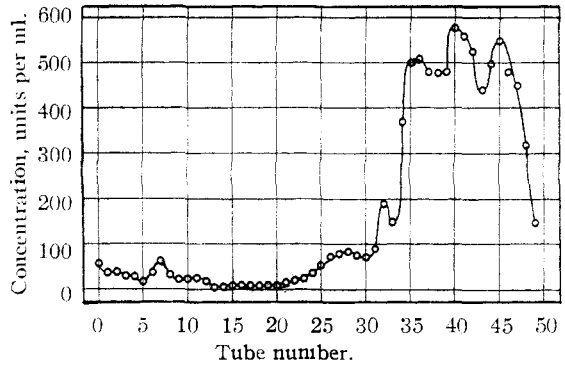

Fig. 4.—Distribution pattern for a 49 transfer distribution, in borate buffer–stearic acid–pentasol at *p*H 7.6, of neomycin sample III.

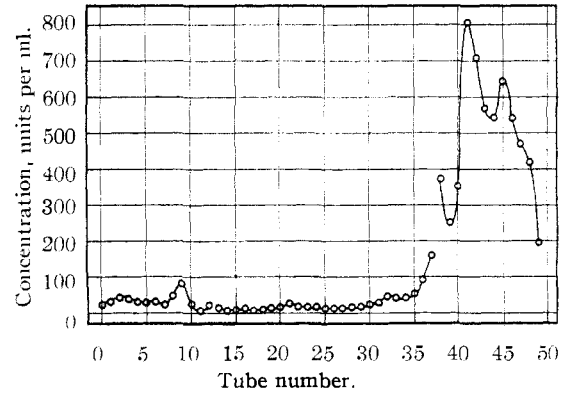

Fig. 5.—Distribution pattern for a 49 transfer distribution, in borate buffer–stearic acid–pentasol at *p*H 7.6, of neomycin sample IV.

The components discussed above were all substances having partition ratios greater than unity, as shown by their position in the distribution curves. Two other components, which were more soluble in the aqueous phase than in the solvent phase, were also detected in all but neomycin A around tubes 2 and 8.

A comparison of the distribution curve (Fig. 5) for neomycin B with the curves (Figs. 2, 3 and 4) obtained for the other neomycin preparations, except that for neomycin A, showed that this substance was no different from those commonly referred to at present as "neomycin" except that it apparently lacked the neomycin A component. The peaks occurring at tubes 37, 41 and 45 in Fig. 5 were analogous to those appearing around these tubes in the other curves. Antibiotic spectra carried out on these fractions agreed with those for corresponding fractions from the other preparations. Hence this material should not be considered as a single substance, and the designation "neomycin B" should be discarded.

Antibiotic spectra were determined for the various samples and their components. The ratio of activity against *E. coli* was determined. The assay values of the five preparations are recorded in Table I. The results show that (a) lots I–IV possess similar activity by the cup method, whereas V has a very high cup activity; (b) lots I–IV have a high activity by the dilution method against *E. coli*, but V has only a low activity; (c) the ratios of activity against different bacteria by different preparations may vary considerably. The activity of all the components of sample II, and of the major component of V are reported in Table II. These spectra are characteristic of those obtained for materials occurring in the corresponding tubes for the other samples. From these results it is believed that the components occurring around tubes 36, 40 and 47 are those typical of neomycin.

TABLE I

ANTIBIOTIC ACTIVITY OF NEOMYCIN SOLIDS BEFORE COUNTERCURRENT DISTRIBUTION

Sample	Potency (*E. coli*, μ/mg.) Cup assay	Potency (*E. coli*, μ/mg.) Dilution assay	Ratio of streak dilution assay against various bacteria to streak dilution assay against *E. coli* for a particular sample: *E. coli*	*B. subtilis*	*S. aureus*	*Mycobacterium 607*
I	138	1120	1	32.5	3.8	2.8
II	133	530	1	25.0	3.6	6.4
III	164	660	1	25.0	2.3	2.5
IV	145	1090	1	14.0	4.2	3.2
V	1160	106	1	30.0	2.0	1.5

TABLE II

RATIO OF ACTIVITY OF THE VARIOUS COMPONENTS OF THE NEOMYCIN COMPLEX TO ACTIVITY AGAINST *E. coli* AS MEASURED BY STREAK DILUTION ASSAYS

Organism	Sample II Tube number 1	7	36	40	47	Sample V 33
E. coli	1	1	1	1	1	1
B. subtilis	6.1	7.1	11	11	10	16.7
S. aureus	1.1	2.4	3.6	2.4	3.3	1
Mycobacterium 607	0.7	2.3	2.4	2.8	4.0	1.3

Critical examination of the data presented in Table II may explain the confusion that has arisen in various laboratories concerning the potency of neomycin preparations. Usually the cup assay procedure[4] with *B. subtilis* as test organism is used with the original reference standard, produced in this Laboratory, or against a new secondary standard. In this procedure[15] the potency is expressed in terms of *E. coli* units, as determined by the diameter of the zone of inhibition: the ratio of activities of the standard against *B. subtilis* and *E. coli* has been previously established by the streak dilution assay technique.[10] If this ratio of activities were not constant, then the results obtained by the cup assay procedure should necessarily be in error. The data presented in Table II show the variability of this ratio among the various components of the neomycin complex; this would lead us to predict that the cup assay on a given sample would depend both on the relative concentrations of the various components it contained and on the concentrations of the components in the standard used. Further, the variability of the streak dilution method, which has been used in obtaining most of these data, is great enough to explain the variance of the results reported in the two tables. The data presented in Table I corroborate this explanation.

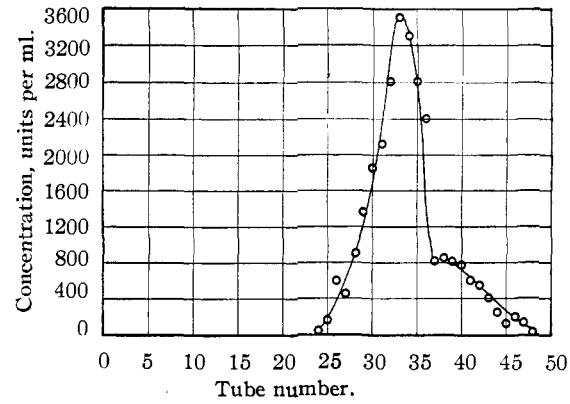

Fig. 6.—Distribution pattern for a 49 transfer distribution, in borate buffer–stearic acid–pentasol at *p*H 7.6, of neomycin sample V.

The high potency of neomycin A, as determined by the cup assay procedure, is probably due to a greater diffusion rate of this material, as compared with the diffusion rates of the major components of neomycin; the explanation given above is also pertinent here.

In conclusion, it may be noted that to date clinical work carried out with neomycin has in effect been carried out with products that are not single chemical entities but mixtures of substances having distribution coefficients that are so similar as to require at least 49 transfers to differentiate one from the others. This clinical work has been carried out with materials that have had for their major components those substances which occurred between tubes 35 and 49 when the original solid was subjected to a 49 transfer distribution in a borate buffer–stearic acid–pentasol system at *p*H 7.6.

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