residue hydrolyzed by refluxing for three hours with 3 cc. of 1 *N* sodium hydroxide. Acidification of the solution with concentrated hydrochloric acid gave 98 mg. of needles which were recrystallized from water. The purified product melted at 207–208°.

Anal. Calcd. for $C_{11}H_{18}O_4N_2$: C, 54.53; H, 7.49; N, 11.56. Found: C, 54.65; H, 7.42; N, 11.87.

***dl*-Hexahydro-2-oxo-4-(6,6-dicarboxyhexyl)-1-furo(3,4) imidazole (XV).**—To a solution of 233 mg. of the above chloride (XIII) in 5 cc. of absolute alcohol, one cc. of an ethyl malonate solution was added which was prepared as described for the preparation of (X), and the solution refluxed for twelve hours. The solvent was removed *in vacuo*, the residue dissolved in ethyl acetate, and the solution washed with water, dried over sodium sulfate, and the solvent removed *in vacuo*. The resulting oil was dissolved in 5 cc. of a 10% aqueous solution of barium hydroxide, and the solution heated on a steam-bath for two hours. The mixture was acidified with 2 *N* sulfuric acid and the barium sulfate removed by filtration. The filtrate was concentrated to a small volume *in vacuo* and the malonic acid (XV) which separated out was purified by recrystallization from water; 114 mg. of material, melting with decomposition at 175–176°, was obtained.

Anal. Calcd. for $C_{13}H_{20}O_6N_2$: C, 51.99; H, 6.71; N, 9.32. Found: C, 52.34; H, 6.79; N, 9.47.

***dl*-Hexahydro-2-oxo-1-furo(3,4)imidazole-4-heptoic Acid (XVI) (*dl*-Bis-homo-oxybiotin).**—Forty-five mg. of the above malonic acid (XV) was decarboxylated in a small test-tube at a temperature of 180–190° and the resulting *dl*-bis-homo-oxybiotin purified by recrystallization from water. Needles melting at 184–185° were obtained.

Anal. Calcd. for $C_{12}H_{20}O_4N_2$: C, 56.24; H, 7.87; N, 10.93. Found: C, 56.33; H, 7.88; N, 10.59.

Biochemical Experiments

The growth-promoting activities of the compounds for *S. cerevisiae* and *L. arabinosus* were determined as described previously.[16] The molar inhibition ratios[11] were calculated from the minimal amounts of substance necessary to completely inhibit growth in the presence of 0.1 millimicrogram (mγ) of *d*-biotin or 0.2 mγ of *dl*-oxybiotin for *L. arabinosus*, and 0.1 mγ of *d*-biotin and 0.4 mγ of *dl*-oxybiotin for *S. cerevisiae*.

Acknowledgment.—The microanalyses reported in this paper were performed in our microanalytical laboratories by Mr. George L. Stragand. The authors wish to express their appreciation to Barbara Flinn, Jean DeWoody, and Audrey Ransom for technical assistance.

Summary

A number of oxybiotin homologs with the same stereochemical configuration as *dl*-oxybiotin have been synthesized. These homologs were tested both as growth promoters and as growth inhibitors for *S. cerevisiae* and *L. arabinosus*. None of the homologs had significant growth-promoting activity, indicating a high degree of specificity of the valeric acid side chain in oxybiotin. Some of the compounds, especially *dl*-homo-oxybiotin, had inhibitory activity which was more pronounced toward oxybiotin than toward biotin.

(16) Winnick, Hofmann, Pilgrim and Axelrod, *J. Biol. Chem.*, **161**, 405 (1945).

PITTSBURGH, PENNSYLVANIA RECEIVED AUGUST 17, 1946

[CONTRIBUTION FROM THE WYETH INSTITUTE OF APPLIED BIOCHEMISTRY]

Purification of Streptomycin Salts by Means of Alumina[1]

BY GEORGE P. MUELLER

Streptomycin obtained from broths by adsorption on charcoal at *p*H 7 to 8, and subsequent elution, varies in potency according to the procedure employed, but generally lies between 150 and 450 γ/mg.[2,3] Carter, *et al.*, have described the purification of 200-γ streptomycin hydrochloride up to a potency of 800 γ/mg. by chromatographic adsorption on acid-washed alumina.[2] These experiments have been repeated in this Laboratory with results that are in excellent agreement with the data published.

Although yields are good and certain fractions of the eluate from such alumina columns yield streptomycin of the highest potency, the application of chromatography to large-scale purifications is, as always, beset with the necessity of working with large quantities of solvents and adsorbent. In order to find a method suitable for the purification of large quantities, it was considered possible to make use of selective adsorption by the alumina of the impurities from aqueous-methanolic solutions of crude streptomycin. This behavior was characteristic of the acid-washed alumina columns.

A method is presented here in which streptomycin concentrates of potencies 290 and 360 γ/mg. may be increased by any amount in keeping with the yield desired, within a practical upper limit of 650 γ/mg. The success of this method depends upon the extent of selective adsorption of impurities, and conditions under which this selectivity may be increased or decreased are described. Besides being economical with solvents and adsorbent, this scheme is easily adapted to large quantities of relatively low-grade streptomycin concentrates and permits a prediction of the yield and potency of the product from any operation, once the characteristics of the adsorbent are known.

In seeking this method, initial trials were made by filtering solutions of impure streptomycin hydrochloride through thin beds of the adsorbent. Decoloration and about 30% increase in potency were obtained with beds only 2 mm. thick. Usually, however, channelling of these thin beds

(1) This paper was presented before the Division of Biological Chemistry at the American Chemical Society Meeting in Chicago, 1946.

(2) Carter, Clark, Dickman, Loo, Skell and Strong, *J. Biol. Chem.*, **160**, 337 (1945).

(3) LePage and Campbell, *ibid.*, **162**, 163 (1946).

occurred. While the mechanical refinements necessary to overcome this problem might have been devised, it was thought that such variables as adsorption and elution of pure streptomycin, adsorption and elution of impurities, and the duration of useful activity of the adsorbent in a dynamic system would be too difficult either for laboratory study or large-scale control.

Satisfactory adsorption isotherms were obtained by the simpler scheme of agitating a solution of streptomycin with the adsorbent, filtering and analyzing the filtrate for total residue and total activity, where both of these values were corrected and recorded as being contained in the original volume of solution. This correction is required by the assumption that any mechanical retention of solution by the adsorbent must be distinguished from the actual adsorption–desorption equilibrium reached during agitation and prior to filtration.

Initially, the same alumina, streptomycin trihydrochloride and 80% methanol as were used in the chromatographic procedure were employed. The entire study encompassed the additional use of a streptomycin phosphate hydrochloride, acid-washed Harshaw alumina, methanol concentrations varying from 30 to 100%, agitation periods from ten to forty minutes, ratios of 10 to 60 grams of alumina per million γ of streptomycin and solution concentrations of 4,000 to 20,000 γ/ml. A comparison was made between the results of a single agitation with alumina and repeated treatments of a single solution with small amounts of alumina.

Experimental

Solvents.—Commercial U. S. I. methanol was treated at reflux for several hours over magnesium turnings and subsequently distilled. As it became available Mallinckrodt analytical reagent grade of absolute methyl alcohol was used.

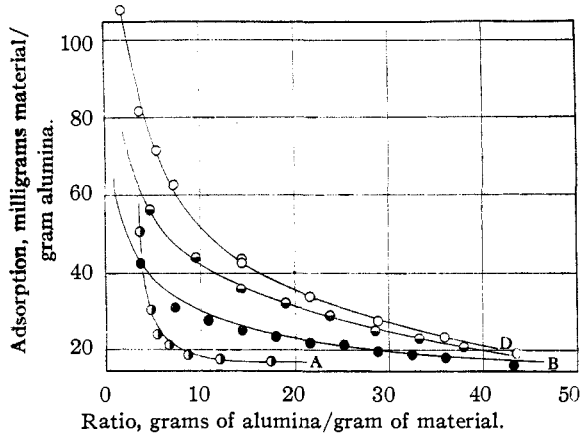


Fig. 1.—Adsorption isotherms of streptomycin trihydrochloride (360γ/mg.) in 80% methanol treated repeatedly with fresh portions of Brockmann alumina (◑), streptomycin trihydrochloride (360γ/mg.) in 80% methanol treated once with Brockmann alumina (●), streptomycin trihydrochloride (360γ/mg.) in 98% methanol treated once with Harshaw alumina (◒), and streptomycin phosphate hydrochloride (288γ/mg.) in 98% methanol treated once with Harshaw alumina (○).

Alumina.—Merck alumina (according to Brockmann) was treated as described previously.[2,4] Harshaw alumina was used which was rated as "passing 80," but had the approximate composition: 5–8% retained on 150-mesh, 20% on 200-mesh, 10–15% on 300-mesh, with the remainder passing 300-mesh. Thirty-five pounds of this was suspended with stirring in 20 gal. of distilled water and acidified with 165 cc. of concd. sulfuric acid. After a short settling period, the supernatant liquid together with much fine alumina was rapidly siphoned away. After being washed in a like manner with nine 10-gal. portions of water, the material was collected and dried in steam-heated ovens.

Streptomycin.—The active material from surface-culture broth was adsorbed on activated carbon and subsequently eluted and precipitated as the trihydrochloride. The phosphate was prepared by treating the amine, freed at *p*H 8, with phosphoric acid.

Procedure.—A solution of the antibiotic, usually 10 cc., together with the adsorbent, was placed in a tightly stoppered 50-cc. centrifuge tube and shaken mechanically. The contents of the tube were then transferred as completely as possible onto a medium-grade Pyrex sintered glass funnel. Slight pressure applied at the mouth of the funnel hastened filtering, and this pressure was eventually raised to 10 or 20 cm. to force all the free liquid through into a graduated tube. The volume of filtrate, measured to the nearest 0.1 cc., was transferred with the aid of methanol into a tared 100-cc. flask. Several of the latter were attached to a manifold and evacuated until the methanol was removed, the residues being then diluted with 3 cc. of water and freeze-dried. After its weight had been determined, the dry streptomycin was subsequently removed, finely powdered and redried in small vials over calcium chloride *in vacuo*. Small portions of each sample weighed on a micro balance were submitted in proper dilution with *M*/50 phosphate buffer, *p*H 7.0, for assay by microbiological (cup plate[5] and disk[6]) and spectrophotometric methods.[7] The activities are reported here in terms of micrograms of free base per milligram weight of any preparation, or simply as γ/mg. The cup plate assays were generally acceptable and the average of all values within ±15% of the gross average was the single value used for calculations. Occasionally, the cup plate assays were checked against values obtained with the other two methods.

(4) Due to the existing difficulty in obtaining Merck alumina prepared according to Brockmann, some of this which had been acid-washed and used previously in chromatographing streptomycin trihydrochloride was washed by stirring with eight changes of water during two hours and dried at 100° *in vacuo*. This material was employed wherever "Brockmann alumina" is mentioned.

(5) Stebbins and Robinson, *Proc. Soc. Exptl. Biol. Med.*, **59**, 255 (1945).

(6) Loo, Skell, Thornberry, Ehrlich, McGuire, Savage and Sylvester, *J. Bact.*, **50**, 701 (1945).

(7) A spectrophotometric assay based on a suggestion of Schenk and Spielman, THIS JOURNAL, **67**, 2276, (1945), has been developed in this laboratory by Dr. Eric T. Stiller, Mr. M. Kuna and Mrs. R. Garwin.

The determination of the potency of a streptomycin preparation is effected by heating 10 cc. of a solution of sodium hydroxide, 0.5 *N*, containing at least 200 γ/ml. of streptomycin. After four minutes in a boiling water-bath, the solution is cooled and acidified to *p*H 2 with hydrochloric acid and extracted twice with chloroform. The chloroform extracts are filtered through filter paper by gravity to remove most of the water and diluted to 50 cc. with chloroform which has been shaken with water and filtered through filter paper. The absorption measurements are read on the chloroform solution using moist chloroform in the compensating cell of the Beckman Spectrophotometer. The density at the maximum, 2750 Å., is used for the determination. Values obtainable are reproducible within ±5% of the mean, and are usually in good agreement with the biological methods.

Results and Discussion

The adsorption isotherms in Fig. 1 show that a decreasing rate of adsorption per gram of alumina is characteristic of an increase in the amount of alumina used. Correlation of these curves with the corresponding activity curves makes evident that the impurities present in streptomycin are very strongly adsorbed and that the specific adsorption of a streptomycin preparation whose activity is greater than 600 γ/mg. must be less than 20 mg. per gram of alumina.

Conditions giving rise to the lower curve in Fig. 1, although not strictly analogous to the counter current process extant in the Tswett column, approach those conditions in that the number of readsorptions of a substance as it passes down the column becomes very great. In this instance the curve has been plotted so that the striking contrast between the adsorbability of the various substances may be noted. It must be realized, however, that in this experiment no less alumina was used than is represented by the other isotherms. Actually the value along the abscissa of any point on this curve is with reference to the streptomycin in the solution which gave rise to the preceding point. Thus the total amount of alumina used with any sample may be seen in Figs. 2 and 3, represented as the *cumulative* alumina–streptomycin ratio.

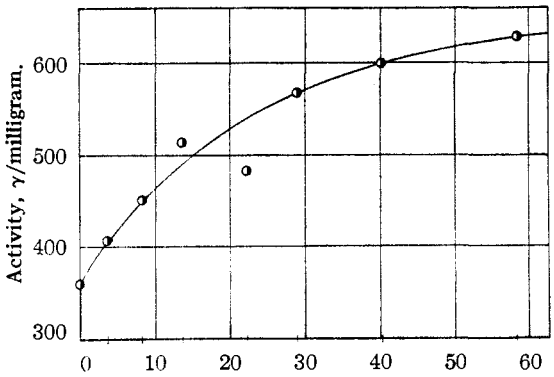


Fig. 2.—Activity curve for solid streptomycin after adsorption of impurities from a solution of streptomycin trihydrochloride (360γ/mg.) in 80% methanol treated *repeatedly* with small amounts of Brockmann alumina. The first point shows the untreated material; the second point was obtained by shaking 100 cc. of a solution containing 1.665 g. of the streptomycin concentrate with 6.0 g. of fresh alumina for ten minutes, sampling and analyzing the resultant solution for solids and activity; the third point from 85.4 cc. of solution remaining from the first treatment, with 5.4 g. of fresh alumina, etc., until 19.3 cc. of solution was shaken with 2.4 g. of alumina for ten minutes to obtain the last point. Fig. 1 shows the corresponding adsorption isotherm.

For any given alumina–streptomycin ratio,

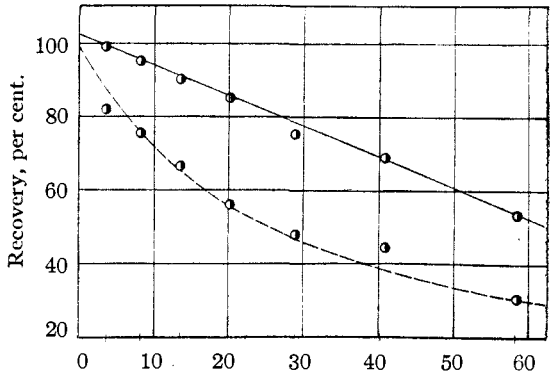


Fig. 3.—Percentage recovery after adsorption of impurities by varying amounts of alumina. Points on the broken curve are solid residue values for which potencies appear in Fig. 2. These solid residue weights are *corrected, i. e.*, filtration from the alumina invariably gave a smaller volume of solution than that of the original. The loss of solution was attributed to mechanical retention by the alumina. It would have been incorrect to attribute loss of the solid contained therein to actual adsorption of material from solution, therefore, the weight of residue in the filtrate was increased by the factor of original volume/collected volume to correct for this loss. Thus the data remained free of any complications which would have resulted from washing the alumina, so destroying the equilibrium established. The *total activity* curve represents the product of values read directly from the curves for activity and for solids. The latter had been drawn through the most probable experimental points: – – –, total solids; —, total activity.

repeated adsorption using small amounts is superior to a single operation with a large amount of alumina. This may be observed from a comparison of the two experiments in which the solvent, alumina and streptomycin were the same, but differed in that one experiment consisted of a single, while the other of multiple adsorptions.

Besides noting that the activity curve for the repetitive procedure (Fig. 2) is higher than that for the single treatment (Fig. 4), it should also be realized that in a property which may be termed *purification factor*, the former far exceeds the latter experiment. The quotient of *the ordinate value* of any point on the total activity curve by that of the point on the solids curve at the same alumina–streptomycin ratio gives the activity of the product after purification with that amount of alumina. This quotient is the purification factor. Thus, under the conditions of the experiment, the amount of adsorbent necessary to obtain a given yield of product of any activity may be predicted. This factor must be as high as possible, since higher yields of purer streptomycin are thereby obtained with less alumina than otherwise. Comparison of Figs. 3 and 5 reveal

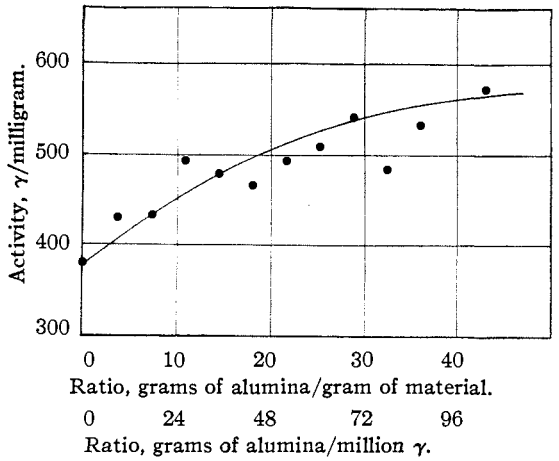


Fig. 4.—Activity curve for single adsorption operations using streptomycin trihydrochloride (360γ/mg.), 0.1667 g. in 10.0 cc. of 80% methanol for each point, and Brockmann alumina, 0.0, 0.6, 1.2, etc., to 6.0, and 7.2 g., with shaking for fifty minutes. The corresponding isotherm appears in Fig. 1.

that multiple purifications are far the more efficient in this respect.

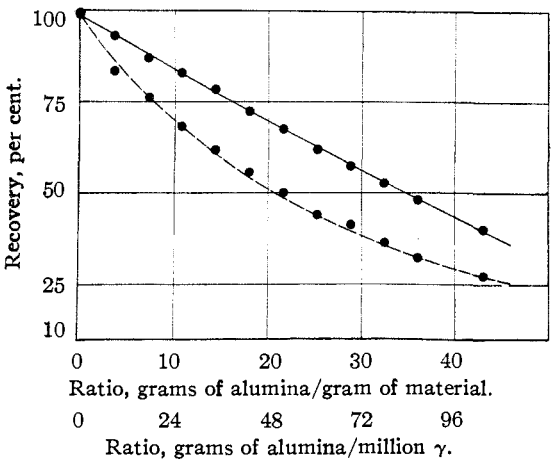


Fig. 5.—Per cent. recovery after adsorption of impurities as described for Fig. 4. Explanation of the broken and solid curves appears under Fig. 3: - - -, total solids; —, total activity.

A study of the effects of changing the methanol concentration revealed that 70% was probably the lower limit of usefulness. In this study, at a ratio of 14.4 g. of alumina per gram of streptomycin, pure methanol proved to give an extremely high purification factor. As was noted during chromatographic adsorption studies, alumina retained very little material in the presence of water.

Harshaw alumina, possibly due to its higher state of subdivision, was more adsorbing than Brockmann alumina. It too was specific toward the impurities in streptomycin and had a greater purification factor in 98% methanol (Fig. 9) than

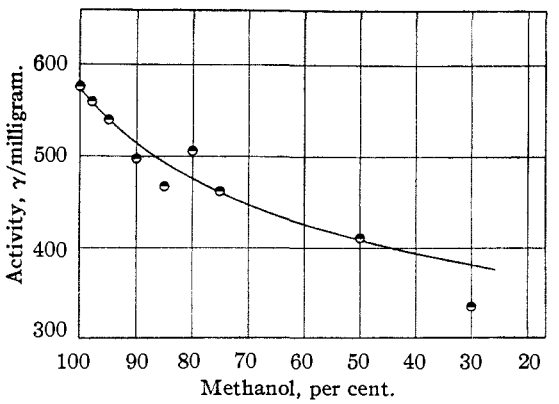


Fig. 6.—Activities, showing the effect of methanol concentration on adsorption of impurities by 2.4 g. of Brockmann alumina from 0.1667 g. of streptomycin trihydrochloride in 10 cc. of solvent during twenty minutes of shaking.

did Brockmann alumina in 80% methanol (Fig. 5). The pure streptomycin phosphate-hydrochloride was adsorbed powerfully by it, as may be seen from the isotherm (Fig. 1). Such adsorption behavior was expected in view of the difficulty previously encountered in eluting this salt with 80% methanol from a column containing Brockmann alumina.

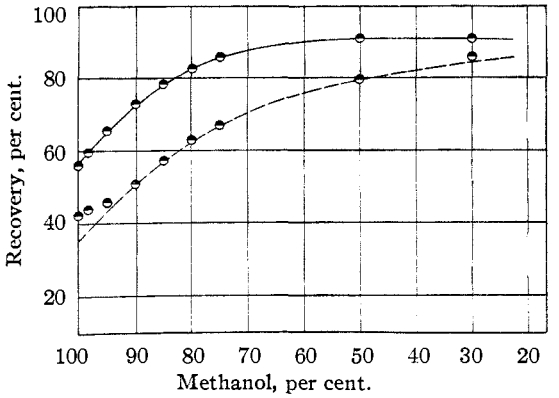


Fig. 7.—Percentage recovery of solids and total activity, *cf.* Fig. 3: - - -, total solids; —, total activity.

The time of shaking within the limits described did not appear to matter, since certain experiments identical in all respects but this gave identical points on the isotherm. The concentration of streptomycin salt in the solution likewise was not critical. Thus, at levels of 4,000, 7,580, 12,000 and 20,000 γ/ml., 10 ml. of streptomycin trihydrochloride (360 γ/mg.) shaken with Harshaw alumina in a constant ratio of 14 g. per gram of salt gave activities of 448, 559, 462 and 500 γ/mg., respectively.

The various isotherms and activity curves given here may well be typical for other streptomycin concentrates. Thus a sample of streptomycin hydrochloride obtained from another firm was

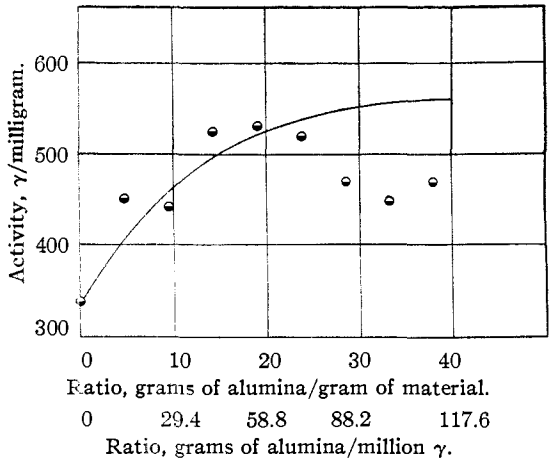


Fig. 8.—Activities of streptomycin trihydrochloride (360γ/mg.) after purification in 98% methanol with Harshaw alumina. Each 10 cc. of solution containing 0.2105 g. was shaken for thirty minutes with one of the following amounts of alumina: 0.0, 1.0, 2.0, etc., to 8.0 g.[8] The adsorption isotherm appears in Fig. 1.

tested. This had been prepared from the broth in a different manner than were the samples described here. Its adsorption isotherm with Harshaw alumina paralleled that in Fig. 1, there being only a small vertical displacement of the entire curve.

In all recovery curves, a correction has been made which assumed that there was no loss of solution occurring with filtration, when actually from 5 to 29% of the solution was retained by the alumina after each operation. To recover

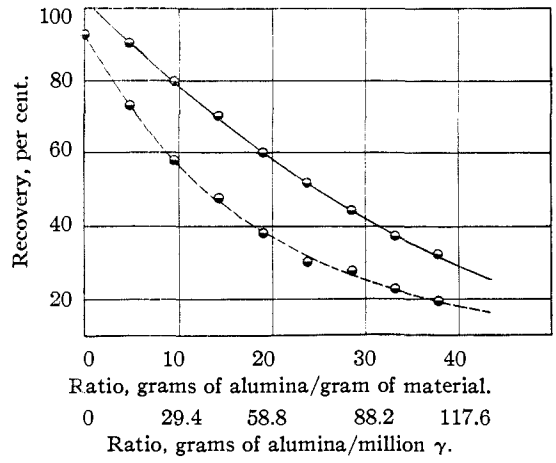


Fig. 9.—Per cent. recoveries of solids and total activity corresponding to Fig. 8, *cf.* Fig. 3: – – –, total solids; —, total activity.

(8) The last three points on this curve as well as the last two points in Fig. 10 are very low. In spite of this, the corresponding points on the adsorption isotherms are clearly in order. These five points, together with seven others not recorded in this report, were obtained from an experiment carried out at a different time than those giving the other points on these curves. All of these twelve samples, obtained concurrently, exhibited low potencies. It appears that some inactivating influence was present during the experiment.

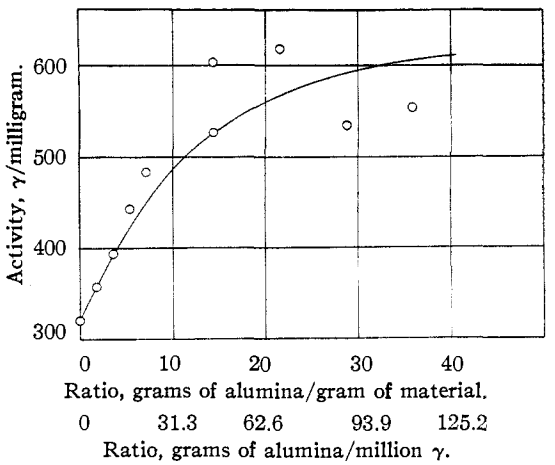


Fig. 10.—Activity curve for the purification of streptomycin phosphate-hydrochloride (288γ/mg.) by shaking for thirty minutes 0.2780 g. in 10.0 cc. of 98% methanol, each solution with 0.0, 0.5, 1.0, 1.5, 2.0, 4.0, 6.0, 8.0, 10.0 g. of Harshaw alumina. The adsorption isotherm appears in Fig. 1.

the retained activity, therefore, in three instances where the losses were 19, 24 and 15% of solution, washing the alumina on the filter with pure solvent brought through 23, 21 and 21% of activity, respectively, represented by material assaying in each instance about 440 γ/mg. As was expected, a pure solvent eluted some impurities and the activities were not as high as in the original filtrates. The apparent losses, for which corrections have been applied, have been accepted experimentally so that the data may be clear of complications; they are not real, however, because the active material may be recovered simply by washing the adsorbent and retreating the washings with fresh alumina.

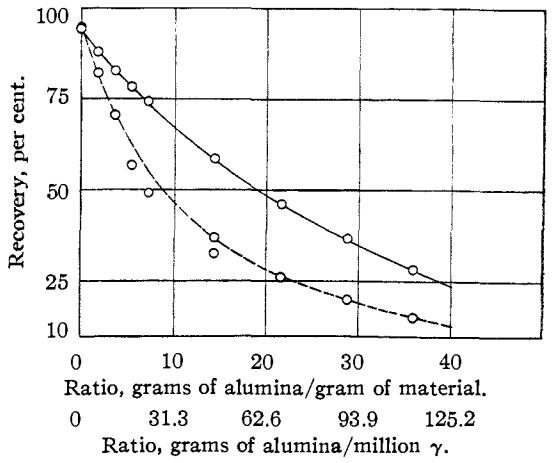


Fig. 11.—Per cent. recovery of solids and total activity corresponding to Fig. 10, *cf.* Fig. 3: – – –, total solids; —, total activity.

The author wishes to thank Drs. E. T. Stiller and W. F. Bruce for their interest in this work and

Dr. Eric G. Snyder for the crude streptomycin salts used. The assistance of Messrs. L. G. Colio and M. Kuna with the assays and Mr. S. V. Lieberman in preparing aluminas is gratefully acknowledged.

Summary

A method which is adaptable to large quantities of material has been proposed and evaluated for increasing the activity of crude streptomycin preparations by partial adsorption on alumina. From initial potencies in the region of 300 γ/mg. increases up to 600 units per mg. were readily obtained by single or multiple agitations of a methanolic solution of the antibiotic with acid-washed alumina.

PHILADELPHIA, PENNSYLVANIA RECEIVED JULY 31, 1946

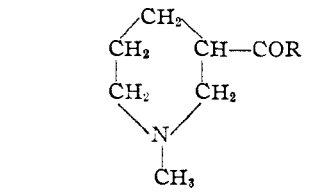
[CONTRIBUTION FROM THE WELLCOME RESEARCH LABORATORIES]

Rearrangements between Primary Ethanolamides of Carboxylic Acids and the Corresponding Aminoethylesters*

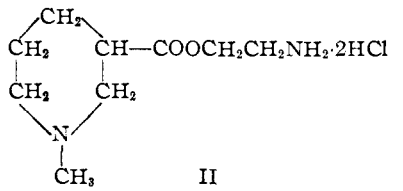
BY ARTHUR P. PHILLIPS AND RICHARD BALTZLY

While it is well known that esters of β-amino alcohols, especially those with primary amino groups, tend to rearrange to ethanolamides and it has been reported that the latter are in turn transformed to salts of aminoesters by hydrogen chloride,[1] the mechanism of these rearrangements and the exact conditions required remain undefined.

In connection with a problem of pharmacological interest, the ethanolamides of certain carboxylic acids were desired. It seemed best to prepare these by heating the corresponding esters with excess ethanolamine, using the latter both as reactant and high-boiling solvent.[2] Pertinent data for the ethanolamides thus prepared are presented in Table I.



I, R = NH—CH_2CH_2OH
Ia, R = OCH_3



II

TABLE I

ETHANOLAMIDES, $RCONHCH_2CH_2OH$

R =	Ratio of moles ethanolamine to moles ester	Reflux time, hours	Yield, %	B. p., °C.	Mm.	M. p., °C.	Analyses, % Carbon Calcd.	Carbon Found	Hydrogen Calcd.	Hydrogen Found
3-(N-Methyl)-hexahydropyridyl	6	2	100	183–185	1	.....	58.03	57.74	9.75[a]	9.83
3-Pyridyl	1.5	2	92–95	210–212	2	89–90	57.80	57.89	6.08	5.85
4-Pyridyl	5	½	90–95	220	1	134–135	57.80	57.83	6.08	6.10
4-Quinolyl	5	2	95	.....		112–113	66.84	66.69	5.60	5.92
4-Hydroxy-3-quinolyl	12	¾	100	.....		253–254	62.04	62.05	5.21	5.24
Phenyl	5	1	95–100	185–187	1	60–61	65.41	65.33	6.72	6.64
2-Hydroxyphenyl	2.5	½	100	210–215	2	113–114	59.64	59.42	6.13	6.07
Benzyl	5	2	100	202–204	1	60–61	67.00	67.14	7.31	7.04
n-Propyl	8	3½	100	150–151	1	.....	54.92	54.72	10.00	10.00

[a] Neutral equivalent: calcd., 186.1. Found: 186.1, 186.2.

In the first attempt to prepare the ethanolamide (I) from dihydroarecoline (Ia) a variation in technique produced an unexpected result. After removal of ethanolamine *in vacuo*, the residual, high-boiling, viscous oil was taken up in ethanol and treated with an excess of ethanolic hydrogen chloride in an attempt to isolate the amide as its hydrochloride. The product, however, was shown by analysis to be the dihydrochloride of a substance isomeric with the ethanolamide. This substance can hardly be formulated as other than the dihydrochloride of the aminoethyl ester (II).

The dihydrochloride was stable during recrys-

* Presented before the Organic Section of the American Chemical Society, Chicago meeting, September, 1946.

(1) (a) Immediata and Day, *J. Org. Chem.*, **5**, 512 (1940); (b) Kanao, *J. Pharm. Soc. Japan*, **48**, 1070 (1928); (c) Cope and Hancock, THIS JOURNAL, **66**, 1448, 1453, 1738 (1944); (d) Reasenberg and Goldberg, *ibid.*, **67**, 933 (1945).

(2) *Cf.* D'Alelio and Reid, *ibid.*, **59**, 111 (1937); Wenker, *ibid.*, **57**, 1079 (1935). Alternative preparations are given by Knorr and Rössler, *Ber.*, **36**, 1278 (1903); Fränkel and Cornelius, *ibid.*, **51**, 1657 (1918).