JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

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VOLUME 75 MARCH 10, 1953 NUMBER 5

[CONTRIBUTION FROM THE KERCKHOFF LABORATORIES OF BIOLOGY, CALIFORNIA INSTITUTE OF TECHNOLOGY]

Some New Naturally Occurring Imidazoles Related to the Biosynthesis of Histidine[1]

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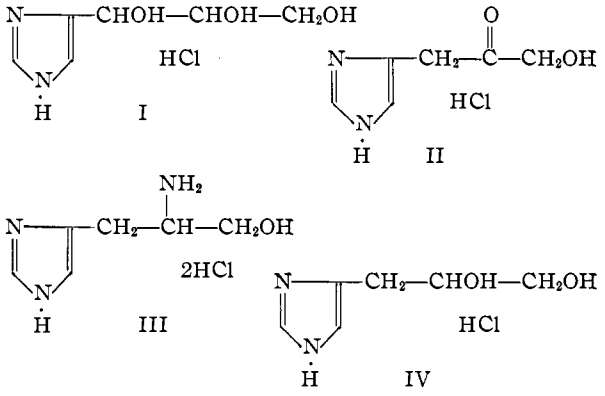
RECEIVED AUGUST 19, 1952

A general method is given for the isolation of a number of imidazole derivatives that are accumulated by mutants of *Neurospora* and *Penicillium*. Methods of degradation and chromatography have been used to identify with reasonable certainty three crystalline products as 4-(trihydroxypropyl)-imidazole, 4-(2-keto-3-hydroxypropyl)-imidazole and L-4-(2-amino-3-hydroxypropyl)-imidazole (L-histidinol). Evidence is presented to show that two other non-crystalline products are phosphate esters of the trihydroxy and ketohydroxy compounds while a sixth product is 4-(2,3-dihydroxypropyl)-imidazole. These findings are discussed in relation to the problem of the biological synthesis of histidine. A chemical synthesis of isomers of 4-(trihydroxypropyl)-imidazole is described.

Previous investigations in this Laboratory on the histidine requiring mutants of *Neurospora*[3,4] have demonstrated that several of these mutants accumulate imidazole derivatives in the medium that are related in some way to the biological synthesis of histidine. Although none of the accumulated imidazoles will support the growth of any of the neurospora mutants, it was hoped that their isolation and identification would provide a clue to the biosynthetic pathway that yields this amino acid. Evidence is presented here that supports the contention that histidine is derived from carbohydrate, probably pentose, in quite a direct manner. This conclusion is in agreement with the results from tracer experiments reported by Levy and Coon.[5]

By means of mercuric chloride precipitations followed by chromatography on Dowex-50, 4-(trihydroxypropyl)-imidazole hydrochloride (I) has been isolated from mutant C84,[6] I and 4-(2-keto-3-hydroxypropyl)-imidazole hydrochloride (II) from mutant C141, and L-4-(2-amino-3-hydroxypropyl)-imidazole dihydrochloride (L-histidinol) (III) from mutant T1710. In addition, two compounds which appear to be the phosphate esters of I and II have been obtained from the mycelium of C141.



Another imidazole accumulated by two penicillium histidine mutants and in small amounts by C141 is chromatographically identical with 4-(2,3-dihydroxypropyl)-imidazole (IV).

Various isomers of I have been synthesized from L-arabinose, D-arabinose, D-ribose and D-xylose by the Parrod reaction of sugars with cuprammonium solution and formaldehyde.

Experimental

Isolation.—Mutant C141 was grown with forced aeration in two 15-liter cultures of Fries minimal medium[7] supplemented with 450 mg. of L-histidine monohydrochloride monohydrate per culture. This is sufficient histidine for half-maximal growth. The mycelium was collected by filtration through cheese-cloth. It was then homogenized in a blendor with several portions of boiling water, filtered and the 3 liters of filtrate obtained was combined with the

(1) This work was supported in part by funds from the Rockefeller Foundation and by funds from the Atomic Energy Commission administered through contract with the Office of Naval Research, U. S. Navy, Contact N6 onr-244, Task Order V.

(2) A. E. C. Predoctoral Fellow in the Biological Sciences.

(3) J. Lein, H. K. Mitchell and M. B. Houlahan, *Proc. Natl. Acad. Sci.*, **34**, 437 (1948).

(4) F. Haas, M. B. Mitchell, B. N. Ames and H. K. Mitchell, *Genetics*, **37**, 217 (1952).

(5) L. Levy and M. J. Coon, *Federation Proc.*, **11**, 248 (1952), (abstract).

(6) Genetic and physiological data for these mutants are given in reference 4.

(7) G. W. Beadle and E. L. Tatum, *Am. J. Bot.*, **32**, 678 (1945).

30 liters of medium in which the mold had been grown. The extracted mycelium was discarded.

The solution (*p*H 4) was then brought to *p*H 8.5 with barium hydroxide. The resulting precipitate, which was allowed to settle overnight, was then removed by filtration and discarded.[8] The alkaline filtrate was treated with 100 g. of mercuric chloride dissolved in 400 ml. of ethanol to give a precipitate containing all the imidazoles. The precipitate was collected and dried. The imidazoles were extracted from the dried precipitate with 800 ml. of 0.5 *N* hydrochloric acid. This extract was treated with hydrogen sulfide and then filtered to remove the mercuric sulfide. The resulting solution was concentrated *in vacuo* to 40 ml. and utilized for chromatography on a Dowex-50 column.

A 2 × 70 cm. column of Dowex-50 resin (250–500 mesh) was prepared and equilibrated with 1.5 *M* hydrochloric acid following the method of Stein and Moore.[9] The sample was eluted with 800 ml. of 1.5 *M* hydrochloric acid followed by 800 ml. of 2.5 *M* hydrochloric acid; 20-ml. samples were obtained during the elution by use of an automatic fraction collector.[10] Samples (0.001 ml.) from each fraction were chromatographed on paper[11] in order to follow the elution process. Appropriate fractions were then combined and evaporated over sodium hydroxide pellets in a desiccator.

The same general procedure as described above has been utilized for isolation of the imidazoles from the mutants other than C141 with the exception that preliminary purifications of histidinol from the culture medium of strain T1710 were carried out by the method of Vogel, *et al.*[12]

A summary of the data on isolations of six natural and one synthetic imidazole is given in Table I.

TABLE I[a]

THE VARIOUS ISOLATED IMIDAZOLES

Substance	Mutant	*Rf* in 3:1 propanol–0.2 *N* NH_3 Whatman no. 1	*Rf* in 3:1 propanol–1 *N* acetic acid Whatman no. 1	Dowex eluate-fraction at which substance starts to appear Ml.	HCl, *N*
Phosphate ester of I	C141	0.04	0.16	200	1.5
Phosphate ester of II	C141	.07	.17	350	1.5
I	C141 also C84	.46	.46	400	1.5
Isomer of I synthesized from L-arabinose		.46	.46	400	1.5
"Penicillium imidazole"	C141	.66	.55	800	1.5
				50	2.5
II	C141	.66	.45	800	1.5
				100	2.5
III	T1710	.62	.10–0.28	500	2.5
				20	4

[a] For details of chromatography on paper see reference number 11.

Identification. I from **Mutants C141 and C84.**[13]—A colorless sirup was obtained when the Dowex fractions containing I, from the C141 isolation, were pooled and evaporated. All attempts at crystallization failed during the first few months of work with the compound. The picrate is extremely soluble and the usual base precipitants were equally unsatisfactory. The compound crystallized spontaneously, however, after a month in the refrigerator. It was triturated with acetone, then with cold absolute ethanol and recrystallized twice from absolute ethanol. Large white rosettes of broad needles were obtained; m.p. 102.5–103° (cor.), $[\alpha]^{23}D$ 11.6° (*c* 12.1, in water).

Anal. Calcd. for $C_6H_{10}O_3N_2 \cdot HCl$: C, 37.10; H, 5.67; N, 14.43. Found:[14] C, 37.53; H, 5.85; N, 14.57.

Periodate Oxidation of I.—One ml. of 0.26 *M* sodium metaperiodate was added to 20 mg. of crystalline I (from C141) in a 10-ml. volumetric flask and the solution was diluted to the mark with distilled water. The volumetric flask was placed in an ice-bath and 2-ml. aliquots were withdrawn 0.5, 1.5 and 2.5 hours after mixing. Periodate consumed was determined by the method of Forrest and Todd.[15] The reaction was found to be at an end within the first half-hour with the consumption of 1.8 moles of periodate per mole of compound I.

A second aliquot of the reaction mixture was titrated with 0.01 *M* sodium hydroxide in order to determine acid produced in the reaction (formic acid). An acid equivalent of 0.9 mole of acid per mole of I was found.

A third aliquot (1 ml.) from the periodate reaction was used for the determination of formaldehyde using a modification of the method of Boyd and Logan.[16] The 1-ml. sample was diluted to 150 ml. and a 3-ml. aliquot was mixed with 0.2 ml. of sodium arsenite solution (25%) and 2.5 ml. of filtered chromotropic acid solution (720 mg. of chromotropic acid in 20 ml. of water mixed gradually in an ice-bath with 30 ml. of concd. sulfuric acid). The mixture was heated with occasional stirring at 100° for 30 min. Known amounts of formaldehyde were treated in the same way and after cooling, optical densities were determined in the Beckman spectrophotometer at a wave length of 580 mμ. The standard curve is linear in the range of 2 to 10 μg. of formaldehyde per sample. By use of this method it was found that the periodate reaction mixture yielded an equivalent of 0.94 mole of formaldehyde per mole of compound I.

A fourth aliquot of the periodate reaction mixture was chromatographed on paper.[11] An ultraviolet absorbing, non-diazo reacting substance corresponding to imidazole aldehyde was observed. Authentic imidazole aldehyde[17] was run alongside and the R_f values were identical.[18] No other imidazole was observed.

Degradation of I to Imidazoleglyoxylic Acid.—A sample of I (150 mg.) from C84 was refluxed for 3.5 hours with 10 ml. of concd. nitric acid. Nitric acid was removed by evaporating the solution to dryness in a stream of air, adding water and evaporating again. The residue was then taken up in a small amount of water and the *p*H adjusted to 3.0 with sodium carbonate solution. The resulting precipitate of imidazoleglyoxylic acid was recrystallized from hot water; yield 57 mg., m.p. 280–290° (dec.); R_f in 3:1 1-propanol–0.2 *N* ammonia solvent, 0.28; absorption spectra: 20 μg./ml. in 0.1 *N* hydrochloric acid, maximum at 285 mμ, optical density 1.92; 20 μg./ml. in 0.1 *N* sodium hydroxide, maximum at 252 mμ, optical density, 0.81.

Anal. Calcd. for $C_5H_4O_3N_2$: C, 42.90; H, 2.86; N, 20.00; neut. equiv., 140. Found: C, 42.95; H, 2.99; N, 20.40; neut. equiv., 141.

The degradation was repeated on a small scale with I from C141 and imidazoleglyoxylic acid was identified chromatographically.

Imidazoleglyoxylic Acid from Histidine.—L-Histidine was oxidized with nitric acid to imidazoleglyoxylic acid[19] and isolated as described above. As demonstrated by chromatography of samples from the oxidation mixture, histidine is first converted to imidazoleacetic acid at a high rate but it is necessary to reflux about 10 hours to effect the oxidation of this compound and a number of side reactions occur. Compounds I and II do not give rise to this resistant intermediate. Calcd., neut. equiv., 140; found, 139; m.p. 280–290° (dec.). Knoop reported 290° (dec.); R_f in 1-propanol–ammonia, 0.28. Absorption spectra are the same as for imidazoleglyoxylic from I.

The Bis-(phenyldiazo) Derivative of I.—A cold solution of phenyldiazonium chloride (from 100 mg. of aniline, 0.3

(8) The barium precipitation removes tartrate and other substances in the medium that would otherwise come down later with the mercury precipitation of the imidazoles. Barium does not precipitate any of the imidazoles or imidazole phosphates.

(9) W. H. Stein and S. Moore, *Cold Spring Harbor Symposia Quant. Biol.*, **14**, 179 (1950).

(10) The authors are indebted to Drs. G. Keighley and H. Borsook for use of this equipment.

(11) B. N. Ames and H. K. Mitchell, THIS JOURNAL, **74**, 252 (1952).

(12) H. J. Vogel, B. D. Davis and E. S. Mingioli, *ibid.*, **73**, 1897 (1951).

(13) Compound I was isolated from C84 several years before the Dowex technique became available. It was never obtained crystalline but was investigated as a sirup.

(14) All microanalyses were done by Mr. G. Swinehart.

(15) H. S. Forrest and A. R. Todd, *J. Chem. Soc.*, 3295 (1950).

(16) M. J. Boyd and M. A. Logan, *J. Biol. Chem.*, **146**, 279 (1942).

(17) A sample was generously supplied by Dr. Peter Lowy of the California Institute of Technology.

(18) An R_f value of 0.75 was obtained in 3:1 *n*-propanol–0.2 *N* ammonia.

(19) F. Knoop, *Beitr. Chem. Physiol. u. Path.*, **10**, 116 (1907).

ml. of concentrated hydrochloric acid, 80 mg. of sodium nitrite and 1.5 ml. of water) was added to 150 mg. of I, (from C84) in 5 ml. of water. The coupling was done in an ice-bath. A concentrated sodium carbonate solution was added to the solution until a red precipitate of the bis-(phenyldiazo) derivative was formed. This precipitate was washed with water and recrystallized from aqueous methanol; m.p. 173–180° (dec.).

Anal. Calcd. for $C_{18}H_{18}N_6O_3$: C, 59.00; H, 4.97; N, 22.94. Found: C, 59.08; H, 5.39; N, 22.30.

Synthesis of 4-(L-*erythro*-Trihydroxypropyl)-imidazole.—Parrod[20] described a method for the preparation of 4-(D-*arabino*-tetrahydroxybutyl)-imidazole from glucose or fructose. This method as modified by Huebner[21] has now been utilized for the preparation of the various optical isomers of 4-(trihydroxypropyl)-imidazoles from different pentoses.

L-Arabinose (10 g.) was dissolved in a solution of 45 g. of copper acetate in 75 ml. of concd. aqueous ammonia. After the addition of 16 ml. of 37% formaldehyde solution the mixture was heated on a water-bath for 30 min. The copper–imidazole precipitate from the cooled reaction mixture was removed, resuspended and treated with hydrogen sulfide. The products were then separated on a Dowex-50 column as described above for the natural imidazoles. The desired product (approx. 0.5 g.) was eluted from the column in the same fractions as compound I. Similar preparations were made on a smaller scale from D-arabinose, D-ribose and D-xylose. None of these products has been obtained in a crystalline form but all have the same chromatographic and chemical properties as I. A comparison of the optical properties of the various products has not yet been carried out since the synthetic compounds have not yet crystallized.

II from Mutant C141.—On evaporating the pooled fractions containing II from the C141 Dowex column, the compound crystallized as white needles. These were recrystallized three times from 95% ethanol; m.p. 171–174° dec. (cor.). The crystals turn brown at 171°.

Anal. Calcd. for $C_6H_8O_2N_2 \cdot HCl$: C, 40.86; H, 5.11; N, 15.90. Found: C, 40.79; H, 4.85; N, 15.58.

II reduces alkaline copper sulfate and Tollens reagent and reacts with phenylhydrazine.

Periodate Oxidation of II.—The periodate oxidation was done by the same method as described for I. Aliquots taken at 1.5, 6.5 and 20 hours all gave a value of 1.1 moles of periodate taken up per mole of II. 1.0 mole of formaldehyde was found. Imidazoleacetic acid[22] was the only imidazole apparent on chromatographing the reaction mixture. The isomeric hydroxyaldehyde, which would also reduce alkaline copper sulfate, would, unlike II, yield imidazoleacetaldehyde and formic acid on periodate oxidation.

Degradation of II to Imidazoleglyoxylic Acid.—Compound II (200 mg.) was refluxed with 20 ml. of concentrated nitric acid. With this compound the maximum yield was obtained within 1 hour. No imidazoleacetic acid could be detected on the chromatograms run at intervals during the oxidation. The carbonyl group apparently activates the methylene group next to the ring with the resultant rapid formation of imidazoleglyoxylic acid in high yield. The isolation was done in the same way as for the product from I; m.p. 280–290° (dec.); R_f value in *n*-propanol–ammonia, 0.28. Absorption spectra are as given for imidazoleglyoxylic from I.

III (L-Histidinol) from Mutant T1710.—On evaporation of the hydrochloric acid eluate from the T1710 Dowex run, III crystallized. It was recrystallized three times from 95% ethanol; m.p. 194–195°. A sample mixed with authentic L-histidinol dihydrochloride[23] (m.p. 193.5–194.5°) melted at 193.5–194.5°. The R_f values of the two samples were identical.

Anal. Calcd. for $C_6H_{11}ON_3 \cdot 2HCl$: C, 33.78; H, 6.10; N, 19.70. Found: C, 33.42; H, 5.87; N, 20.04.

Phosphate Ester of I.—Evaporation of the first fraction from the Dowex chromatogram of imidazoles from mutant C141 (Table I) yielded 500 mg. of sirup. Chromatography of this material on paper gave rise to a spot which reacted with both the diazo reagent and the phosphate ester reagent of Hanes and Isherwood (as utilized by Bandurski and Axelrod[24]). Hydrolysis of the material with 6 *M* hydrochloric acid at 100° for 1 hour or with alkaline phosphatase (Armour) at *p*H 7.8 for 2 hours at 35° yielded products containing orthophosphate and an imidazole that is chromatographically identical with I in several solvents.

II Phosphate Ester from Mutant C141.—The fractions containing the second imidazole from the C141-Dowex run also gave a sirup when evaporated. The substance gave a diazo reaction and a phosphate ester test at the same R_f value on a chromatogram (Table I). Warming this material in dilute hydrochloric acid solution caused complete hydrolysis to yield a substance that is chromatographically identical with II. In addition both II and the hydrolysis product gave an orange ninhydrin test, reduced alkaline copper sulfate and gave a slightly rose colored diazo reaction at the same R_f value on a chromatogram.

This ester hydrolyzed spontaneously over a period of several months. An α-keto group is known to labilize phosphate esters of this type, as can be seen by the extreme lability of dihydroxyacetone phosphate ester.

"Penicillium Imidazole Compound" IV.—This imidazole from C141 runs slightly faster than II on the Dowex column. Unlike II, to which it is very similar in chromatographic properties (Table I), it does not reduce copper nor does it give a ninhydrin reaction. It is chromatographically identical both with the imidazole accumulated by two penicillium histidine-less mutants and with 4-(2,3-dihydroxypropyl)-imidazole obtained by nitrous acid treatment of III. Only a small amount is accumulated by C141 and insufficient material for characterization was obtained from the Dowex run. Two of the penicillium mutants accumulate IV in large amounts, but it has not yet been isolated in large quantities.

Discussion

Structural Evidence.—The degradations of I and II to imidazoleglyoxylic acid establishes the imidazole ring and the position of the non-branched side chain at carbon atom 4. The results of the periodate oxidations furnish evidence for structures[25] which are fitted by only I and II. The analyses, derivatives and preliminary synthetic work in the case of I all confirm the structure presented. As yet there is no evidence indicating whether I is the D-*erythro*, L-*erythro*, D-*threo* or L-*threo* isomer. II has no asymmetric carbon atoms and its structure is therefore uniquely determined. III is a known compound, synthesized by Karrer, *et al.*,[26] and isolated from a histidine-less mutant of *E. coli* by Vogel, Davis and Mingioli.[12] The determination of its structure is therefore based on a comparison with authentic III. Agreement of various physical properties, analysis and a mixed melting point seem to establish conclusively the identity of III.

The phosphate esters of I and II have not been crystallized and the evidence for their structure is chromatographic and enzymic. The position of the phosphate group on I is unknown, though by analogy with the ester of II which only has one hydroxyl, the primary hydroxyl group seems likely, but a phospho-enol linkage is possible in II. The relative stability of the ester of I to acid hydrolysis suggests a primary ester.

The evidence for the identity of IV with the imidazole accumulated by two of the penicillium histidine-less mutants and in small amounts by C141 is solely chromatographic. It is mentioned because

(20) J. Parrod, *Ann. chim.*, **19**, 233 (1933).

(21) C. F. Huebner, THIS JOURNAL, **73**, 4667 (1951).

(22) The R_f value in *n*-propanol–ammonia is 0.31.

(23) A generous sample was obtained through the courtesy of Drs. H. J. Vogel and B. D. Davis.

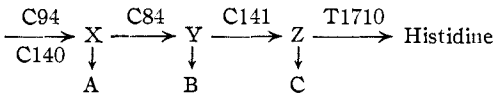
(24) R. S. Bandurski and B. Axelrod, *J. Biol. Chem.*, **183**, 405 (1951).

(25) A discussion of the applicability and specificity of this reagent is given by E. L. Jackson in "Organic Reactions," Vol. 2, John Wiley and Sons, Inc., New York, N. Y., 1944, p. 341.

(26) P. Karrer, M. Suter and P. Waser, *Helv. Chim. Acta*, **32**, 1936 (1949.)

of the apparent structural relationships with the other compounds and its accumulation by both fungi.

The Biosynthesis of Histidine.—A previous publication[4] presented genetic and biochemical evidence that the histidine mutants of neurospora are related from the standpoint of biosynthesis of histidine according to the scheme



A, B and C refer to the compounds accumulated by the various mutants and they were not considered to be intermediates in the biosynthesis. These compounds have now been identified as: A = I, B = II and C = III. These compounds do not support the growth of any of the mutants and the discovery of the phosphate esters of I and II in the mycelium of strain C141 suggests the possibility that these esters correspond to the actual intermediates X and Y indicated in the above scheme. Since phosphate esters in general are not taken up by growing neurospora, it is necessary to use enzymatic methods to determine whether these esters are actually intermediates in histidine biosynthesis. Experiments of this kind are in progress. Since the first compound accumulated in the series contains a trihydroxypropyl side chain, it is a particularly attractive hypothesis that the carbon chain of histidine is derived in quite a direct fashion from pentose phosphate.

Compound III isolated from an *E. coli* histidine mutant by Vogel, *et al.*,[12] provided an important clue as to the nature of the biosynthetic pathway. It was found that his isolated L-histidinol was utilized slowly by another histidineless *E. coli* mutant. By selection from this second mutant, a strain was obtained which utilized L-histidinol 75% as well as histidine. If not histidinol, but its phosphate ester is the true intermediate, results such as this can be easily explained. Perhaps what was being selected for was an organism with a histidinol phosphorylating enzyme.

Acknowledgment.—We are grateful to Dr. Adolph Abrams for his many helpful suggestions concerned with chromatography on Dowex and to Dr. Hugh Forrest for his interest in the work and his aid in the periodate titrations. We are indebted to Dr. Felix Haas for mutants C140 and C141 and to Dr. C. E. Harrold for mutant T1710.

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[CONTRIBUTION FROM THE RESEARCH LABORATORIES OF MERCK & CO., INC.]

Streptomyces Antibiotics. XXV. Isolation of Neomycin A

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RECEIVED AUGUST 4, 1952

Neomycin A has been isolated and characterized as the crystalline free base and as the crystalline picrate and *p*-(*p'*-hydroxyphenylazo)-benzene sulfonate. Neomycin A sulfate and hydrochloride were obtained as amorphous white solids. The steps leading to the isolation of neomycin A comprised chromatography of "neomycin complex" on alumina, formation of picrate, conversion to hydrochloride, rechromatography on alumina, and formation and recrystallization of the *p*-(*p'*-hydroxyphenylazo)-benzene sulfonate. An alternative procedure involved acid-treatment of "neomycin complex," formation and recrystallization of the picrate. Acetylated and benzoylated neomycin A as amorphous solids were prepared. Catalytic deacetylation yielded crystalline N-acetylneomycin A. Neomycin A may occur as a minor constituent of the "neomycin complex." Acid hydrolysis forms additional amounts of neomycin A, showing the presence of conjugated neomycin A.

Some of the chemical and biological properties of crystalline salts of neomycin A have been reported.[1] The methods used for separation of neomycin A from crude concentrates and for the preparation of pure salts of this antibiotic are described herein.

Early studies of neomycin[2,3] indicated that the biological activity of culture filtrates of *Streptomyces fradiae* was due to a single antibiotic entity. Very shortly, however, it was recognized that this activity was in reality due to a mixture of at least three antibiotic components, and the term "neomycin complex" was suggested to describe the crude concentrates obtained from culture filtrates.[4] Another antibiotic substance, active mainly against fungi, was also recognized as an elaboration product of *Streptomyces fradiae* and was later designated fradicin.[4,5] The latter substance is not considered as part of the neomycin complex, as it is separated at an early stage of purification. Purification studies on the neomycin complex have thus far resulted in the isolation of three antibiotic entities which have been designated neomycin A,[1] B[6,7] and C.[7]

Our early purification results led us to believe that crude concentrates of neomycin were yielding at least two antibiotically active substances. Chromatographic fractionation was followed by means of the cup assay employing *B. subtilis*. Since neomycin A appeared to diffuse more readily in agar than did other active components of the neomycin complex, this assay facilitated our work. The steps leading to pure neomycin A comprised the following: chromatography of crude concen-

(1) R. L. Peck, C. E. Hoffhine, Jr., P. H. Gale and K. Folkers, THIS JOURNAL, **71**, 2590 (1949).

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

(3) S. A. Waksman, H. A. Lechevalier and D. A. Harris, *J. Clin. Invest.*, **28**, 934 (1949).

(4) E. A. Swart, D. Hutchinson and S. A. Waksman, *Arch. Biochem.*, **24**, 92 (1949).

(5) E. A. Swart, A. H. Romeo and S. A. Waksman, *Proc. Soc. Exp. Biol. Med.*, **73**, 376 (1950).

(6) P. P. Regna and F. X. Murphy, THIS JOURNAL, **72**, 1045 (1950).

(7) J. D. Dutcher, N. Hosansky, M. N. Dorien and O. Wintersteiner, *ibid.*, **73**, 1384 (1951).