[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, UNIVERSITY OF WISCONSIN]

Production, Isolation and Components of the Antibiotic Streptolin[1]

BY L. M. LARSON, HELEN STERNBERG AND W. H. PETERSON

RECEIVED DECEMBER 19, 1952

Streptolin broth was prepared by deep culture fermentation of streptomyces 5-11. The crude antibiotic was isolated by a method which involved carbon adsorption, elution with aqueous acetone followed by precipitation with acetone. It was crystallized as the reineckate and converted to a sulfate which assayed 43,000 u./mg. A system of ion-exchange paper chromatography showed that the broth had five active components and that the purified sulfate was a mixture of two components designated streptolins A and B. Streptolin A was isolated in pure form by fractional crystallization of the helianthates. Streptolin A sulfate assayed 32,000 u./mg. Analytical data indicate that streptolins A and B are very similar compounds.

Streptolin was first reported by Rivett and Peterson[2] as an antibiotic isolated from culture filtrates of a species of streptomyces designated S-11. It was of special interest because of its activity against a wide range of gram positive and gram negative bacteria. The deep culture fermentation of the organism was studied and a crystalline helianthine salt of the antibiotic was isolated. It was found to be different from any of the known antibiotics but was too toxic to be of practical significance. This early work did not establish the homogeneity of the product nor did it reveal much of its chemical nature.

In the present work the product has been found to be a mixture of the two very similar antibiotics which we have called streptolins A and B. The purpose of this paper is to describe the isolation and characterization of streptolin A, the identification by means of a new system of ion-exchange paper chromatography of the active components produced by the organism, and the preparation of substantial amounts of material suitable for chemical characterization of the antibiotics.

Large amounts of broth were prepared by the submerged fermentation of the organism in a 50-gallon fermentor. Yields of around 40,000 *E. coli* dilution units per milliliter were obtained. A crude sulfate I was isolated from the culture filtrate by adsorption of the antibiotic on activated carbon, elution with dilute acetone and precipitation with solvents. This sulfate assayed around 30,000 units per milligram.

The antibiotic was further purified by crystallization as the reineckate II. This assayed 19,000 units per milligram and was converted to an amorphous sulfate III which assayed 43,000 units per milligram. A helianthate IV was prepared from III and crystallized from methanol. This assayed 19,500 units per milligram and yielded a sulfate V which again assayed 43,000 units per milligram.

Craig analyses, with a butanol–water–*p*-toluenesulfonic acid system, of the purified sulfates (III and V) showed no inhomogeneity. However, paper chromatography showed these products as well as the purest preparation described by Rivett and Peterson[1] to consist of essentially two active components, streptolins A and B. Quantitative chromatograms indicate that about 24% of the activity and 37% of the weight of this mixture is due to streptolin A.

Streptolin A helianthate (V) was isolated in presumably pure form by repeated crystallization of the helianthate mixture, IV, from 50% methanol. As shown in Table I, streptolin A which forms the more insoluble helianthate, reaches a potency of around 14,700 units per milligram and does not change on further crystallization. Streptolin A sulfate (VI), prepared from V, assayed 32,000 units per milligram.

Streptolin B, which has a much higher potency, has not been isolated in pure form. The helianthate (Table I, Crop 2-L) obtained from the crystallization liquors, had a potency of 24,000 units per milligram and yielded a sulfate which assayed 55,000 units per milligram. It was however still contaminated with considerable amounts of streptolin A.

Elemental analyses of crystalline streptolin A helianthate (V) are in good agreement for a free base with the minimum formula $C_{17}H_{31}N_5O_8$ or $C_{24}H_{45}N_7O_{11}$. The infrared absorption spectrum of streptolin A sulfate is shown in Fig. 1.

It is quite probable that streptolins A and B have very similar chemical composition. The elemental analyses of the mixture, IV, are in close agreement with those of pure A. This is particularly noticeable in case of the helianthates corresponding to crops 1, 5 and 7 in Table I. The optical rotations of the sulfates corresponding to the mixture and pure A are very similar. Like-

TABLE I

FRACTIONAL CRYSTALLIZATION OF STREPTOLIN HELIANTHATE

Crop no.[a]	Weight, g.	Potency, u./mg.	Elemental analyses, % C	H	N	S
1	1.00	19,500	52.23	5.82	14.54	6.91
2	0.36	16,500				
2L	.42	24,000				
3	.27	15,800				
4	.22	15,000				
5	.19	14,500	52.45	5.78		6.80
6	..	....				
7	.15	14,700	52.5	5.90	14.46	6.85
Calcd. for $C_{17}H_{31}N_5O_8 \cdot 3C_{14}H_{15}N_3O_3S$			52.52	5.67	14.53	7.11
$C_{24}H_{45}N_7O_{11} \cdot 4C_{13}H_{15}N_3O_3S$			52.53	5.79	14.55	7.01

[a] The starting material, crop 1, was streptolin helianthate IV which had been crystallized from methanol. The subsequent crystallizations were from 50% methanol. Crop 2L was isolated by evaporation of the crystallization liquors from crop 2. Crop 6 was not weighed and assayed.

(1) Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by grants from Public Health Service, National Institutes of Health, and Parke, Davis and Co. Presented at the 121st Meeting, American Chemical Society, Milwaukee, Wis., 1952.

(2) R. W. Rivett and W. H. Peterson, THIS JOURNAL, **69**, 3006 (1947).

wise the infrared spectrum of the mixture is apparently identical with that of pure A.

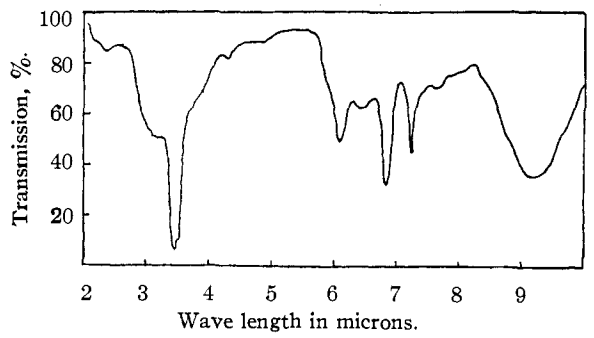

Fig. 1.—Infrared absorption spectrum of streptolin A sulfate in a nujol mull.

Acid hydrolysis of both A and the mixture, followed by chromatographic separation on a Dowex 50 column, yields the same four ninhydrin-positive products. One of these has been identified as ammonia and another as isolysine (3,6-diaminohexanoic acid).[3] Preliminary reports on the separation and identification of these components has been given[4] and will be covered in more detail elsewhere.

Paper Chromatography.—The active components found in the broth and in the various preparations which have been described were studied by means of an ion-exchange paper chromatography system. This was developed as an extension to paper strip chromatography of the methods which have been used so widely for column chromatography with ion-exchange resins.

A paper cation exchanger was effected by impregnating paper strips with a salt (sodium sulfate) which is relatively insoluble in a suitable developing solvent (75% ethanol). The anion of this salt is also capable of forming a similarly insoluble salt with the antibiotic cation. The developing solvent contains a second salt (sodium chloride) which can exchange with the insoluble antibiotic salt to yield a relatively soluble salt which can migrate. Buffer capacity was achieved by using a sodium sulfate–bisulfate buffer for impregnating the strips.

The use of this and other variations of this general method for resolving and identifying a number of common antibiotics and other compounds will be described elsewhere.

After downflow development, the strips were analyzed by plating them on agar seeded with *B. subtilis*. Because of very slow diffusion, the streptolin inhibition zones were difficult to interpret except when sodium chloride was added to the agar. This effect is particularly evident in the first two strips shown in Fig. 2.

Strips 1 and 2 are longitudinal halves of the same chromatogram of a broth sample. The first was plated on agar which contained no salt. It shows two discrete rapidly moving zones and one very elongate, ill-defined zone. The fastest moving zone has been identified as streptothricin, the next is unidentified, and the elongate zone contains the streptolin activity.

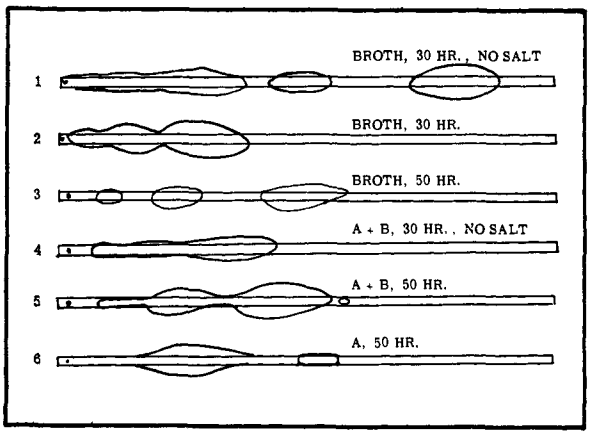

Fig. 2.—Ion-exchange paper chromatograms of streptolin preparations. The sample applied, time of development, and whether or not the assay agar contained salt is indicated by the individual labels. The sample was applied at the small spot at the left. Development was from left to right. Strips 1 and 2 represent 300 units of streptolin broth; strip 3, 150 units of broth; strip 4, 15 γ of streptolin sulfate which was prepared from the crystalline reineckate; strip 5, 15 γ of streptolin A sulfate.

When the other half of the strip was plated on agar containing salt (strip 2), the two faster moving zones were no longer apparent. Presumably they had diffused to such an extent that there was no longer an inhibiting concentration in a defined zone. These conditions, however, revealed concentration gradients of the elongate zone such that three components were evident. The faster-moving zone is streptolin B, the next streptolin A and the slowest is unidentified. More effective separation of these antibiotics was obtained by longer development of the chromatogram as is shown in strip 3.

All the antibiotics found in the broth except streptolins A and B are removed by the purification steps leading up to the crystalline reineckate. Chromatographic analyses of streptolin sulfate III prepared from the reineckate is shown in strips 4 and 5 of Fig. 2. The first of these was developed for only a short time and plated on agar which contained no salt to show the absence of streptothricin and the other faster-moving antibiotic. Strip 5 is a similar chromatogram, after further development, plated on sodium chloride agar. It shows that the slowest moving antibiotic has also disappeared and only streptolins A and B are present.

The last chromatogram shows the analyses of a streptolin A sulfate (IV) prepared from helianthate which had been fractionally crystallized. It appears to still contain a trace of B activity.

Quantitative chromatograms were run on the mixture of streptolins A and B. Strips similar to 4 in Fig. 2 were cut into quarter-inch squares and the antibiotic concentration on each square determined by assaying on diffusing agar with pure streptolin A as a standard. The results of these analyses are shown in Fig. 3. The streptolin A fraction accounted for 37% of the weight of the mixture and 24% of the activity. The total recovery

(3) E. E. van Tamelen and E. E. Smissman, THIS JOURNAL, **75**, 2031 (1953).

(4) E. E. Smissman, R. W. Sharpe and E. E. van Tamelen, Abstracts 121st Meeting Am. Chem. Soc., Milwaukee, Wis., 8c (1952).

of activity was 110%. This is probably due to unequal diffusion rates for the two antibiotics. This composition is also characteristic of the streptolin prepared by Rivett and Peterson.[1]

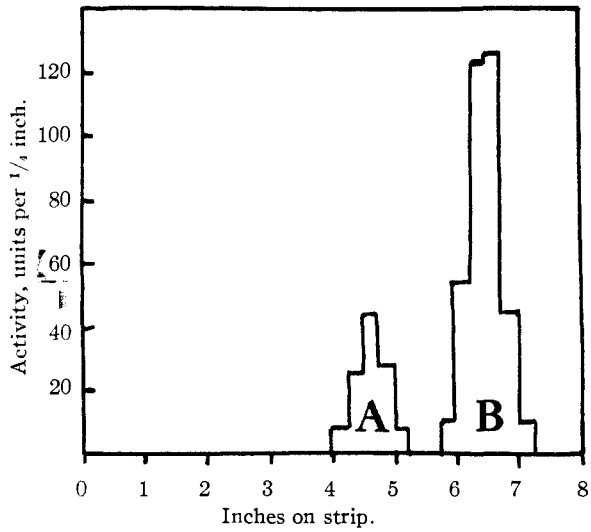

Fig. 3.—Quantitative assay of a sectioned paper chromatogram of the streptolin sulfate mixture III with streptolin A sulfate as a standard: 430 units (10 γ), of the mixture was applied; 115 units (3.6 γ) of A and 360 units of B was recovered.

Antibiotic No. 136 reported by Bohonos, *et al.*[5] and found by Peterson and Reineke[6] to contain five components by their papergram method has been analyzed by our system of chromatography and found to contain the same five components as are present in broths from our culture, S-11. It is probable that cultures S-11 and 136 are similar if not identical microörganisms. A systematic bacteriological study of both microörganisms will have to be made to establish their identity or non-identity.

Acknowledgment.—The authors wish to acknowledge the assistance of R. W. Sharpe and E. E. Smissman in the production of streptolin and that of Margaret Larson in the *coli* assays. They are indebted to D. J. Johnson for the infrared absorption data and to Dr. M. J. Johnson for many helpful suggestions on the chromatography work.

The animal toxicity of all the streptolin preparations appears to be about equivalent on an activity basis. Levels of around 200,000 units (6 mg. of streptolin A sulfate) per kilo of body weight were lethal intravenously to mice.

Experimental

Assay and Standards.—The *E. coli* turbidimetric assay and standards described by Rivett and Peterson[1] were generally used for determination of streptolin activity. The streptomycin disc assay described by Loo, *et al.*,[7] was also satisfactory if 1% sodium chloride was added to the *B. subtilis* agar.

Fermentation.—Fermentation broths were produced by the general deep culture procedure which Rivett and Peterson had used in 30-liter fermentors. A fifty-gallon glass lined Pfaudler fermentor was used. It was equipped for agitation, sterile air supply and automatic temperature control. This fermentor is described in detail elsewhere.[8]

Optimum aeration and agitation conditions were translated from the 30-liter fermentors to the large fermentor by using conditions which gave equivalent sulfite oxidation rates as determined by the method of Cooper, *et al.*[9] This rate was 0.8 millimole of oxygen per liter per minute and corresponded to an air flow of 1.25 vol. per vol. per minute with agitation of 140 r.p.m. in the large fermentor.

The fermentor contained 25 gallons of medium (1.5% soy bean meal, 1.5% corn steep solids, 1.5% glucose, 0.5% sodium chloride and 0.2% calcium carbonate). A 2%, 72-hr. vegetative inoculum was used for seeding, and 2% octadecanol in lard oil was added at intervals to control foaming.

The broths were harvested around 96 hr. after inoculation. Typical broths assayed 30,000 to 50,000 *E. coli dilution* units per ml.

Isolation of Crude Streptolin.—A crude product was isolated by a procedure similar to that described for recovery of streptothricin.[10] One hundred liters of broth which assayed 30,000 units per ml. was acidified to *p*H 2 with sulfuric acid, mixed with 250 g. of Darco G-60 and filtered with Hyflo (Johns-Manville Co., filter aid), through a plate and frame filter press. The filtrate was neutralized to *p*H 7.5 with sodium hydroxide and again filtered. The antibiotic was then adsorbed on 1500 g. of Darco G-60, 500 g. of Hyflo was mixed with the adsorbate. The mixture was collected in the filter press and washed with 10 gallons of water. About 5% of the activity was lost in these steps.

The cake was eluted by cycling 18 liters of 7% acetone through the press for 30 minutes and adding dilute sulfuric acid such that *p*H 2 was maintained. This was repeated twice with new batches of acetone. The third eluate was held for elution of subsequent batches. The first two eluates, which contained about 75% of the adsorbed activity, were mixed with two volumes of acetone. The gum which settled on standing was dissolved in 600 ml. of water, adjusted to *p*H 2 with sulfuric acid and precipitated with 3 liters of methanol.

Forty-five grams of crude sulfate I which assayed 30,000 units per milligram was obtained, giving an over-all recovery of 45%. In all about 200 g. of this material was prepared.

Crystalline Streptolin Reineckate.—Twenty-five grams of I was precipitated from water with 50 g. of ammonium reineckate. The precipitate was crystallized slowly from 400 ml. of warm 40% ethanol. Twenty-four grams of crystalline streptolin reineckate II assaying 19,000 units per milligram was obtained and analyzed. *Anal.* Calcd. for $C_{17}H_{31}N_5O_8 \cdot 3C_4H_7N_6S_4Cr$: C, 25.03; H, 3.76; N, 23.15; Cr, 11.12. Found: C, 25.25; H, 4.1; N, 22.91; Cr, 11.06. The analytical data fit equally well other minimum formulas such as $C_{24}H_{45}N_7O_{11} \cdot 4C_4H_7N_6S_4Cr$. The hydrochloride of streptolin has also been prepared and analyzed.[11] The data fit either the C_{17} or the C_{24}-formulas. To date adequate data are not available for distinguishing among the possible minimum formulas of streptolin.

Ten grams of reineckate was converted to the sulfate by dissolving in 250 ml. of methanol and precipitating with 8 ml. of 2.6 *N* sulfuric acid. The precipitate was dissolved in water, decolorized with carbon and reprecipitated with methanol. Four grams of a white amorphous sulfate III which assayed 43,000 units per milligram was obtained, $[\alpha]^{25}D$ −20° (*c* 1.8).

Crystalline Streptolin Helianthate.—By the method of Rivett and Peterson a helianthate was prepared from III and crystallized from methanol. Three grams of III yielded 6.1 g. of helianthate IV which assayed 19,500 units per mg. It melted with decomposition at 203°. The analytical data for IV is given as that of the first crop in Table I.

One gram of IV was converted to the sulfate by slurrying with three successive 100-ml. portions of 0.5 *M* triethylamine sulfate in 90% methanol. The resultant yellow

(5) N. Bohonos, R. L. Emerson, A. J. Whiffen, M. P. Nash and C. DeBoer, *Arch. Biochem.*, **16**, 215 (1947).

(6) D. H. Peterson and L. M. Reineke, THIS JOURNAL, **72**, 3598 (1950).

(7) Y. H. Loo, P. S. Skell, H. H. Thornberry, J. Erhlich, J. M. McGuire, G. M. Savage and J. C. Sylvester, *J. Bact.*, **50**, 701 (1945).

(8) G. H. Buelow and M. J. Johnson, *Ind. Eng. Chem.*, **44**, 2945 (1952).

(9) C. M. Cooper, G. A. Fernstrom and S. A. Miller, *ibid.*, **36**, 504 (1944).

(10) M. J. Vander Brook, A. N. Wick, W. H. DeVries, R. Harris and G. F. Cartland, *J. Biol. Chem.*, **165**, 463 (1945).

(11) E. E. Smissman, R. W. Sharpe, B. F. Aycock, E. E. Van Tamelen and W. H. Peterson, THIS JOURNAL, **75**, 2029 (1953).

product was washed with methanol, dissolved in 10 ml. of water, decolorized with carbon and precipitated with 50 ml. of methanol; 0.41 g. of amorphous white sulfate which was apparently identical with III was obtained.

Isolation of Streptolin A.—One gram of IV was dissolved in 500 ml. of 50% methanol at 55° and allowed to cool slowly to room temperature. The crystals which separated were similarly recrystallized five times. The results of this procedure and the analytical data for the product are given in Table I. The more insoluble helianthate V which had constant properties for the last three crystallizations appears to be a homogeneous product and is designated streptolin A, m.p. 206° (dec.). This procedure was repeated with similar results on a larger batch of material.

Streptolin A helianthate V yielded an amorphous sulfate VI which assayed 32,000 units per milligram, $[\alpha]^{25}D$ −20° (*c* 1.0).

The elemental analyses of the amorphous sulfates do not agree with that expected from the corresponding crystalline products. This is presumed to be due to formation of mixed salts (*i.e.*, tritetrabasic), and possible contamination with triethylamine.

Evaporation of the 2nd crystallization liquors gave a helianthate with higher biological activity (product 2-L, Table I). This yielded a sulfate VII which assayed 54,000 units per milligram, $[\alpha]^{25}D$ −22° (*c* 2). This has been shown by paper chromatography to be still a mixture of streptolins A and B.

The infrared spectra of streptolin A sulfate (VI) and the mixed sulfate VII, were determined in a nujol mull. A Baird recording infrared spectrophotometer with a sodium chloride prism was used. The spectrum for streptolin A is given in Fig. 1. The spectrum for the mixture was within experimental error of that from streptolin A.

Chromatographic Data.—The general procedure and equipment described by Karnovsky and Johnson[12] was used for developing and analyzing downflow chromatograms. One-half inch strips of Eaton-Dikeman No. 613 paper were soaked in a solution containing 0.95 *M* sodium sulfate and 0.05 *M* sodium acid sulfate and air dried. Strips were cut such that twenty inches was allowed for development of the chromatogram. After application of the antibiotic sample, the strips were placed in the developing chamber such that the point of sample application was about three inches from the liquid level in the reservoir. Development was carried out at 25° for 30 to 50 hr. The solvent front went beyond the end of the strip.

At the end of the development period, the tapes were air-dried, split lengthwise and analyzed by plating on agar inoculated with *B. subtilis*. The agar medium was the same as the streptomycin assay medium of Loo, *et al.*, except that when diffusion of the streptolin antibiotics was desired, 1% sodium chloride was added. The plates were incubated at 25°.

Quantitative estimations of the chromatogram zones were made by cutting the strips into one-fourth inch squares and assaying these against squares containing known amounts of streptolin A on the streptolin assay agar. The analyses of the various preparations are given in Figs. 2 and 3.

(12) M. L. Karnovsky and M. J. Johnson, *Anal. Chem.*, **21**, 1125 (1949).

MADISON 6, WISCONSIN

[CONTRIBUTION FROM THE DEPARTMENT OF AGRICULTURAL BIOCHEMISTRY, THE OHIO STATE UNIVERSITY]

Hydrolysis of Proteins by Ion Exchange Resin Catalysis[1]

BY JACK C. PAULSON, FRED E. DEATHERAGE AND EMORY F. ALMY

RECEIVED NOVEMBER 24, 1952

The hydrolysis of casein by ion exchange resin catalysis proceeds very near to the point of completion after refluxing the protein with 0.05 *N* hydrochloric acid and Dowex 50 for a period of 48 hours. Low recoveries of certain amino acids are believed to be due to the presence of unhydrolyzed peptide residues. The rate of protein hydrolysis by ion exchange resin catalysis is affected by the nature of the resin and the nature of the protein. Among the proteins investigated, this type of hydrolysis indicates a peptide bond selectivity for certain amino acids.

Introduction

It has been shown that rates of protein hydrolysis by dilute acid depend not only on temperature and acidity, but also on the nature of the anion of the acid used.[2] It was demonstrated that certain high molecular weight sulfonic acids, at concentration levels less than 0.2 *M*, are more effective catalysts than the common mineral acids. The mechanism of catalysis was described as an increased basicity of the amide and peptide bonds as a result of a combination of these bonds with the large anion of the catalyst.

The practical difficulty of catalyst removal is overcome by using an insoluble sulfonic acid polymer such as an ion exchange resin. In the case of a cation exchange resin, the large nucleophilic anion should be effective in labilizing the protein peptide bond. Underwood and Deatherage[3] reported that coffee bean proteins can be hydrolyzed by refluxing with water and acid regenerated Dowex 50, a polystyrenesulfonic acid ion exchange resin. The present work is concerned with the extent of hydrolysis of casein by Dowex 50, an investigation of catalytic activity of certain other ion exchange resins, and the effect of the nature of the protein.

Experimental Procedure

Hydrolysis Procedure.—The casein was isolated from skim milk according to Cohn and Hendry.[4] Bovine serum albumin, egg albumin, gelatin and lactalbumin were commercial samples. Casein: moisture, 11.9%; ash, 0.75%; bovine serum albumin: moisture, 4.32%; ash, 0.53%.

Before use, the cation exchange resins were regenerated in the hydrogen cycle by repeated suspension and decantation with 4 *N* hydrochloric acid. The resin was then rinsed with water until the filtrate was chloride free, drained free of excess water, and stored in the moist form. The anion exchange resins were regenerated in the same manner with 5% sodium hydroxide.

The hydrolysis of casein was carried out by refluxing at atmospheric pressure 3 g. of casein with 300 ml. of 0.05 *N* hydrochloric acid and 15 g. of 200–400 mesh approximately 12% cross linked Dowex 50. Since the rate of resin-catalyzed hydrolysis depends upon the hydrogen ion concentration, hydrogen ions were added within the limits of stability of the resin. Provisions must be made for stirring in order to prevent bumping and to permit good contact be-

(1) Presented before the Biological Division of the American Chemical Society at Atlantic City, N. J., September 18, 1952.

(2) J. S. Steinhardt and C. H. Fugitt, *J. Research Natl. Bur. Standards*, **29**, 315 (1942).

(3) G. E. Underwood and F. E. Deatherage, *Science*, **115**, 95 (1952); *Food Research*, **17**, 425 (1952).

(4) E. J. Cohn and J. L. Hendry in Blatt, "Organic Syntheses," Coll. Vol. II, John Wiley and Sons, Inc., New York, N. Y., 1943.