II. The National Institutes of Health has established criteria of purity for some of these properties and this information is included in the table.

Stability.—The final product meets National Institutes of Health requirements for stability when heated as a 25% solution and stabilized with either 0.04 *M* acetyl DL-tryptophanate or 0.02 *M* acetyl DL-tryptophanate and 0.02 *M* sodium caprylate. A study of the effect of added zinc on the stability of solutions of final albumin in 0.02 *M* tryptophanate plus 0.02 *M* caprylate showed no effect for amounts of zinc as high as 65 μg./ml. in tests lasting 144 hours at 57°.

Safety.—Safety tests[38] showed that the albumin had no demonstrable toxicity in the guinea pig or mouse, and in the pyrogen tests with rabbits, there were no toxic symptoms in any case other than a temperature rise with pyrogenic samples.

Zinc.—The data given in Table II indicate that the zinc levels in the placental albumin are well within the range of those occurring in the albumin now being distributed for human use. It appears from the review of Hegsted[39] that there is no danger of chronic toxicity resulting from these concentrations of zinc, since it can be absorbed and excreted in large quantities with no evidence of tissue damage.

Acknowledgment.—We wish to acknowledge the assistance of the staff of the Michigan Department of Health Plasma Fractionation Laboratory which included, besides the authors, F. C. Coryell, W. Gay, P. Jordan, A. Kennedy, G. Nonhof, Dr. O. Olson, L. Rasmussen, J. Regan and J. Sgouris. For assistance in the many analytical determinations and biological testing we are indebted to A. Burger, E. Carr, J. Collins, A. Forell, V. Ku, J. Mapes, T. Misenheimer, S. Mizuba, L. Roberts, H. Samaras and C. D. Siders.

(38) These tests were carried out in the manner prescribed by the National Institutes of Health for Normal Serum Albumin.

(39) D. M. Hegsted, J. M. McKibbin and C. K. Drinker, U. S. Pub. Health Repts., Suppl. No. 179 (1945).

LANSING, MICHIGAN

[CONTRIBUTION FROM THE RESEARCH LABORATORIES OF THE UPJOHN COMPANY]

The Isolation and Purification of Amicetin[1]

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Amicetin, a new antitubercular antibiotic, is produced in deep culture fermentation by *Streptomyces vinaceus-drappus* on a soya meal-cerelose-yeast medium and extracted by a simple butanol process using a Podbielniak extractor. The antibiotic has been obtained in crystalline form as the free base and various salts. It has been characterized on the basis of its physical, chemical and biological properties.

Amicetin[2] is produced by a previously unidentified microörganism which has now been characterized and given the name *Streptomyces vinaceus-drappus*.[3,4] Representative yields of amicetin produced by *S. vinaceus-drappus* on a soya meal-cerelose-yeast medium in different sized fermenters are given in Table I. These yields are expressed in streptomycin units as measured by a disc-plate assay procedure using *Mycobacterium avium* as the test organism.[5] By this method pure streptomycin sulfate assays at 800 units per mg., and crystalline amicetin free base, at about 1000 units per mg.

TABLE I

Fermenter (capacity)	Vol. medium	Yield, *M. avium* units/ml.
Erlenmeyer flask (500 ml.)	100 ml.	350
Glass fermenter (5 gal.)	8 l.	300
Stainless steel tank (100 gal.)	75 gal.	250
Stainless steel tank (2000 gal.)	1500 gal.	250

The clarified fermentation liquor from the larger fermenters was conveniently extracted with about one-quarter its volume of 1-butanol at *p*H 8.5–9.5 using a Podbielniak extractor. The butanol solution was extracted with approximately one-quarter its volume of dilute sulfuric acid of such strength to give a final aqueous extract of *p*H 2. These operations were repeated until the aqueous volume was sufficiently small that when the solution was brought to *p*H 8.5 and seeded, amicetin crystallized as the hydrated free base.

The crude crystals so obtained were tan to white in color, had the appearance of matted needles, and melted at about 160–165°. Material of this quality assayed 850–950 *M. avium* units per mg. When examined by the countercurrent distribution method of Craig[6] in an all-glass machine using 60 to 65 transfers with a system of 1-butanol, di-*n*-butyl ether and water (2:1:3 by volume), material at this stage of purification was found to contain 70–90% of one component, depending somewhat on the quality of the starting fermentation liquor.

Further purification was accomplished by dissolving the antibiotic in dilute hydrochloric acid, stirring with activated carbon, filtering and reprecipitating the free base by adjusting the filtrate to *p*H 8–8.5. The slurry of hydrated needle crystals was heated with constant stirring to 60–65° to convert the antibiotic to the granular anhydrous crystal form which melted at 244–245°. The low-melting needle crystals could also be converted to the granular form by warming a methanol suspen-

(1) Presented before the Division of Medicinal Chemistry at the National Meeting of the American Chemical Society at Los Angeles, California, March 15–19, 1953.

(2) C. DeBoer, E. L. Caron and J. W. Hinman, THIS JOURNAL, **75**, 499 (1953).

(3) C. DeBoer, manuscript in preparation.

(4) In the Lilly Research Laboratories, amicetin is produced by an organism known as *Streptomyces fasciculatis*.

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

(6) L. C. Craig and H. O. Post, *Anal. Chem.*, **21**, 500 (1949).

sion. The purified product assayed 1000–1200 *M. avium* units per mg. A representative sample of amicetin of this quality was found to contain 99% of one component by solubility analysis.

The molecular formula of amicetin has not yet been rigorously established, but elemental analyses and titration data are in keeping with the formula $C_{29}H_{44}N_6O_9$. Amicetin is soluble in aqueous mineral acid and alkali, but only slightly soluble (1–2 mg. per ml.) in water at room temperature. Of the common organic solvents, amicetin is most soluble in water-saturated 1-butanol. Titration studies show that amicetin contains weakly basic groups with pK_a's of *ca.* 7, and a weakly acidic group of pK_a' 10–11. The low-melting needle form of crystal melts over the range 160–165° and the granular high-melting form, sharply at 244–245°; $[\alpha]^{24}D$ +116.5° (*c*, 0.5 in 0.1 *N* hydrochloric acid). The ultraviolet absorption spectrum of amicetin is characterized by a strong maximum which shifts in wave length with changes in *p*H: when dissolved in water, the maximum appears at 305 mμ and $E^{1\%}_{1\,cm.}$ 465; in 0.1 *N* hydrochloric acid the maximum is at 316 mμ and $E^{1\%}_{1\,cm.}$ 433; in 0.1 *N* sodium hydroxide, the maximum appears at 322 mμ, $E^{1\%}_{1\,cm.}$ 470. Curves of these spectra are shown in Fig. 1.

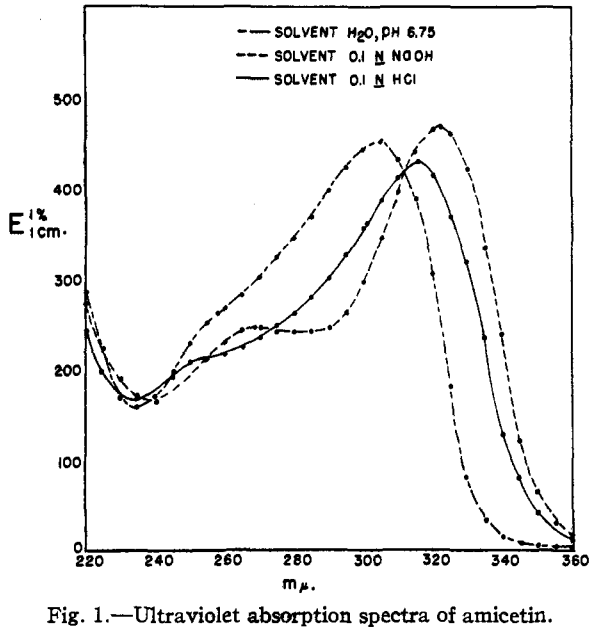

Fig. 1.—Ultraviolet absorption spectra of amicetin.

The infrared absorption spectrum of a liquid-petroleum mull of the high-melting crystal form of amicetin is shown in Fig. 2. The bands at about 3405 and 3230 cm.$^{-1}$ are characteristic of O–H or N–H groups. The bands at about 2900, 1380 and 1460 cm.$^{-1}$ are due to the liquid petrolatum. Mono-substituted amide carbonyl and/or conjugated carbonyl are indicated by the bands at 1684, 1654, 1614, 1567, 1522 and 1491 cm.$^{-1}$. At about 1252, 1176, 1104, 1072 and 1046 cm.$^{-1}$ there occur bands which may be due to C–O or C–O–C linkages. The bands at 692, 758 and 790 cm.$^{-1}$ are indicative of a substituted aromatic nucleus.

Amicetin can be detected by paper chromatography. The position of the antibiotic on the chromatogram was determined by the use of an ultraviolet absorption scanner or by employing the bioautograph technique using *M. avium* as the test organism. On 16-hour descending chromatograms using 90% 1-butanol–10% water as the solvent system, amicetin has an R_f value of about 0.22. Using 1-butanol saturated with water at room temperature, the R_f value is about 0.46.

By virtue of its basic character, amicetin forms salts with many acidic substances. Mineral-acid salts such as the sulfate and hydrochloride are readily formed and are considerably more water-soluble than the free base. With citric acid, amicetin forms a highly water-soluble complex which has been used in studying the pharmacology of this antibiotic. Ascorbic acid gives a similar water-soluble complex. The crystalline hydrochloride was obtained by dissolving the free base in 0.05 *N* hydrochloric acid and diluting the solution with methanol and acetone. The colorless crystals melted at 190–192°. Amicetin reacts with such dyes as methyl orange and orange II to form water-insoluble salts which can be used for purification and identification. Benzoylation of amicetin under mild Schotten–Baumann conditions yielded an antibiotically-inactive compound whose elemental

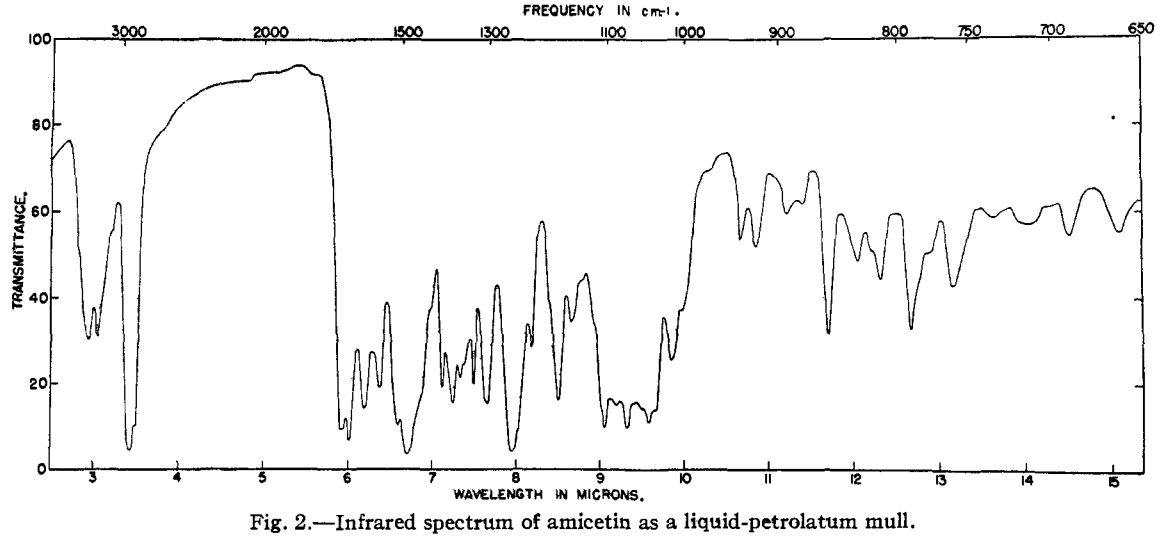

Fig. 2.—Infrared spectrum of amicetin as a liquid-petrolatum mull.

analyses were in agreement with those for a tribenzoyl derivative.

The antibacterial action of amicetin has been described.[2] Studies on the pharmacology and *in vivo* efficacy of amicetin will be reported elsewhere. Further information on the chemistry of amicetin and its degradation products is given by Flynn, *et al.*[7]

Acknowledgment.—The authors are indebted to Dr. H. A. Nelson and Messrs. C. M. Large and G. D. Mengel for the preparation of amicetin on a pilot-laboratory scale. We wish to thank Drs. J. L. Johnson and E. R. Garrett, Mr. J. E. Stafford and Mrs. A. E. Fonkin for certain physico-chemical data on crystalline amicetin, and Mr. W. A. Struck and his associates for the microanalytical work. Also we are indebted to Mr. R. J. Herberg of the Lilly Research Laboratories for the solubility analysis of purified amicetin. Appreciation is expressed to Drs. J. S. Evans and G. F. Cartland for their interest and assistance in carrying out this work.

Experimental

Fermentation.—In a typical example, the surface growth of *S. vinaceus-drappus* was transferred from a casein–starch–agar slant in order to inoculate 500-ml. erlenmeyer flasks each containing 100 ml. of yeast extract–peptone–glucose medium. After 48 hours of incubation at 24° on a reciprocating shaker moving at 90 four-inch strokes per minute, the growth in one of the flasks was used to inoculate a 5-gallon seed fermenter containing 12 l. of beef extract–peptone medium. Air was supplied at the rate of 6 l. per minute and the agitator rate was 280 r.p.m. After 48 hours of fermentation at 24°, the growth in this 5-gallon fermenter was used to seed a 100-gallon tank. The growth from the 100-gallon tank was, in turn, used to seed a 2000-gallon fermenter containing 1500 gallons of medium of the following composition: cerelose, 2.5%; yeast, 0.25%; soya flour, 0.7%; ammonium sulfate, 0.5%; calcium carbonate, 0.8%; sodium chloride, 0.4% and potassium dihydrogen phosphate, 0.04%. The fermentation was carried out at 24° using an aeration rate of 0.5 to 2 volumes of air per volume of broth per minute for approximately four days.

Extraction.—The fermentation liquor from a 2000-gallon tank was filtered on a plate-and-frame filter using 1–2% Dicalite 4200[8] as the filter aid. The clear broth, 1400 gallons containing 783 million units, was extracted at *p*H 9.5 with one-quarter its volume of 1-butanol in a Podbielniak extractor.[9] The butanol concentrate was recycled in the extractor against aqueous sulfuric acid of such strength as to give an aqueous effluent of *p*H 2.0–2.5. The aqueous solution, 38 gallons, was adjusted to *p*H 9.5 with sodium hydroxide and extracted three times with butanol, affording a combined butanol extract of 8 gallons. This concentrate was extracted batchwise three times with aqueous sulfuric acid to give a final aqueous solution of about 26 l. This solution was adjusted to *p*H 8.5, clarified and concentrated *in vacuo* to remove dissolved butanol. After holding the concentrated solution, 20 l., overnight in the cold room, the crystals were collected and dried to yield 342 g. of the low-melting form of amicetin which assayed 950 *M. avium* units per mg. This material had a rather indefinite melting point[10] in the neighborhood of 160°.

Purification.—Recrystallization and conversion to the high-melting crystal form was accomplished in the following manner: 160 g. of the crude crystalline product was dissolved in approximately 1 l. of 0.5 *N* hydrochloric acid and enough water to give a solution of *p*H 3. The solution was stirred with 160 g. of Darco G-60 for 30 minutes and filtered through a pad of Super-Cel. The filtrate, containing a trace of colloidal carbon, was swirled with a few drops of 1-butanol to coagulate the carbon, then filtered again through a pad of Super-Cel. The clear, yellow filtrate was made alkaline to *p*H 8 by addition of 6 *N* sodium hydroxide, with vigorous stirring and the resulting thick slurry was heated to 65° with continued stirring to convert the antibiotic to the high-melting crystal form. While still warm, the crystals were collected and washed with hot water. The mother liquor was treated with more alkali to bring the *p*H back to 8.0 and a second crop of crystals was obtained to provide a total of 127 g. (74.5%) of the colorless granular crystals which melted at 244–245°; $[\alpha]^{24}D$ +116.5° (*c*, 0.5 in 0.1 *N* hydrochloric acid).

Anal. Calcd. for $C_{29}H_{44}N_6O_9$: C, 56.11; H, 7.15; N, 13.54. Found: C, 55.98; H, 6.92; N, 13.18.

Distribution Studies.—Most of the countercurrent distribution work was done with a solvent system consisting of 1-butanol, di-*n*-butyl ether (peroxide-free), and water in the proportions 2:1:3 by volume. The low solubility of the antibiotic in this solvent system necessitated introduction of the sample into the first two to four tubes of the all-glass machine. Samples of from 0.5 to 1.0 g. were subjected to 60 to 65 transfers. At the end of the run, selected tubes were diluted to 40 ml. with methanol to give a single phase. The analysis was made on the basis of solids, ultraviolet absorption, *M. avium* plate assay and paper chromatography. The peaks coincided well, and occurred at tube number 44 for a 63-plate distribution. Comparison of the observed values with the theoretical values calculated by the methods of Craig[11] indicated the crude hydrated crystalline preparations to contain 70–90% of one component.

This distribution-analysis method was difficult to apply in the case of the granular anhydrous crystals because a portion of the sample became hydrated and appeared to behave as a second component in the system.

Preparation of Amicetin Salts and Solutions.—Details for the preparation of crystalline amicetin helianthate and hydrochloride are given by Flynn, *et al.*[7] The crimson-red orange II salt, m.p. 204–206°, was prepared using the helianthate procedure. Solutions suitable for parenteral administration in concentrations up to 10 mg. per ml. were prepared by dissolving amicetin in 0.05 *N* hydrochloric acid and adding dilute sodium hydroxide solution to *p*H 6. Aqueous solutions at *p*H 6 containing more than 10 mg. per ml. of amicetin were prepared by dissolving the solid in an aqueous solution of oxalic, tartaric, lactic, citric or ascorbic acids and adjusting to *p*H 6 by addition of sodium hydroxide. Citric acid was particularly effective in solubilizing amicetin. Solutions as concentrated as 150 mg. of amicetin per ml. were prepared and found to be stable at *p*H 6 when stored in the refrigerator. These solutions were prepared using 4 mg. of citric acid for each 10 mg. of amicetin. Solutions of up to 100 mg. of amicetin per ml. remained clear when stored in the refrigerator, but at 140 mg. per ml., solid precipitated. However, on warming to room temperature, a clear solution was obtained.

Benzoylation of Amicetin.—Two hundred and fifty mg. of anhydrous amicetin was dissolved in 9 ml. of 0.1 *N* sodium hydroxide to give a clear solution of *p*H 12.0. A total of 0.4 ml. of benzoyl chloride was added to the solution in 0.1-ml. portions. The reaction mixture was stirred mechanically and sufficient 0.5 *N* sodium hydroxide was added to hold the solution at *p*H 11.3–11.6. As the reaction progressed, solid separated. This precipitate was centrifuged, washed with water and dissolved in 3A alcohol.[12] Water was added to reprecipitate the product. After being refrigerated overnight, the partially crystalline material was collected and dried to yield 170 mg. After recrystallization from hot 3A alcohol, the benzoyl derivative melted at 177–179°. When determined in 3A alcohol the ultraviolet absorption spectrum showed two distinct maxima: $E^{1\%}_{1\,cm.}$ 501 at 227.5 mμ and $E^{1\%}_{1\,cm.}$ 351 at 305 mμ. The elemental analyses indicated that a tribenzoyl derivative was formed.

Anal. Calcd. for $C_{50}H_{56}N_6O_{12}$: C, 64.36; H, 6.05; N, 9.01. Found: C, 64.59; H, 6.02; N, 9.13.

KALAMAZOO, MICHIGAN

(7) E. H. Flynn, J. W. Hinman, E. L. Caron and D. D. Woolf, Jr., THIS JOURNAL, **75**, 5867 (1953).

(8) Dicalite 4200 is a diatomaceous earth supplied by the Great Lakes Carbon Corporation.

(9) Supplied by Podbielniak, Incorporated, Chicago, Illinois.

(10) All melting points were determined using a Kofler micro hot stage with a rate of heating of 2 to 3° per minute at the melting point.

(11) L. C. Craig and D. Craig, Chapt. IV in "Technique of Organic Chemistry," Vol. III, A. Weissberger, Editor, Interscience Publishers, Inc., New York, N. Y., 1950.

(12) 3A Alcohol is a denatured alcohol which contains 5 gallons of commercially pure methyl alcohol for every 100 gallons of 190° proof ethyl alcohol.