shape. Half the original charge of the dimer is lost since one Hg^{II} is bound to every two nucleotides. This means that the dimer has not only about the same size and shape as the unreacted SDNA molecule, but also the same charge and hence the same mobility. The loss of charge in the monomer on the other hand, is apparently compensated by its reduction in size.

There remains one further point of interest. The aggregation of SDNA by mercuric chloride does not continue into precipitation such as occurs, for example, in the case of polyacrylic acid[36] to which increasing amounts of barium, strontium or calcium ion are added. The most likely explanation appears to be that one is dealing with a competition for mercuric ion by phosphate groups on the SDNA and chloride ion itself. Mercuric chloride is a poor electrolyte but mercuric nitrate, which is a strong electrolyte, is capable of providing sufficient mercuric ion to precipitate SDNA from solution. Mercuric acetate, a half-strong electrolyte in the sense used by Sidgwick[37] also furnishes more mercuric ion than the chloride and is also capable of precipitating SDNA.

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The author is also indebted to E. I. du Pont de Nemours and Co. which supported this research by a du Pont Postdoctoral Fellowship in Chemistry.

(36) F. T. Wall and J. W. Drenan, *J. Polymer Sci.*, **7**, 83 (1951).

(37) N. V. Sidgwick, "The Chemical Elements and Their Compounds," Vol. I, Clarendon Press, Oxford, 1950, p. 324.

MADISON, WISCONSIN RECEIVED DECEMBER 1, 1951

[CONTRIBUTION FROM THE RESEARCH LABORATORIES OF CHAS. PFIZER AND CO., INC.]

Mycomycin. I. Isolation, Crystallization and Chemical Characterization

BY WALTER D. CELMER AND I. A. SOLOMONS

Mycomycin, a previously described unstable antibiotic, has been isolated in crystalline form and characterized as a highly unsaturated, optically active, straight-chain carboxylic acid having the empirical formula $C_{13}H_{10}O_2$. Thermal instability and sensitivity to oxygen are characteristic of the pure compound. Infrared absorption data suggest that the structure of mycomycin contains acetylenic and allenic groups. The methyl ester has been prepared and the reduction product of mycomycin has been identified as *n*-tridecanoic acid.

Mycomycin is an unstable antibiotic isolated by Johnson and Burdon[1] from the elaboration products of *Norcardia acidophilus*. It is active *in vitro* against numerous microörganisms, including streptomycin sensitive and resistant strains of *Mycobacterium tuberculosis* and a variety of pathogenic fungi.[2] A unit of activity has been defined as "the least amount which will inhibit the growth of *Bacillus mesentericus* in tryptose phosphate broth for 16 hours at 37°."[2]

In the original isolation method[3] the broth was filtered, acidified and extracted with hexane. The antibiotic was then re-extracted from the hexane solution into sodium phosphate buffer. The aqueous concentrates exhibited absorption maxima at 267 and 281 mμ which could be correlated with the microbiological activity. Extreme sensitivity to heat was evidenced by rapid loss of both ultraviolet absorption and microbiological activity unless the concentrates were stored at Dry Ice temperatures.

In order to facilitate further studies on the biological and chemical properties of mycomycin, it seemed important to improve the method of isolation and to seek some means of stabilizing the antibiotic. Preliminary observations indicated that the stability of mycomycin was greatly enhanced by working with relatively dilute solutions at low temperatures in an inert atmosphere. In an eight-plate countercurrent distribution of a dilute solution of the antibiotic between chloroform and cold 2% *p*H 7.0 phosphate buffer, the experimental curve showed little divergence from a calculated curve (Fig. 1). The antibiotic was finally obtained as white, crystalline needles. Repeated low temperature crystallizations from various solvents did not alter the characteristic ultraviolet spectrum of the material. The crystals exploded at about 75° in a capillary tube sealed with nitrogen. Though enhanced stability was observed with crystalline mycomycin, complete retention of activity is possible only by storage at a temperature of −40° or lower.

Mycomycin is an acid having a neutral equivalent of about 200 as determined by titration in aqueous methanol. It was found to contain carbon, hydrogen and oxygen, corresponding to the empirical formula $C_{13}H_{10}O_2$. Mycomycin is levorotatory with a specific rotation $[\alpha]^{25}D$ −130 in absolute ethanol Molecular extinction coefficients in methanol at 281 and 267 mμ are 67,000 and 61,000, respectively (see Fig. 2). The infrared absorption spectrum is shown in Fig. 3. The microbiological potency of the crystalline compound is about 50,000 U./mg. Attempts to prepare crystalline salts were unsuccessful due to decomposition.

When the crystals are stored at 27° in a nitrogen atmosphere or *in vacuo*, the half-life is only three

(1) E. A. Johnson and K. L. Burdon, *J. Bact.*, **54**, 281 (1947).

(2) D. E. Jenkins, Trans. 9th Streptomycin Conference on the Chemotherapy of Tuberculosis, Vet. Adm., St. Louis, Missouri, Apr. 18, 1950, p. 179.

(3) Private communication from Dr. E. A. Johnson.

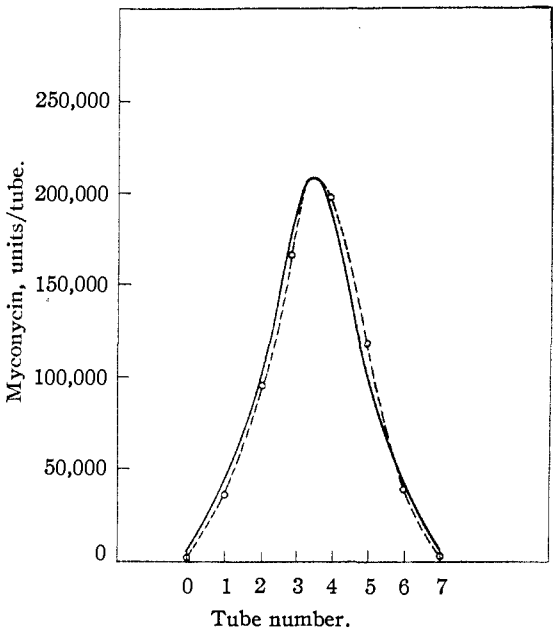


Fig. 1.—Countercurrent distribution of crude mycomycin between chloroform and *p*H 7.0 buffer: O- - - -O, experimental curve; ——, theoretical curve.

hours. Kinetic studies indicated that the inactivation reaction is complex and cannot be classified as to order. The brown product resulting from exposing mycomycin to the atmosphere at room temperature is quite insoluble in organic solvents and in strongly acid or alkaline solutions, and is attacked by oxidizing agents only under drastic conditions. Elemental analysis gave lower carbon values than found in mycomycin, indicating that combination with oxygen is one of the inactivation reactions.

Mycomycin reacts rapidly with oxidizing agents such as potassium permanganate, sodium dichromate and chromic oxide in acetic acid yielding dark resinous products, probably polymeric in nature. The addition of alcoholic silver nitrate to mycomycin results in the immediate formation of a heavy precipitate that darkens on standing at room temperature. Most chemical tests for typical functional groups were of no value because of the rapid decomposition of mycomycin under test conditions. When mycomycin is dissolved in liquid ammonia, instantaneous reaction occurs as indicated by a series of color changes progressing through yellow, green, blue, purple and finally red. Evaporation of the ammonia leaves a black residue insoluble in strong acid or alkali and in ordinary organic solvents.

When mycomycin in ethyl acetate solution is hydrogenated, eight moles is consumed and an almost quantitative yield of *n*-tridecanoic acid is obtained.

The microbiologically active mycomycin methyl ester was prepared by the addition of an ether solution of diazomethane to a cold ether solution of mycomycin. The crystalline ester has the composition $C_{14}H_{12}O_2$ and darkens at room temperature within five minutes after exposure. The methyl ester also forms an unstable, insoluble silver salt with methanolic silver nitrate indicating the presence of a terminal acetylenic grouping.[4] Complete hydrogenation of mycomycin methyl ester in ethyl acetate solution over Adams platinum oxide catalyst at 27° required eight moles of hydrogen. The reduction product was characterized as methyl *n*-tridecanoate.

Examination of the infrared spectrum (Fig. 3) indicates the presence of acetylenic (2210 cm.$^{-1}$) and allenic (1930 cm.$^{-1}$) groupings. The high yield of a single reduction product, *n*-tridecanoic acid, in conjunction with the 1930 cm.$^{-1}$ absorption band infers that the optical activity of mycomycin is due to asymmetrically substituted allenic bonds. Further studies on the structure of this interesting antibiotic are in progress and will be described in a subsequent publication.

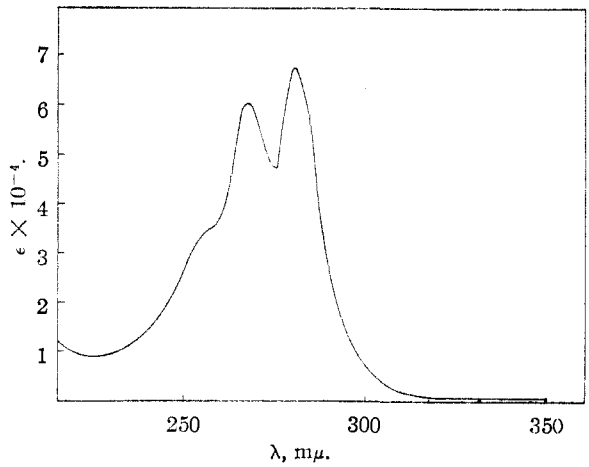


Fig. 2.—Absorption spectrum of mycomycin in methanol.

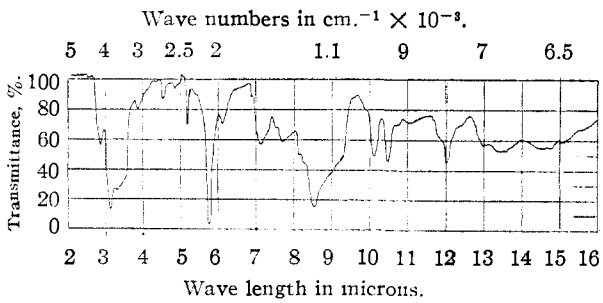


Fig. 3.—Infrared absorption spectrum of mycomycin in dioxane.

Methods

Biological Assays.—The plate assays were carried out with slight modifications of the method described by Schmidt and Moyer.[5] The medium was Difco Bacto streptomycin agar and the test plates were seeded with *B. subtilis* P.C.I. 219 culture. Samples for assay were diluted to approximately 10 U./ml. with 1.3% *p*H 7.0 phosphate buffer. The master standard was a buffer solution of mycomycin, obtained through the kindness of Dr. Johnson, diluted to 100 U./ml. and stored in Dry Ice. Secondary standards were prepared by comparison of the ultraviolet absorption with the master standard.

Ultraviolet Absorption Assay.—Even in relatively crude solutions of mycomycin, such as solvent extracts of acidified fermentation broth, the ultraviolet absorption was found to parallel the biological activity. The absorption

(4) A. Behal, *Ann. chim.*, **15**, 408 (1888).

(5) W. H. Schmidt and A. J. Moyer, *J. Bact.*, **47**, 199 (1944).

spectrum did not shift with changes in solvent or *p*H. Since in dilute solution the absorption follows Beer's law, ultraviolet absorption was routinely used for assaying mycomycin.

Inert Atmosphere, Low Temperature, Dry Box.—A stainless steel apparatus, similar to conventional dry boxes, was supplemented with cork insulation throughout and a double plate glass for viewing. A blower was attached to the box together with an air circulating tube which included a tray for desiccant or Dry Ice.

Prior to conducting an experiment within the box, all required apparatus, including a tray filled with calcium chloride or some other satisfactory desiccant, was placed inside and the door was closed. Nitrogen was introduced through a stopcock and vented through another, maintaining the box under positive nitrogen pressure throughout the experiment. The blower was then started, circulating the nitrogen in a closed circuit over the drying agent, through the box, and back through the blower again. After allowing time for the relative humidity of the atmosphere of the box to be reduced as low as possible, the tray was rapidly filled with Dry Ice and nitrogen circulation continued until the desired temperature was reached. After the blower was stopped, this temperature could be maintained for several hours. Temperatures as low as −65° were attained.

Experimental

Isolation of Aqueous Concentrates of the Sodium Salt of Mycomycin.—Submerged fermentations of *Norcardia acidophilus* were harvested at a potency of 360 U./ml. and filtered. Ten per cent. of the potency was lost during this operation. The filtered broth (7670 l.) was cooled to 5°, adjusted to *p*H 2.5 with dilute phosphoric acid and extracted countercurrently in a Podbielniak extractor with 3370 l. of hexane previously cooled to 5°. The spent aqueous phase, containing 60 U./ml., was discarded. The hexane extract, which was cooled to 2° upon leaving the extractor, was re-extracted with 151 l. of cold 0.5% *p*H 7.5 sodium phosphate buffer giving 1.54×10^9 units of mycomycin activity (55% over-all yield). Prior to the development of the crystallization techniques described below, the cold buffer solution was subdivided into 50-ml. test-tubes, frozen in an acetone–Dry Ice-bath and subsequently stored at −80° until desired for use in further chemical work or for animal studies. Larger containers were unsatisfactory, since at concentrations over 10,000 U./ml. the freezing and subsequent thawing is sufficiently slow to allow considerable inactivation to occur. When the solutions at this concentration are thawed and allowed to stand at 27°, the half-life of the antibiotic is found to be about three hours.

Countercurrent Distribution of Mycomycin.—The Craig countercurrent procedure[6] was applied to a sample of a phosphate buffer solution of mycomycin prepared as described above. Five hundred milliliters (18,000 U./ml.) was cooled in an ice-bath to 5°, acidified with phosphoric acid to *p*H 2 and extracted with two 55-ml. portions of cold chloroform. The mycomycin (780,000 units) in 100 ml. of the chloroform phase was distributed in an eight-plate chloroform–2% aqueous *p*H 7.0 phosphate buffer system, using 100 ml. of previously equilibrated portions of each phase. Throughout the distribution, the solutions were kept at 5° to minimize decomposition. At the completion of the distribution, the antibiotic content of each phase of each tube was determined by both ultraviolet and microbiological assay which closely paralleled each other. When total concentration per tube (ultraviolet assay) was plotted against the corresponding tube number, a typical distribution curve was obtained. This curve showed no detectable deviation from a calculated curve (Fig. 1).

Crystallization of Mycomycin.—Twenty-five liters of an aqueous buffer sulution of mycomycin, assaying 12,000 U./ml. cooled to 5°, was stirred with 3 l. of methylene chloride at 5° and acidified with 350 ml. of 10% phosphoric acid to *p*H 2. After stirring for five minutes, the two layers were allowed to settle for five minutes. Approximately 2.4 l. of an amber methylene chloride solution that assayed 120,000 U./ml. was recovered, a yield of 96%. A second extraction with 500 ml. of cold methylene chloride yielded 400 ml. assaying 30,000 U./ml. All subsequent operations concerned with the crystallization of mycomycin were conducted within an inert atmosphere, cold, dry box. The first methylene chloride extract was dried with 100 g. of anhydrous sodium sulfate, decolorized with 5 g. of boneblack and filtered. The light yellow filtrate was placed in a 4-liter flask and cooled in a Dry Ice–acetone-bath. Within ten minutes, a mass of small needles had separated from the solution and was collected on a buchner funnel. The product (7.2 g.) was light tan in color and assayed 30,000 U./mg. (72% over-all yield). The mother liquor which was discarded assayed 20,000 U./ml. Attempts to recover mycomycin from the mother liquors resulted on several occasions in spontaneous combustion of the filter cake even when handled in the relatively inert atmosphere of the cold box at −40°.

Recrystallization of Mycomycin.—Two grams of the product prepared above was dissolved in 200 ml. of methylene chloride at 20°. The resulting solution was dried with 20 g. of anhydrous sodium sulfate and shaken with 1.0 g. of boneblack. The solution was then cooled to 0° and rapidly filtered. The temperature of the box was lowered to −40° and after about 15 minutes the solution was sufficiently cold to deposit a crop of fine white crystals. These crystals were filtered by suction and dried in a stream of cold, dry nitrogen (weight 1.0 g., assaying 50,000 U./mg.). A second crop of less pure crystals was obtained by further cooling the mother liquor. Additional recrystallizations of the first crop mycomycin from chloroform or ethyl chloride did not increase the potency. The optical rotation was $[\alpha]^{25}D$ −130° (*c* 0.4% absolute ethanol). Elemental analysis was difficult since at 75° the antibiotic explodes; however, by first passing a stream of oxygen over the sample for combustion and then slowly raising the temperature, the following analyses were obtained.

Anal. Calcd. for $C_{13}H_{10}O_2$: C, 78.76; H, 5.08; one C-methyl, 7.6; neut. equiv., 198. Found: C, 78.17; H, 5.36; C-methyl (Kuhn-Roth), 0.5; neut. equiv. in aqueous methanol, 200.

Hydrogenation of Mycomycin: Characterization of *n*-Tridecanoic Acid.—Mycomycin (200 mg., 1.01 millimoles) was hydrogenated in 10 ml. of ethyl acetate at atmospheric pressure and 27° over 200 mg. of previously reduced Adams platinum oxide catalyst. The initial uptake was quite rapid and after 50 minutes the hydrogenation was complete with a consumption of 175 ml. of hydrogen (S.T.P.) or 7.8 millimoles. The platinum was removed by filtration and the solvent removed by distillation at reduced pressure. The residue (215 mg.) crystallized on standing and was recrystallized to constant freezing point (40.2°) from acetone; mixed freezing point with *n*-tridecanoic acid,[7] 40.4°.

Anal. Calcd. for $C_{13}H_{26}O_2$: C, 72.80; H, 12.13; neut. equiv., 214.3. Found: C, 72.90; H, 12.33; neut. equiv. in 50% methanol–water, 215.

The *p*-phenylphenacyl ester prepared according to Price and Griffith[8] melted at 86.8–87.0°; reported for *p*-phenylphenacyl *n*-tridecanoate, 86.5–87° (mixed melting point undepressed). The 2-alkylbenzimidazole derivative prepared according to Pool, Harwood and Ralston[9] melted at 109.0–110.0°; reported for 2-*n*-dodecylbenzimidazole 109.0–109.5° (mixed melting point undepressed).

Mycomycin Methyl Ester.—Mycomycin (300 mg.) was dissolved in 100 ml. of cold ether and the resulting solution was treated with a slight excess of diazomethane in ether. After standing for 15 minutes at 0°, the solution was extracted with cold aqueous sodium bicarbonate, washed with water and then cooled in an acetone–Dry Ice-bath and filtered. Hexane (50 ml.) was added to the ether solution and the ether removed under vacuum. The crystalline ester that precipitated upon cooling was filtered, washed and then dried by passing a stream of cold dry nitrogen over the product; yield 320 mg. Recrystallization was effected by dissolving 300 mg. of crude mycomycin methyl ester in 10 ml. of hexane at −10°, treating with a small amount of boneblack and cooling the filtrate to −60°. The crystals were filtered, washed with hexane, then butane and dried as described above. The ester gave $[\alpha]^{25}D$ −120° (*c* 0.1% methanol) and m.p. 44° (with decomposition). The

(6) L. C. Craig, *J. Biol. Chem.*, **150**, 33 (1943).

(7) Obtained through the courtesy of Dr. H. J. Harwood of Armour and Company.

(8) D. Price and R. Griffith, THIS JOURNAL, **62**, 2884 (1940).

(9) W. O. Pool, H. J. Harwood and A. W. Ralston, *ibid.*, **59**, 178 (1937).

ultraviolet spectrum of mycomycin methyl ester exhibited absorption peaks at 267 and 281 mμ of approximately equal intensity, ε 70,000.

Anal. Calcd. for $C_{14}H_{12}O_2$: C, 79.24; H, 5.66; CH_3O, 14.62. Found: C, 79.47; H, 5.82; CH_3O, 14.34.

Because of its low water solubility, mycomycin methyl ester could only be assayed in very dilute solutions. However, under these conditions, the activity was that calculated from its mycomycin content.

Mycomycin methyl ester (212 mg., 1.0 millimole) was hydrogenated in 30 ml. of ethyl acetate at atmospheric pressure and 27° over 200 mg. of previously reduced Adams platinum oxide catalyst. The hydrogenation was complete after 60 minutes with a consumption of 179 ml. of hydrogen (S.T.P.) or 8.0 millimoles. The catalyst was removed by filtration and the solvent removed by distillation at reduced pressure. A light yellow, oily residue (228 mg.) was obtained. The oil possessed the same infrared absorption spectrum as methyl *n*-tridecanoate. Saponification of hydrogenated mycomycin methyl ester (200 mg.) with 1.0 *N* sodium hydroxide gave after acidification a crystalline solid (165 mg.), m.p. 39–40°, identified as *n*-tridecanoic acid.

Acknowledgment.—The authors wish to express their appreciation to Dr. B. Sobin for his studies of the fermentation and preliminary extraction and to Mr. P. Guercio for the pilot plant scale broth extractions. We are indebted to Dr. J. Means for microanalyses and to Mr. R. Kersey for biological assays.

BROOKLYN 6, NEW YORK RECEIVED OCTOBER 15, 1951

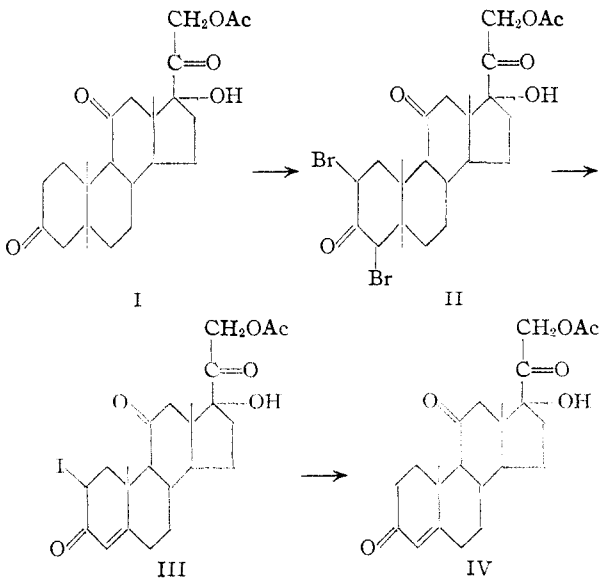
[CONTRIBUTION FROM THE CHEMICAL RESEARCH DIVISION OF SCHERING CORPORATION]

Allopregnan-17α,21-diol-3,11,20-trione-21-acetate

BY EUGENE P. OLIVETO, CORINNE GEROLD AND E. B. HERSHBERG

Allopregnan-17α,21-diol-3,11,20-trione-21-acetate (I) has been prepared by two different procedures: (a) direct hydrogenation of cortisone acetate (IV) in neutral solution using palladium on charcoal catalyst and (b) hydrogenation of cortisone acetate enol ether (V) in neutral solution with palladium-on-charcoal catalyst, followed by hydrolysis of the intermediate enol ether VI. No evidence was obtained for the formation of compounds of the normal series in either process.

Recent reports[1] have outlined the synthesis of cortisone acetate from steroidal sapogenins, ergosterol, stigmasterol, and similar $\Delta^{5,6}$-steroids, which are potentially more abundant than the bile acids now used. In the course of each of these newer syntheses the 5,6-double bond is reduced to give the allo configuration of the A–B rings in the common intermediate, allopregnan-17α,21-diol-3,-11,20-trione-21-acetate (I). The conversion of this compound to cortisone acetate involves the formation of the 2,4-dibromide (II), followed by treatment with sodium iodide to form the 4-double bond, and to replace the 2-bromine with iodine. Removal of the iodine by reduction with bisulfite yields cortisone acetate (IV).



While no yields have as yet been stated for these halogenation–dehalogenation reactions, they are reported[1a] to be lower than those previously obtained with analogous reactions[2] in the androstane series (*ca.* 60%).

In order to study this important reaction in the cortisone series and to avoid carrying out the lengthy synthesis using incompletely described reactions to prepare I from sapogenins, ergosterol, or similar steroids, we decided to begin with cortisone acetate which was more readily available. This was transformed into allopregnan-17α,21-diol-3,11,20-trione-21-acetate by two different routes. In the first and more obvious method, that of direct hydrogenation, the literature gave no clear-cut picture on the likely course of the reduction of this 3-keto-Δ^4-system. Thus, cholestenone[3] yields coprostanone on reduction with palladium in ether, testosterone and androstendione[4] give androstane compounds and progesterone[5] gives almost equal amounts of pregnane and allopregnane derivatives.

Evidently, no generalizations can be drawn from the above examples with respect to the proportion of isomers which will be obtained in a specific case and the nature of the products is dictated by the remainder of the molecule. Tishler and co-workers[1b] hydrogenated cortisone acetate with palladium-in-methanol containing potassium hydroxide to obtain allopregnan-17α,21-diol-3,11,20-trione in unspecified yield. However, besides causing hydrolysis of the 21-acetate group, the alkali may also cause secondary reactions on the sensitive ketol side-chain.

We have found that good yields of the desired allopregnane product (I), may be obtained by hy-

(1) For leading references, *cf.* (a) G. Rosenkranz, C. Djerassi, R. Yashin and J. Pataki, *Nature*, **168**, 28 (1951); (b) J. M. Chemerda, E. M. Chamberlain, E. H. Wilson and M. Tishler, THIS JOURNAL, **73**, 4052 (1951).

(2) G. Rosenkranz, St. Kaufmann, J. Pataki and C. Djerassi, *ibid.*, **72**, 1046 (1950); G. Rosenkranz, O. Mancera, J. Gatica and C. Djerassi, *ibid.*, **72**, 4077 (1950).

(3) H. Grasshof, *Z. physiol. Chem.*, **223**, 249 (1934).

(4) A. Butenandt, K. Tscherning and G. Hanisch, *Ber.*, **68**, 2097 (1935).

(5) A. Butenandt and G. Fleischer, *ibid.*, **68**, 2094 (1935).