g.) was distilled under diminished pressure into fractions which completely solidified within a few days. The purest fractions were redistilled, b.p. 116°/0.14 mm., n_D^{25} 1.5101, (IV-5B) which resolidified. A representative portion of the solidified product, 4.3 g., was readily soluble in warm *n*-hexane and was purified by fractional precipitation of high melting material by cooling until the melting point range narrowed to 1°. At this point it was recrystallized once to yield 0.15 g. of a white solid, m.p. 94–95° (IV-5C). Infrared spectra indicated that the liquid, B, was probably a mixture of the two solids, A and C. IV-5C was thought to predominate in this mixture due to the solubility difference in *n*-hexane between IV-5A and IV-5C and the solubility similarity in *n*-hexane between IV-5B and IV-5C.

N-Methyl-2,6-disubstituted 3,5-thiomorpholinediones. Series V). These were most conveniently prepared by treating 0.02 mole of the corresponding Series IV thiomorpholinedione with 0.05 mole (2.8 g.) of potassium hydroxide in 90 cc. of acetone while stirring at 0°. To this was added 1.25 cc. (2.84 g., 0.02 mole) of methyl iodide. The stirred mixture was allowed to warm to room temperature. After 1 hr. the liquid was decanted from the solid and the acetone was evaporated. The residue was diluted with water and the oil was extracted with ether yielding 2.0 g. of the cyclic imide after evaporation of the ether. This oil was insoluble in sodium bicarbonate solution.

The aqueous solution from above was acidified with concd. hydrochloric acid and the oil thus precipitated was extracted with ether and washed with sodium bicarbonate solution. This aqueous solution was washed with ether and then acidified to reprecipitate the oil which was extracted once more with ether, 4.7 g. after the ether was removed. Distillation of this acid portion under diminished pressure gave the identical cyclic imide.

n-Butylethyl(2-aminoethylmercapto)acetic acid (VI-2). This compound was prepared from 8.28 g. (0.047 mole) of *n*-butylethylmercaptoacetic acid dissolved in a cold 10% solution containing an equimolecular amount of sodium hydroxide (1.88 g., 0.047 mole). To this cold solution were added simultaneously an aqueous solution of 9.6 g. (0.047 mole) of 2-bromoethylamine hydrobromide and a 10% solution containing 3.76 g. (0.047 mole) of sodium hydroxide. The addition took place during a period of 45 min. with constant stirring which was continued for 1 hr. The solution was neutralized with glacial acetic acid and evaporated under diminished pressure to precipitate 7.6 g. (73.8%) of a crystalline product which was recrystallized from a 1/1 mixture of ethanol and water, m.p. 180–181° dec.

Anal. Calcd. for $C_{10}H_{21}NO_2S$: C, 54.76; H, 9.65; N, 6.38. Found: C, 54.75; H, 9.43; N, 6.34.

Diethyl(2-aminoethylmercapto)acetic acid (VI-1). This was prepared similarly in a 97.7% yield and was recrystallized from 95% ethanol, m.p. 233–234° dec.

Anal. Calcd. for $C_8H_{17}NO_2S$: C, 50.22; H, 8.96; N, 7.32. Found: C, 50.17; H, 9.10; N, 7.23.

2-n-Butyl-2-ethyl- and 2,2-diethyl-3-thiomorpholone (VII-1Y *and* VII-2). These were prepared from 2-bromoethylamine hydrobromide[12] with the results as shown in Table III.

2-n-Butyl-2-ethyl-3-thiomorpholone (VII-2Z). In a manner similar to that described above, 5.19 g. (0.029 mole) of *n*-butylethylmercaptoacetamide was treated with 6.0 g. (0.029 mole) of 2-bromoethylamine hydrobromide. The reaction mixture was made strongly basic with sodium hydroxide to precipitate the product which acted like an extremely deliquescent solid and was not purified but was immediately pyrolyzed to produce 3.58 g. (60.1%) of VII-1Z which was redistilled, b.p. 143–145°/1.1 mm., n_D^{25} 1.5138. Infrared spectra showed this to be identical with VII-1Y.

NEWARK, DEL.

[CONTRIBUTION FROM THE DIVISION OF STEROID RESEARCH, THE JOHN HERR MUSSER DEPARTMENT OF RESEARCH MEDICINE, UNIVERSITY OF PENNSYLVANIA, AND THE RESEARCH LABORATORIES, THE UPJOHN CO.]

Investigations on Steroids. XXXI. Preparation of 19-Hydroxycorticosterone[1,2]

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By the action of the fungus *Cunninghamella blakesleeana*, 19-hydroxycortexone (II) has been converted into a crystalline compound interpreted to be 19-hydroxycorticosterone (III). Contrary to expectations, acetylation of III gave a mixture of products, from which as main component the crystalline 11β,19,21-triacetate (VI B) was isolated. Benzoylation of III gave the crystalline 19,21-dibenzoate (V). III possesses little, if any, glucocorticoid activity and is devoid of mineralocorticoid action. Although there appears to be no doubt that III and its benzoylation product are identical with compounds described in the literature,[9] discrepancies exist regarding the acetylation and the physiological activity of III.

The syntheses of 19-hydroxy analogs of a number of steroid hormones were done at the University of Pennsylvania.[5a-d] The preparation of 19-hydroxycortexone[5a,6] (II) coincided with the elucidation of the structure of aldosterone by the combined efforts of a Swiss-British team.[7] II was subsequently found to occur in adrenal tissue.[8,9] We immediately considered it desirable to synthesize

(1) This investigation was supported by research grants (C757-C3, C757-C4, CY757-C5, and CY757-C6) from the National Cancer Institute of the National Institutes of Health, Public Health Service. A part of the K-Strophanthin used in this investigation was kindly donated by S. B. Penick & Co., New York, N. Y.

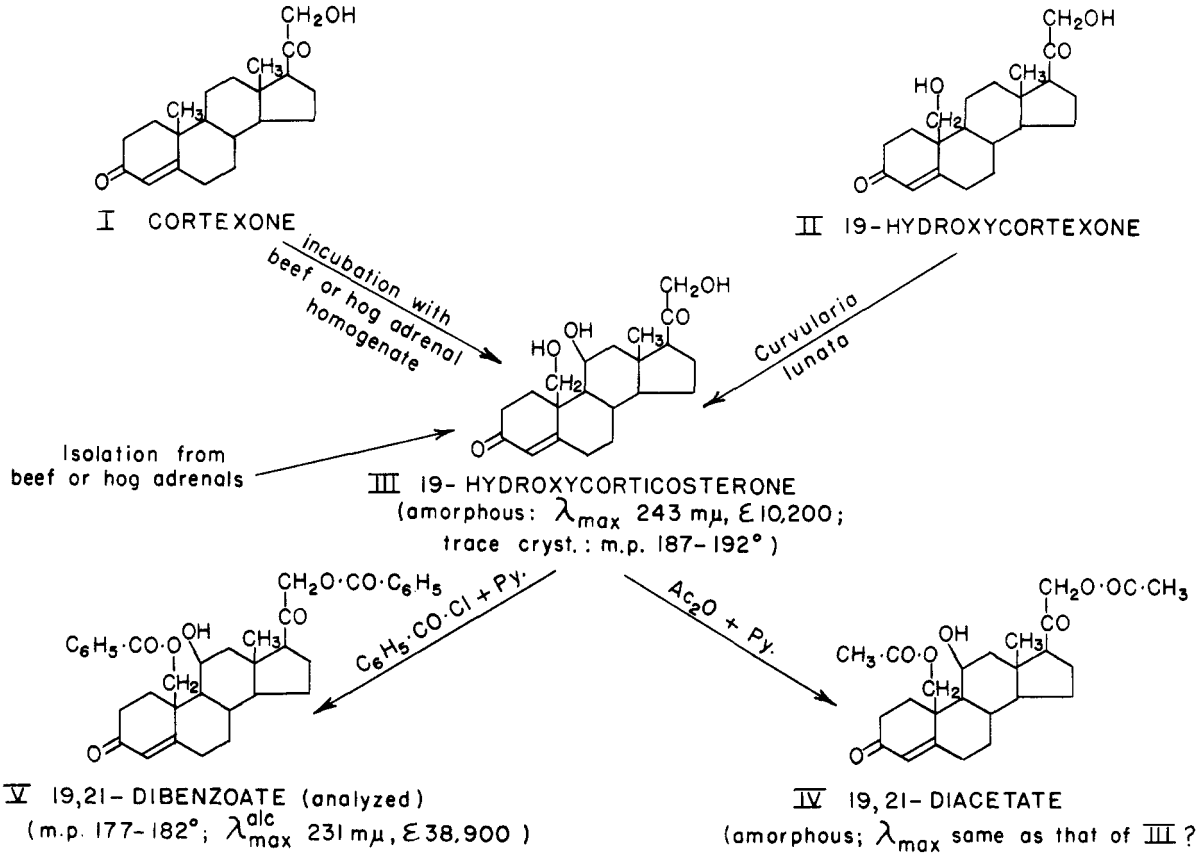
(2) The findings of this paper were presented on September 5, 1958, at the 4th International Congress of Biochemistry in Vienna {*cf.* Maximilian Ehrenstein: Biochemistry of the Corticoids, Proceedings of the Fourth International Congress of Biochemistry, Vol. 4 [Symposium: Biochemistry of Steroids], Pergamon Press, p. 259 (1959)}.

(3) University of Pennsylvania.

(4) The Upjohn Co.

(5) (a) G. W. Barber and M. Ehrenstein, *J. Am. Chem. Soc.*, **76**, 2026 (1954); G. W. Barber and M. Ehrenstein, *J. Org. Chem.*, **19**, 1758 (1954). (b) M. Ehrenstein and M. Dünnenberger, *J. Org. Chem.*, **21**, 774 (1956). (c) M. Ehrenstein and M. Dünnenberger, *J. Org. Chem.*, **21**, 783 (1956) (d) M. Ehrenstein and K. Otto, *J. Org. Chem.*, **24**, 2006 (1959).

R. Neher and A. Wettstein, Helv. Chim. Acta, 39, 2062 (1956).



a structural isomer of aldosterone in which carbon atom 19 rather than carbon atom 18 is linked with carbon atom 11 by way of a hemiacetal bridge. As the first step in this direction, we aimed at preparing 19-hydroxycorticosterone (III).

The microbiological hydroxylation[10] of 19-hydroxycortexone (II) appeared to us as the method of choice. The microbiological procedure was carried out in the Research Laboratories of the Upjohn Co., whereas the isolation and identification of the reaction product were done at the University of Pennsylvania. Hydroxylation of II was achieved by applying the fungus *Cunninghamella blakesleeana Lendner* which is known to hydroxylate in the 11β- position.[11] In two out of three major experiments, using in each case as starting material several hundred milligrams of II, we were able to secure a yield of 25–30% of a hydroxylation product which crystallized after repeated chromatography. In the third fermentation experiment, for unexplained reasons neither the starting material nor a conversion product could be isolated. In this instance, apparently extensive enzymatic degradation took place which was perhaps facilitated by the presence of the 19-hydroxyl group. Obviously, the conditions for the microbiological hydroxylation of II have to be studied further.[12] On the basis of the analysis and also in view of the molecular rotation,[13] the crystalline reaction product was tentatively assigned the structure of 19-hydroxy-

(6) In agreement with the proposals of Fieser, the previous name 19-hydroxy-11-desoxycorticosterone is replaced by 19-hydroxycortexone. *Cf.* "Steroids" by Louis F. Fieser and Mary Fieser, Reinhold Publishing Corp., New York, N. Y., 1959; see pp. 602, 706.

(7) S. A. Simpson, J. F. Tait, A. Wettstein, R. Neher, J. v. Euw, O. Schindler, and T. Reichstein, *Helv. Chim. Acta*, **37**, 1200 (1954).

(8) V. R. Mattox, Proc. Staff Meetings Mayo Clinic, **30**, 180 (1955).

(9) R. Neher and A. Wettstein, *Helv. Chim. Acta*, **39**, 2062 (1956).

(10) For the pertinent literature *cf.* the following reviews: (a) A. Wettstein: Conversion of Steroids by Microorganisms, *Experientia*, **11**, 465–479 (1955). (b) S. H. Eppstein, P. D. Meister, H. C. Murray, and D. H. Peterson: Microbiological Transformations of Steroids and Their Applications to the Synthesis of Hormones, *Vitamins and Hormones*, **14**, 359–432, Academic Press, New York (1956). (c) D. H. Peterson: Microbiological Transformation of Steroids and Their Application to the Synthesis of Hormones, Proceedings of the Fourth International Congress of Biochemistry, Vol. 4 [Symposium: Biochemistry of Steroids], Pergamon Press, 83–119 (1959).

(11) For specific literature, *cf.* references: 10a, pp. 474, 475; 10b, p. 390; 10c, p. 87.

(12) The 21-monoacetate of II[5a] was screened with *Cunninghamella blakesleeana*. The reaction product contained only traces of steroidal material (papergram analysis). In the same fashion, discouraging results were obtained on screening 19-oxocortexone.[23]

corticosterone (III). The infrared spectrum, determined in March 1955 in the laboratory of Dr. T. F. Gallagher at the Sloan-Kettering Institute for Cancer Research, was in agreement with this assumption. Acetylation yielded an amorphous product which initially resisted all attempts at crystallization, even after repeated column chromatography. The analysis of the chromatographed, yet amorphous material was in good agreement with a triacetate. This was difficult to explain in view of the normally unreactive 11β-hydroxyl group. On the other hand, the infrared spectrum showed hydroxyl absorption. Paper chromatography of the amorphous acetate indicated that it consisted of one major and at least two minor components. Further purification was temporarily suspended.

In December 1956 Neher and Wettstein[9] published a comprehensive paper on the isolation of new pregnane compounds from adrenal glands. Among the new products was an amorphous substance, λ_{max} 243 mμ, ϵ_{max}, 10,200 (only traces were obtained in crystalline form; m.p. 187–192°) also considered to be 19-hydroxycorticosterone (III), which was isolated in small amounts from various sources, namely: (1) from beef and hog adrenal glands; (2) from experiments in which cortexone had been incubated with homogenates obtained from beef or hog adrenal glands; (3) from experiments in which 19-hydroxycortexone (II) had been aerobically incubated with the fungus *Curvularia lunata (Wakker) Boedijn*. The identity of the products obtained from these different sources was established by comparison of the crystalline dibenzoates (V) (mixture melting points; paper chromatography; infrared spectra). For comparison with our crystalline III, a sample of amorphous III was kindly supplied by the laboratory of Dr. Wettstein. The infrared spectrum of each of these products was examined in the Sloan-Kettering Institute for Cancer Research through the courtesy of Dr. T. F. Gallagher. The spectra were measured in chloroform solution on a Model 21 Perkin-Elmer double beam infrared spectrometer and the spectra were identical[14] in all respects in the regions of 4000 to 2750; 1800 to 1600; 1500 to 1280; and 1150 to 800 cm.$^{-1}$ (*cf.* also Experimental). On acetylating their amorphous compound, the CIBA group obtained a product which remained amorphous after chromatography over alumina. Apparently it was considered to be uniform. Largely based on paper chromatographic data, this material was assigned the structure of the 19,21-diacetate (IV). Surprisingly this acetate is reported to possess the same ultraviolet characteristics as the free compound. On acetylating a 19-hydroxyl group in Δ^4-3-keto steroids, normally a distinct hypsochromic shift of the absorption maximum is observed.[15]

In view of the nonuniformity of our amorphous acetate, more recently a renewed effort at purification was made. By subjecting it to partition chromatography, the major component was finally obtained in crystalline form. According to the infrared spectrum no hydroxyl group is present. This is in agreement with the analysis of the amorphous acetate which had indicated a triacetate (see above). In our opinion several possibilities exist for the formation of an 11β,19,21-triacetate (VI B) from a 19,21-diacetate (IV). This probably involves the shift of an acetyl group from the 19-position to the vicinal 11β- position, possibly through the intermediary stage of an orthoacetate (VII). In accordance with expectations, the crystalline triacetate and also the minor acetylation products showed the typical hypsochromic shift of the ultraviolet absorption maxima connected with the acetylation of 19-hydroxyl[15] and 11β-hydroxyl[16] groups in Δ^4-3-keto steroids. On the other hand, the molecular rotation of the crystalline triacetate wholly disagrees with the calculated value.[17] This may be due to vicinal effects which do not become manifest in the free compound. If the triacetate contains a 19,11-bridge, as depicted by formula VIII, the presence of the additional asymmetric carbon atom (*) would prohibit a prediction of the molecular rotation. The various acetylation products of III pose an attractive stereochemical problem which will be investigated further.

Neher and Wettstein[9] characterized their amorphous 19-hydroxycorticosterone (III) by the crystalline 19,21-dibenzoate (V). On benzoylating our crystalline (III), a crystalline product also resulted. Since the supplies of the CIBA compound were exhausted, a direct comparison of the two substances was not possible. The recorded infrared spectrum, kindly supplied by Dr. Wettstein, was very similar to that of our compound. This criterion alone does not necessarily establish the identity of

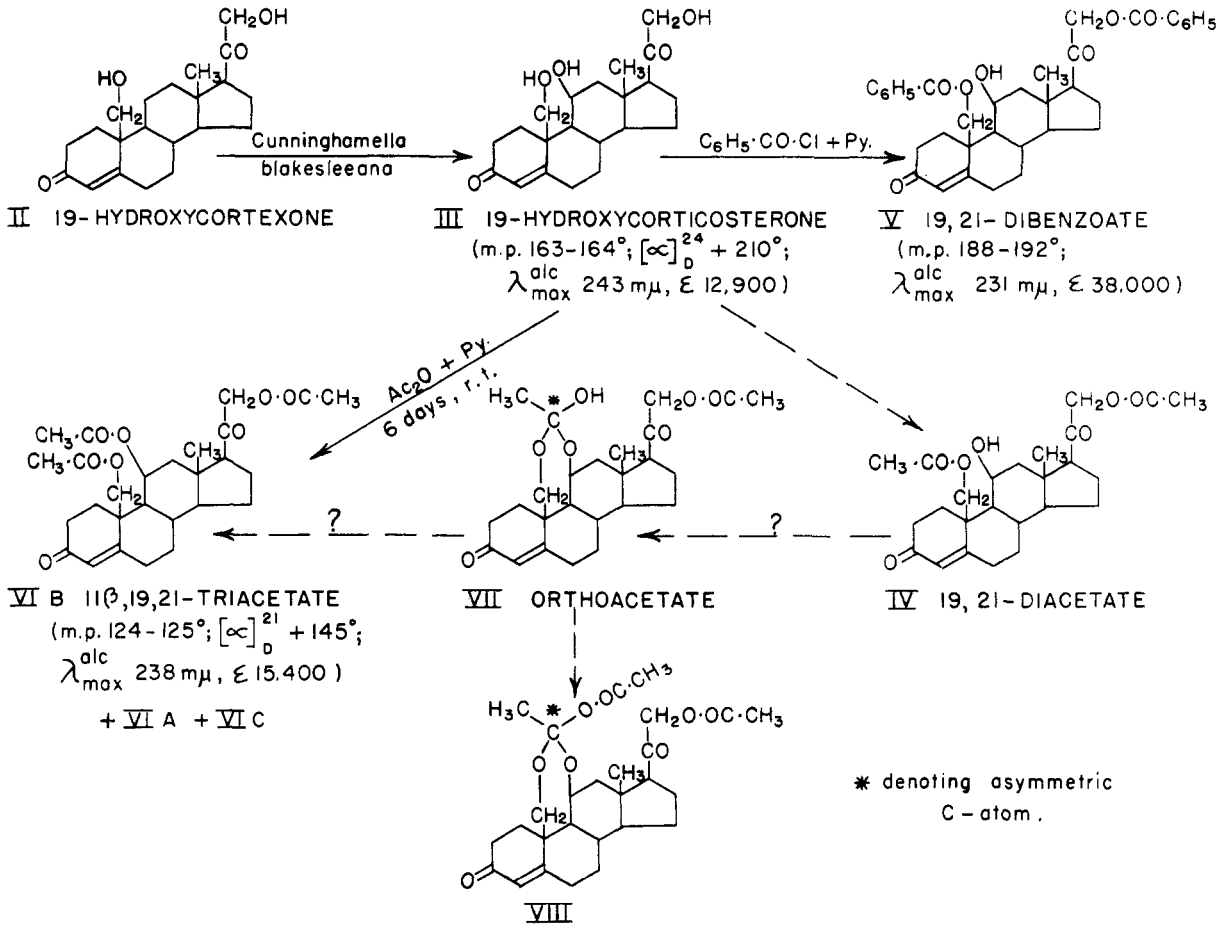
(13) Molecular rotation reported for 19-hydroxycortexone: +640° (Ref. 5a, second paper, see p. 1761). Increment for the introduction of an 11β-hydroxyl group: +110° (average of eight cases of Δ^4-3-oxo steroids; range +41° to +181°; the rotation values recorded in the literature were utilized without regard to certain differences of solvents). Calcd. for 19-hydroxycorticosterone (III): M_D +750°. Found: M_D +761° ± 7°.

(14) February 1957.

(15) Lit. *cf.* E. J. Becker and M. Ehrenstein, *Liebigs Ann. Chem.*, **608**, 54 (1957), see p. 59.

(16) A moderate hypsochromic shift of the absorption maximum has been observed on acetylating 11β-hydroxy-Δ^4-3-keto steroids; *cf.* E. P. Oliveto, C. Gerold, L. Weber, H. E. Jorgensen, R. Rausser, and E. B. Hershberg, *J. Am. Chem. Soc.*, **75**, 5486 (1953).

(17) Molecular rotation reported for 19-hydroxycorticosterone 19,21-diacetate: +902° (Ref. 5a, second paper, see p. 1761). Increment for the introduction of an 11β-acetoxyl group: +189° (value obtained by utilizing pertinent data of the paper cited in ref. 16). Calcd. for 19-hydroxycorticosterone 11β,19,21-triacetate (VI B): M_D +1091°. Found: M_D +708° ± 35°.



the two products, because benzoxy groups impart similar characteristics to the spectrum anyway. However, in view of the similarity of the other constants (melting point, ultraviolet absorption spectrum), there appears to be little doubt regarding their identity.

Physiological activity. The amorphous 19-hydroxycorticosterone (III) described by the CIBA group is claimed to possess notable physiological activity. "Das neue 19-Hydroxycorticosteron besitzt sowohl mineral - als auch glucocorticoide Wirkungen, jedoch schwächere als diejenigen von Aldosteron bzw. Cortison."[9] ("It has both glycogen-deposition and mineralocorticoid activity, but weaker than cortisone in the first property and weaker than aldosterone in the second.")[18]

The crystalline 19-hydroxycorticosterone (III) was subjected to various bioassays.

A. Glucocorticoid acitivity. Two different tests were applied. (1) In the Ingle work test (4 rats), performed by Dr. E. H. Morley in the Research Laboratories of the Upjohn Co., this compound was considerably less active than corticosterone. (2) In the eosinophil depletion assay, conducted through the courtesy of Dr. Ralph I. Dorfman at the Worcester Foundation for Experimental Biology, the substance was less than one third as active as corticosterone. Broadly speaking, the findings in these two assays parallel those in the liver glycogen deposition test which was not done with III. One may conclude, therefore, that III possesses little, if any, glucocorticoid activity.

B. Mineralocorticoid activity. Crystalline (III) was tested by two different groups. (1) In bioassays performed by Dr. Robert H. Curtis in the laboratory of Dr. John A. Luetscher, Jr., at Stanford University School of Medicine, III was not sodium-retaining in a dose of 10 μg. Since rats are usually sensitive to as little as one microgram of cortexone or cortexone acetate,[19] these bioassays show that the mineralocorticoid activity of III, if any, is probably less than one tenth that of cortexone acetate. (2) In assays carried out at the Worcester Foundation for Experimental Biology through the courtesy of Dr. Ralph I. Dorfman III in doses of 6 to 50 μg. had no significant effect on the excretion of sodium or potassium in salt loaded adrenalectomized rats. One must conclude, therefore, that III is devoid of mineralocorticoid activity.

(18) English translation quoted from book mentioned in ref. 6; see p. 706.

(19) *Cf.* John A. Luetscher, Jr., and Quentin B. Deming: "Bioassay of sodium-retaining corticoids and some changes in excretion of these substances in disease" in "Renal Function," Transactions of the Second Conference, 155–178, Josiah Macy, Jr. Foundation, New York, 1951, see p. 59.

EXPERIMENTAL

Melting points were determined with the Fisher-Johns melting point apparatus and are uncorrected. *Ultraviolet* spectra were determined in 95% ethanol with a Beckman Model DU spectrophotometer. The *infrared* studies pertaining to this paper were carried out on a Perkin-Elmer Model 21 double beam spectrometer in the Division of Steroid Metabolism of the Sloan-Kettering Institute for Cancer Research in New York through the courtesy of Dr. Thomas F. Gallagher. The correlations are based upon those summarized in the publication of Jones and Herling.[20] Only those bands are mentioned which appear to have a direct bearing upon the structure of the particular compound. Details of other correlations between spectrum and structure will be summarized at a later time by the group at the Sloan-Kettering Institute.

Unless stated otherwise, the *microanalyses* were performed by Dr. E. W. D. Huffman, Wheatridge, Colo., on samples which were dried to constant weight *in vacuo* (phosphorus pentoxide; 80°) according to Milner and Sherman.[21] The percentage loss of weight on drying is recorded; there was in no instance a gain of weight on exposure of the dried sample to the atmosphere. For *optical rotations* no correction for crystal solvent has been made. The sample was dissolved in chloroform to make 2 cc. of solution and the rotation was determined in a 2-dm. semimicro tube. The *adsorbents* alumina,[22] silica gel,[22] and Florisil[23] used for chromatography have been described.

Conversion of 19-hydroxycortexone (II) *into 19-hydroxycorticosterone* (III). *A. Fermentation procedure.*[24] In trial runs, batches of 5 mg., 10 mg., 15 mg., and 15 mg. of II, m.p. 169–172°, were incubated with the mold *Cunninghamella blakesleeana* for a period of 24 hr. The procedure is described in detail in the main experiment (see below). Examination of the isolated extracts by paper chromatography indicated, respectively, the presence of 50, 50, 50, and 30% of starting material (II) and 8, 8, 8, and 38% of a more polar compound. In all instances there was present about 1 to 2% of a second transformation product which moved slower than the starting material.

On the basis of these trial runs, the first experiment on a larger scale was performed (November 1954). Ten 250 cc. flasks, each containing 100 cc. of a medium (composition: 10 g. of technical dextrose, 20 g. of corn steep liquor solids, and 1000 cc. of tap water; *p*H 5.6–5.8),[25] were inoculated with spores of *Cunninghamella blakesleeana* and incubated (25°) with shaking for 24 hr. Thereafter 10 mg. of II, m.p. 169–172°, was added in 1.0 cc. of ethanol to each flask and the aerobic incubation (25°) was continued for 48 hr. The beer and mycelium from each flask were combined and extracted by the procedure of Peterson *et al.*[26] The extract was examined by paper chromatography using the Bush B-5 system[27] and was estimated to contain 18% of starting material (II) and 45% of a newly formed more polar compound showing similar maximum ultraviolet absorption.

B. Isolation of the reaction product.[28] Except for small amounts of material withdrawn for paper chromatographic studies, all extracts resulting from the trial runs and the main experiment (see A) were combined. The brownish resinous residue (180.1 mg.), originating from a total of 145 mg. of II and estimated to contain 37.5 mg. of unchanged II and 53.1 mg. of the main reaction product, was chromatographed over 10 g. of silica gel (16 × 75 mm.). The following eluates were collected: (a) benzene (200 cc.), benzene-chloroform, 1:1 (200 cc.), and chloroform (400 cc.); 18.8 mg. of yellow oil; (b) chloroform-ether, 9:1 (200 cc.), and 3:1 (200 cc.); 15.9 mg. of semicrystalline material; (c) chloroform-ether, 1:1 (200 cc.) and ether (1200 cc.); 48.1 mg. of crystalline fractions, m.p.'s between 159 and 165°, considered to be starting material (II); (d) ether-methanol, 199:1 (200 cc.); 5.9 mg. of semicrystalline residue; (e) ether-methanol, 99:1, 197:3, 39:1, 19:1, 9:1, 3:1, and 1:1 (200 cc. each); 74.0 mg. of yellow resin, representing the crude reaction product (III). The latter was rechromatographed over 20 g. of silica gel (16 × 140 mm.). The material eluted with ether-methanol, 39:1 (600 cc.) and 19:1 (1000 cc.) was a colorless resin; total wt.: 58.8 mg. This was chromatographed over 20 g. of Florisil (16 × 180 mm.). The first twenty eluates (200 cc. each of chloroform-acetone; ratios gradually changing from 199:1 to 7:3) gave a total of 8.5 mg. of resin. The following ten eluates (200 cc. each of chloroform-acetone; ratios gradually changing from 13:7 to 1:3) yielded exclusively crystalline residues; total: 33.9 mg. of crude 19-hydroxycorticosterone (III). The fractions eluted subsequently with chloroform-acetone, 3:17, acetone, acetone-methanol and methanol (total vol., 1200 cc.) gave 10.6 mg. of resin. Total recovery in this chromatogram was 53.0 mg.

Recrystallization of the crude crystalline III (33.9 mg.) from acetone-hexane gave 31.6 mg. of globular clusters of minute needles, m.p. 161–162°. Additional recrystallization from acetone-water (m.p. 97–100°; resolidification about 130°; remelting at 160–161°) and acetone-hexane yielded 20.9 mg. of colorless rods, m.p. 163–164°. $[\alpha]_D^{24}$ +210° ± 2°; $[M]_D^{24}$ +761° ± 7° (10.45 mg.; α + 2.20° ± 0.02°). λ_{max}^{alc} 243 mμ; ε12,900.

Anal. Calcd. for $C_{21}H_{30}O_5$ (362.45): C, 69.58; H, 8.34. Found: C, 69.38; H, 8.55. Residue, 0.34. (Corr. for residue: C, 69.62; H, 8.58).

The infrared spectrum of III was determined[29] in chloroform solution. There is strong associated hydroxyl absorption. The carbonyl region shows a band at 1708 cm.$^{-1}$ which is due to the C=O stretching vibration of the 20-ketone group. There is weak absorption at 1688 cm.$^{-1}$ which suggests the presence of a small amount of impurity. Bands at 1666 and 1618 cm.$^{-1}$ are due to the Δ^4-3-ketone system.

A second incubation experiment on a larger scale was performed (May 1955) in identical fashion with a total of 248 mg. of 19-hydroxycortexone (II).[30] In this instance, extraction of the beer and mycelium gave as much as 2.18 g. of a dark brown oil. Papergram analysis of the crude extract indicated the presence of approximately 75 mg. of III (~30% conversion) and 25 mg. (~10%) of unchanged II. Chromatography over 25 g. of silica gel (18 × 150 mm.); benzene, benzene-chloroform and chloroform eluted a total of 1.71 g. of waxy and oily material, probably of non-steroid nature; elution with chloroform-ether and ether gave 153.6 mg. of yellow resin, probably containing some starting material (II); ether-methanol mixtures eluted 238.4 mg. of yellow resin. Two chromatograms of the latter material on Florisil (25 g., 18 × 240 mm.; 10 g., 18 × 85 mm.)

(20) R. N. Jones and F. Herling, *J. Org. Chem.*, **19**, 1252 (1954).

(21) R. T. Milner and M. S. Sherman, *Ind. Eng. Chem., Anal. Ed.*, **8**, 427 (1936).

(22) Ref. 5a, second paper.

(23) G. W. Barber and M. Ehrenstein, *J. Org. Chem.*, **20**, 1253 (1955).

(24) In collaboration with H. C. Murray (microbiology) and L. M. Reineke (paper chromatography), Research Laboratories, The Upjohn Co.

(25) *Cf.* ref. 10b, p. 422.

(26) D. H. Peterson, H. C. Murray, S. H. Eppstein, L. M. Reineke, A. Weintraub, P. D. Meister, and H. M. Leigh, *J. Am. Chem. Soc.*, **74**, 5933 (1952).

(27) I. E. Bush, *Nature*, **166**, 445 (1950); *Biochem. J.*, **50**, 370 (1952).

(28) Experiments by G. Winston Barber.

(29) March 1955. Interpretation by Friederike Herling. The fingerprint region is different from any spectrum in the collection of the Sloan-Kettering Institute.

(30) Pooled material; m.p.'s between 160 and 173°. Papergram analysis indicated the presence of approximately 5% of a second component which was not affected by the bioconversion.

finally led, by eluting with chloroform-acetone, to a total of 89.9 mg. of twelve consecutive crystalline fractions whose melting points indicated uniformity throughout. Recrystallization of this material from acetone-hexane (75.1 mg.; rosettes of colorless needles; m.p. 101–103°, no resolidification) and acetone-water yielded 33.7 mg. of fan-like clusters of flat needles representing 19-hydroxycorticosterone (III); m.p. 100–105°, resolidification about 130°, final melting at 159–160°; no depression of melting point when mixed with the analytical sample of III (see above), λ_{max}^{alc} 243 mμ; ε14,000. The infrared spectrum of this product was determined[31] in chloroform solution and was found to be identical in all respects with that of the reference compound (see above) in the regions of 4000 to 2750, 1800 to 1600, 1500 to 1280, and 1150 to 800 cm.$^{-1}$ Surprisingly, no additional crystalline material could be secured from the mother liquors of the crystallizates, even after chromatography.

A third large scale incubation experiment was carried out in analogous fashion (April-May 1957) with 540 mg. of 19-hydroxycortexone (II), m.p. 158–160°. Papergram analysis of the extracted material indicated the following composition: starting material (II), 45%; 19-hydroxycorticosterone (III), 10%; two additional products, showing greater polarity than the starting material, 14% and 10%, respectively. The crude extracted material (solids, 1.62 g.) was refermented with the result that, in addition to traces of the starting material (II) and of several products more polar than II, only very small amounts (1–3%) of III could be detected by paper chromatography. Subsequent chromatography (solid, 1.51 g.) once over silica gel and twice over Florisil failed to yield any crystalline product.

Acetylation of 19-hydroxycorticosterone (III). *First experiment (February 1955).*[28] A solution of 10 mg. of pure crystalline (III), m.p. 163–165° (analytical sample), in 2 cc. of pyridine and 1 cc. of acetic anhydride was kept at room temperature for 16 hr. After evaporating most of the pyridine *in vacuo*, the residue was taken up in 25 cc. of 1*N* hydrochloric acid and, after standing for 5 min., the mixture was extracted with ether. After washing with 1*N* hydrochloric acid, 1*N* sodium carbonate, and water, the ether phase was dried over sodium sulfate and evaporated to dryness leaving 11.3 mg. of a white brittle foam. Attempts to crystallize this product failed and hence it was chromatographed over 2 g. of alumina (activity I-II; 6 × 85 mm.); petroleum ether, petroleum ether–benzene, benzene, benzene-ether, and ether eluted nothing; 50 cc. of ether plus 0.2 cc. of methanol, 50 cc. of ether plus 0.5 cc. of methanol, and 49 cc. of ether plus 1 cc. of methanol eluted, respectively, 2.7 mg., 4.0 mg., and 2.1 mg. of colorless resin. Attempts to crystallize the combined material (VI) were unsuccessful. It was finally dissolved in 1 cc. of benzene, and the solution was evaporated to dryness from the frozen state *in vacuo*. The resulting powdery amorphous residue was triturated with 1 cc. of petroleum ether, yielding after decanting and drying 8.0 mg. of powdery material. $[\alpha]_D^{22}$ + 201° ± 4° (5.07 mg.; α + 1.02° ± 0.02°). λ_{max}^{alc} 239 mμ.

Anal. Calcd. for $C_{25}H_{34}O_7$ (446.52) (diacetate): C, 67.24; H, 7.68. Calcd. for $C_{27}H_{36}O_8$ (488.56) (triacetate): C, 66.37; H, 7.43. Found[32]: C, 66.43; H, 7.97. Weight loss, 3.74 (Did not melt on drying at 70°).

The infrared spectrum of this product was determined[33] in carbon disulfide and in carbon tetrachloride solution. It shows associated hydroxyl absorption which can be tentatively interpreted as indicative of the presence of a hydroxyl group at C-11β associated with the 19-acetate. Carbonyl absorption at 1754 and 1732 cm.$^{-1}$ (carbon tetrachloride) is evidence for a 21-acetoxy-20-ketone group and at 1744 cm.$^{-1}$ suggests the presence of the 19-acetoxy group. Bands at 1678 and 1624 cm.$^{-1}$ (carbon tetrachloride) are due to the Δ^4-3-ketone system. There is a broad band at 1421–1416 cm.$^{-1}$ (carbon tetrachloride) which is due to the C—H scissoring vibrations of the unsubstituted methylene groups at C-2 adjoining the 3-ketone group and at C-21 in 21-acetoxy-20-ketones, and probably at C-19 in 19-acetates. A broad band at 1375 cm.$^{-1}$ (carbon tetrachloride) is due to C—H bending vibrations of the acetate methyl groups. C—O stretching vibration at 1231 cm.$^{-1}$ (carbon disulfide) confirms the presence of acetate groups.

Papergram analysis of this material[34] was conducted using Carbitol (diethylene glycol monoethyl ether) as stationary phase and methylcyclohexane saturated with Carbitol as mobile phase (development time: 12 to 20 hr.). A separation into three spots (ultraviolet scanner) was observed: The middle spot (about 9 cm. from the starting point) represents about 70% of the total steroid; the more polar spot (about 6 cm. from the starting point) about 20%; and the rapid moving component (about 16 cm. from the starting point) about 5–10%.

Second experiment (July 1955 and February 1958).[28] A solution of 20 mg. of crystalline III, double m.p. 100–105° and 159–160°, in 2 cc. of dry pyridine and 1 cc. of acetic anhydride was allowed to stand at room temperature for 6 days. The reaction mixture was evaporated *in vacuo* at room temperature and the residue was taken up in ether. After washing with 1*N* hydrochloric acid, 1*N* sodium carbonate, and water, the ether was dried and evaporated, and the residual colorless resin (30.6 mg.) was chromatographed over 10 g. of Florisil (10 × 230 mm.). Chloroform containing 6–10% of acetone eluted 18.7 mg. of colorless resin as a single peak (VI).

The composition of this material was investigated by chromatographing 0.1 mg. on a 1/2-inch strip of Whatman No. 1 filter paper in the system propylene glycol-methylcyclohexane. For comparison, 0.05-mg. samples of 19-hydroxycorticosterone (III), 19-hydroxycortexone (II), and the 21-monoacetate and 19,21-diacetate of II were chromatographed on parallel 1/2-inch strips (length: 26 cm.) at the same time. Development was continued for 10 days, 5 cc. of eluate being collected per strip.

The dried strips were scanned in a model DU Beckman spectrophotometer at 240 mμ. The quantities were estimated from the areas under the peaks when the extinction values are plotted against the distance along the paper strip. In this way, three ultraviolet absorbing components of VI were detected and the composition of VI was estimated as approximately 10% of VI A, a rapidly moving component found in the eluate;[35] approximately 60% of VI B, which had moved about 7.5 cm. from the starting point; and approximately 25% of VI C, which had moved hardly at all. Of the comparison compounds, III, II, and the 21-monoacetate of II had not moved at all, whereas the 19,21-diacetate of II had moved 21 cm. from the starting point.

The acetylation product (VI) was now chromatographed[36] on an intimate mixture of 100 g. of Florisil and 50 g. of redistilled propylene glycol (26 × 190 mm.) with redistilled methylcyclohexane as the mobile phase. Substance VI A was apparently eluted by the first 500 cc. (residue: 2.6 mg.) and was not investigated. The next 4500 cc. of methylcyclohexane eluted nothing, so the methylcyclohexane was gradually replaced by benzene, the following fractions being collected and evaporated to dryness *in vacuo:* (1) 400 cc. of

(31) April 1958. Interpretation by Beatrice S. Gallagher.

(32) The sample (2.104 mg.) was blocked between two samples of known composition and of the same size. The error should be within ±0.3%.

(33) March 1955. Interpretation by Friederike Herling.

(34) Courtesy of L. M. Reineke, Research Laboratories, The Upjohn Co.

(35) The eluate was evaporated to dryness and the residue was subjected to another papergram analysis (shorter time of development).

(36) To a solution of the acetylation product VI in a few drops of acetone was added a pinch of Florisil. The dried mixture was placed on top of the column. All eluents of this chromatogram were saturated with propylene glycol.

methylcyclohexane plus 100 cc. of benzene, residue: 2.6 mg.; (2) 300 cc. of methylcyclohexane plus 200 cc. of benzene, residue: 4.0 mg.; (3) 200 cc. of methylcyclohexane plus 300 cc. of benzene, residue: 2.3 mg.; (4) 100 cc. of methylcyclohexane plus 400 cc. of benzene, residue: 4.7 mg.; (5) 500 cc. of benzene, residue: 3.0 mg.

These five fractions were subjected to paper chromatographic investigation as described above, 0.06 mg. portions of the residues being run simultaneously on parallel 1/2-inch strips (length: 26 cm.; time: 6 days; total eluent: 43 cc.). Fractions (1) and (2) were found to contain only substance VI B and were therefore combined and briefly chromatographed over 2 g. of Florisil (8 × 100 mm.). Chloroform containing 2–6% of acetone eluted 5.3 mg. of colorless resin, representing the triacetate of 19-hydroxycorticosterone [$C_{27}H_{36}O_8$ (488.6)] (VI B). Crystallization from acetone-water gave 3.0 mg. of cubic crystals, m.p. 124–125°. $[\alpha]_D^{21}$ +145° ± 7°; M_D^{21} +708° ± 35° (2.41 mg.; α + 0.35° ± 0.01°). λ_{max}^{alc} 238 mμ; ϵ 15,400.

The infrared spectrum of the crystalline triacetate (VI B) was determined[37] in carbon disulfide and in carbon tetrachloride solution. It is similar to but not identical with that of the amorphous acetylation product (*cf.* first experiment). There is *no hydroxyl absorption* and a relatively more intense acetate-carbonyl band at 1744 cm.$^{-1}$ Because of the resemblance in the "fingerprint" region (1400–650 cm.$^{-1}$) of the crystalline (second experiment) and amorphous product (first experiment) and because the carbonyl region (1800–1600 cm.$^{-1}$) of both products differs only in band intensities and not band positions, the spectra are interpreted to mean that the amorphous material (first experiment) is a mixture which contains the crystalline compound (second experiment). Assuming that the latter is pure, the bands that characterize it in the region 1800–1600 cm.$^{-1}$ are as follows: 1754 cm.$^{-1}$ (shoulder) 20-ketone–21-acetate (a-band);[38] 1744 cm.$^{-1}$ acetate-carbonyl; 1736 cm.$^{-1}$ (shoulder) 20-ketone–21-acetate (b-band);[38] 1686 cm.$^{-1}$ (shoulder); 1679 cm.$^{-1}$ 3-ketone in Δ^4-3-ketone; 1627 cm.$^{-1}$ C=C stretching vibration in Δ^4-3-ketone (this value is higher than average, probably due to the influence of the adjacent acetate group at C-19).

Fractions 3 and 4 of the above chromatogram were found to be mixtures of VI B and VI C, whereas fraction 5 contained only substance VI C. On these papergrams, VI C was itself found to be resolved into three components, each of which, on elution from the paper and examination of the ultraviolet absorption spectrum had maximum absorption at 240 mμ, thus suggesting the hypsochromic shift of the absorption maximum which has been generally observed on acetylating 19-hydroxy-Δ^4-3-keto steroids.[15,16] Each of these components also reduced triphenyltetrazolium chloride after a short delay, indicating the presence of the ketol acetate grouping at position 17. When compared with II and III by paper chromatography in the system toluene propylene glycol, all three components of VI C ran considerably faster than either II or III.

Benzoylation of 19-hydroxycorticosterone (III).[28] The mother liquor residues from the recrystallization of III (microbiological hydroxylation of II, first experiment, see above) were combined and chromatographed over 10 g. of Florisil. The fractions eluted by chloroform containing 25–60% of acetone were combined (12 mg.) and dissolved in 0.6 cc. of dry pyridine. To this was added 0.8 cc. of a 10% solution of benzoyl chloride in dry benzene. After standing for 40 hr. and subsequently adding 1 cc. of water, the reaction mixture was evaporated to dryness *in vacuo*. The residue was taken up in ethyl acetate and the solution was shaken with 3*N* hydrochloric acid and 1*N* sodium carbonate, dried and evaporated, leaving 22.1 mg. of colorless resin. This was chromatographed over 2 g. of alumina (activity I–II; 8 × 55 mm.); petroleum ether and petroleum ether–benzene eluted nothing; the fractions eluted by benzene and by benzene containing 5–30% of ether were combined (5.3 mg.). Crystallization from benzene–petroleum ether gave 1.6 mg. of yellowish granular crystals, m.p. 188–192°. λ_{max}^{alc} 231 mμ; ϵ (for mol. wt. 570.6) 38,000. The infrared spectrum was determined[31] in methylene chloride solution and showed the following absorption bands: (1) 3680 and 3600 cm.$^{-1}$ (hydroxyl absorption); (2) 1718 (20-ketone and benzoate), 1667, (1620, 1602, 1583 cm.$^{-1}$ obscured by solvent absorption); (3) 1179, 1115, 1098, 1073, 1042, 1028, 875 cm.$^{-1}$

The corresponding data obtained by Neher and Wettstein[39] on a compound interpreted to be the 19,21-dibenzoate of 19-hydroxycorticosterone (V) are given for comparison purposes: m.p. 177–182°; λ_{max}^{alc} 231 mμ; ϵ 38,900. The infrared spectrum, as recorded in the CIBA laboratories, showed the following absorption bands (in CH_2Cl_2): (1) 3690 and 3610 cm.$^{-1}$; (2) 1721, 1672, 1626, 1605, 1587 cm.$^{-1}$; (3) 1172, 1114, 1093, 1070, 1039, 1026, 879 cm.$^{-1}$

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(37) April 1958. Interpretation by Beatrice S. Gallagher.

(38) *Cf.* footnote in table, p. 37 of Glyn Roberts, Beatrice S. Gallagher and R. Norman Jones: "Infrared Absorption Spectra of Steroids. An Atlas. Volume II." Interscience Publishers, Inc., New York, 1958.

(39) Ref. 9, see p. 2085.