lyzed by the hydroxamic acid–ferric chloride procedure (Rapport and Alonzo, 1955).

The aqueous phase representing the formed glycerylphosphorylcholine was analyzed for P. The rate curves showing the appearance of glycerylphosphorylcholine and fatty acid esters are shown in Figure 4.

The combined experiments I and II have thus given analytical data of the reactant and all the products of this system. In order to extend these analyses and to learn more about which ester group on the lecithin molecule was hydrolyzed, the individual fatty acid methyl esters were analyzed by gas chromatography.

C. Gas Chromatographic Analysis of the Fatty Acid Methyl Esters.—The fatty acid methyl esters liberated after 3, 6, and 24 minutes of hydrolysis in experiment II were analyzed by gas chromatography with a Perkin-Elmer Model 154D Vapor Fractometer. Thirty μl of an *n*-heptane solution of the fatty acid esters (3 to 5 mg of fatty acid esters) was injected into the machine by means of a Hamilton syringe. Fractionation was carried out by use of a Perkin-Elmer "P" column consisting of polyethyleneglycol succinate on chromosorb. The temperature of the run was 207°. The carrier gas was helium (at 30 lb pressure, flow rate of about 145 cc per minute). A thermistor detector was employed, and the areas of the peaks were measured by means of an automatic printing integrator. The data from these analyses are shown in Table I.

TABLE I

GAS CHROMATOGRAPHIC ANALYSIS OF THE FATTY ACID METHYL ESTERS RELEASED BY SODIUM METHOXIDE HYDROLYSIS OF EGG LECITHIN AT 0°

Hydrolysis Time (min.)	% of Total Fatty Acids[a]					
	Myristic	Palmitic	Palmitoleic	Stearic	Oleic	Linoleic
3	0.24	41.4	2.51	14.7	28.4	12.9
6	0.44	38.1	1.94	14.5	30.0	15.2
24[b]	0.12	35.1	1.71	14.5	32.1	16.1

[a] The details of the analytical method are given in the experimental section. A very small amount of longer chain fatty acids was also detected, but these emerged as a broad peak which was difficult to quantitate; hence these fatty acids are not included in the above data. [b] The reaction is complete after this time, and hence the analysis represents the total fatty acids of the egg lecithin.

REFERENCES

Baer, E., and Buchnea, D. (1959), *J. Am. Chem. Soc. 81*, 1758.
Baer, E., and Maurukas, J. (1955), *J. Biol. Chem. 212*, 25.
Baer, E., Maurukas, J., and Russell, M. (1952), *J. Am. Chem. Soc. 74*, 152.
Borgstrom, B. (1954), *Acta Physiol. Scand. 30*, 231.
Dawson, R. M. C. (1954), *Biochim. Biophys. Acta 14*, 374.
Dawson, R. M. C. (1960), *Biochem. J. 75*, 45.
de Haas, G. H., and van Deenen, L. L. M. (1961), *Biochim. Biophys. Acta 48*, 215.
de Haas, G. H., Mulder, I., and van Deenen, L. L. M. (1960), *Biochem. Biophys. Res. Communs. 3*, 287.
Hanahan, D. J., Brockerhoff, H., and Barron, E. J. (1960), *J. Biol. Chem. 235*, 1917.
Hirsch, J., and Ahrens, E. H. (1958), *J. Biol. Chem. 233*, 311.
Hubscher, G., Hawthorne, J. N., and Kemp, P. (1960), *J. Lipid Res. 1*, 433.
Marinetti, G. V., Erbland, J., and Kochen, J. (1957), *Fed. Proc. 16*, 837.
Marinetti, G. V., Erbland, J., and Stotz, E. (1959), *Biochim. Biophys. Acta 33*, 403.
Marinetti, G. V., Erbland, J., and Stotz, E. (1960a), *Biochim. Biophys. Acta 38*, 534.
Marinetti, G. V., Erbland, J., Temple, K., and Stotz, E. (1960b), *Biochim. Biophys. Acta 38*, 524.
Rapport, M. M., and Alonzo, N. (1955), *J. Biol. Chem. 217*, 193.
Tattrie, N. H. (1959), *J. Lipid Res. 1*, 60.

16α-Hydroxysteroids. XIII.* Carbonyl Reduction by *Streptomyces roseochromogenus*

LELAND L. SMITH,† THEODORE FOELL,† AND JOSEPH J. GOODMAN

From the Lederle Laboratories Division, American Cynamid Company, Pearl River, New York

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Streptomyces roseochromogenus fermentations of 9α-fluorohydrocortisone result in a small amount of C_{20}-carbonyl reduction, the 20β-dihydro derivatives of both 9α-fluorohydrocortisone and 16α-hydroxy-9α-fluorohydrocortisone being formed. Fermentations of 9α-fluorohydrocortisone-21-aldehyde result in rapid and complete reduction of the aldehyde to 9α-fluorohydrocortisone, which is then altered characteristically by the microorganism.

In our studies of the 16α-hydroxylation of 9α-fluorohydrocortisone (I) by *Streptomyces roseochromogenus* a variety of reducing (Smith *et al.*, 1960b; Smith *et al.*, 1961) and non-reducing (Smith *et al.*, 1962) steroids have been isolated and identified. Among the non-reducing steroids regularly detected in such fermentations is a polar steroid with paper chromatographic behavior indistinguishable from 20β-dihydro-9α-fluorohydrocortisone (II) and a still more polar component with the mobility of 16α-hydroxy-20β-dihydro-9α-fluorohydrocortisone (III).

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† Present address: Wyeth Laboratories, Inc., Philadelphia, Pa.

Both steroids fail to reduce alkaline tetrazolium blue but are readily detected by their reaction with isonicotinic acid hydrazide on paper chromatograms (Smith and Foell, 1959). An instrumental evaluation of the intensity of fluorescence of these two components in fermentation broth extracts indicated that the levels of II were of the order of 3% of substrate charged, and the levels of III, less than 1%.

Isolation of II from a selected steroid-rich broth extract concentrate previously described (Smith *et al.*, 1961) was accomplished by paper chromatography, and the steroid thus obtained was identified as 20β-dihydro-9α-fluorohydrocortisone[1] by comparison of infrared spectra, chromatographic behavior, and color tests. Attempts at isolation of the very polar III did not give a satisfactory preparation for characterization. Comparison of the purified preparation of III with authentic 16α-hydroxy - 20β - dihydro - 9α - fluorohydrocortisone (Smith *et al.*, 1960a) on extended chromatographic irrigation in several solvent systems established identity of the two samples.

Carbonyl reduction by *S. roseochromogenus* is more pronounced with steroidal aldehydes. 9α-Fluorohydrocortisone 21-aldehyde hydrate (IV) (9α - fluoro - 11β,17α,21,21 - tetrahydroxy - 4 - pregnene-3,20-dione) is reduced rapidly (half-life, *ca.* 1 hr.) to 9α-fluorohydrocortisone, which in turn is subject to the same series of bioconversions characteristic of the action of this organism on 9α-fluorohydrocortisone.

The complex spate of steroidal products elaborated by *S. roseochromogenus* with the aldehyde IV as substrate is identical with that obtained when 9α-fluorohydrocortisone is used as substrate. This identity of chromatographic patterns in effect serves to establish that the aldehyde IV is in fact reduced to 9α-fluorohydrocortisone. Paper chromatographic analyses at different times clearly showed the disappearance of aldehyde, the appearance of 9α-fluorohydrocortisone, and, at later times, the appearance of the several elaboration products derived from 9α-fluorohydrocortisone, the main product being 16α-hydroxy-9α-fluorohydrocortisone.

It was desirable to obtain further evidence for the identities assigned. The initial reduction product 9α-fluorohydrocortisone could be conveniently trapped in non-aerated fermentations. Reduction occurred in non-aerated systems, but further alteration products were not formed. After the aldehyde disappeared the single reducing product obtained was compared with 9α-fluorohydrocortisone chromatographically in the usual manner and identity of the two samples was established. Acetylation of the reduction product on filter paper (Rahandraha and Ratsimamanga, 1956) gave a single product identified chromatographically as 9α-fluorohydrocortisone 21-acetate. Chromic acid oxidation on paper gave a major product with chromatographic properties of 9α-fluorocortisone. In each case, careful control experiments were run with authentic 9α-fluorohydrocortisone. Thus, although classical identification procedures were not employed, the identity of the initial reduction product with 9α-fluorohydrocortisone by means of modifications of the procedures of Rahandraha and Ratsimamanga is established.

As a further step in characterizing the elaboration products derived from the aldehyde the major product, 16α-hydroxy-9α-fluorohydrocortisone, was so recognized on the basis of rigorous paper chromatographic comparisons and by conversion on paper (Smith and Foell, 1960) to the cyclic acetonide by reaction with perchloric acid and acetone. The acetonide product was identified chromatographically as 16α-hydroxy-9α-fluorohydrocortisone 16α,17α-acetonide.

Acetylation on paper followed by chromatographic resolution gave unaltered steroid plus a major reducing component recognized as 16α-hydroxy-9α-fluorohydrocortisone 16α,21-diacetate. Identical results were obtained with authentic 16α-hydroxy-9α-fluorohydrocortisone treated similarly at the same time.[2] Oxidation with chromic acid after acetylation (both reactions conducted on paper according to Rahandraha and Ratsimamanga, 1956) gave a major reducing component recognized as 16α-hydroxy-9α-fluorocortisone 16α,21-diacetate by paper chromatography. Again known 16α-hydroxy - 9α - fluorohydrocortisone treated the same way gave identical results.

Microbiological reduction of steroidal 20-ketones by normally hydroxylating organisms is known (McAleer *et al.*, 1958; Gould *et al.*, 1957), and steroidal aldehydes have been reduced both microbiologically (Murray and Peterson, U. S. Patent No. 2,602,769, July 8, 1952; Meister *et al.*, 1954; Peterson, 1956) and by mammalian liver preparations (Schneider, 1953; Monder and White, 1961).

The rapid reduction of the 21-aldehyde by *S. roseochromogenus* lessens the likelihood that such aldehydes are formed during aerated fermentations of 9α-fluorohydrocortisone. Examination of normal aerated fermentation systems via paper chromatography using isonicotinic acid hydrazide and tetrazolium blue combinations (Smith and Foell, 1959) and the Porter-Silber reagent (Birmingham, 1959) (on separate chromatograms) failed to reveal any evidence for the 21-aldehyde. As such

[1] The authentic sample of 20β-dihydro-9α-fluorohydrocortisone, prepared by sodium borohydride reduction of 9α-fluorohydrocortisone, was kindly made available to us by Dr. N. Rigler of these laboratories.

[2] Acetylation of 11β,16α,17α,21-tetrahydroxy-20-ketones on Whatman No. 1 filter paper with our modification of the technique of Rahandraha and Ratsimamanga (1956) may give the 16α,21-diacetate together with both the 16α- and 21-monoacetates and unreacted parent steroid, or, for as yet not understood reasons, may give mainly the 21-monoacetate together with small amounts of the 16α-monoacetate (or the 16α,21-diacetate) and unaltered parent steroid. Authentic tetraols must be aceylated at the same time as samples under test in order that these minor variations be controlled. In all cases substantial amounts of unaltered alcohol remain. The characteristic pattern of unaltered tetraol, 16α-monoacetate, 21-monoacetate, and 16α,21-diacetate (in increasing order of chromatographic mobility), becomes a very specific means of recognition of such steroids.

the aldehyde IV does not appear to be a biological intermediate in the formation of 21-acetylamino-9α - fluoro - 11β,17α - dihydroxy - 4 - pregnene-3,20-dione from I by *S. roseochromogenus* (Smith *et al.*, 1962). The aldehyde IV is not hydroxylated by *S. roseochromogenus;* neither 16α- nor 2β-hydroxylated derivatives could be detected.[3]

Experimental

9α-Fluoro-11β,17α,20β,21-tetrahydroxy-4-pregnen-3-one (II).—The steroid-rich broth extract concentrate obtained from *S. roseochromogenus* fermentations of 9α-fluorohydrocortisone already described (Smith *et al.*, 1961) was used for the isolation of 20β-dihydro-9α-fluorohydrocortisone. An initial separation of some non-steroidal materials was accomplished by adsorption of the extract concentrate onto silica gel from ethyl acetate. After being washed with ethyl acetate to remove coloration, the steroids were eluted with methanol. The methanol eluates were concentrated and applied to eighteen sheets of Whatman No. 1 filter paper, which were then chromatographed using benzene-ethanol-water, 2:1:2 (4 hours). Four bands were detected by ultraviolet light absorption on the paper at R_F 0.4, 0.36, 0.29, and 0.15. Only the most mobile and the least mobile zones were examined. From the R_F 0.4 zone (after elution and rechromatography) 21-acetylamino-9α-fluoro-11β, 17α - dihydroxy - 4 - pregnene - 3,20 - dione (Smith *et al.*, 1962) was isolated.

The most polar regions of the chromatogram with R_F <0.2 were eluted with hot methanol, the eluates concentrated and rechromatographed in a benzene-acetone-water, 2:1:2, system for 4–6 hours. Four zones were detected, a reducing zone at R_F 0.50, a non-reducing zone at R_F 0.36, a reducing zone at R_F 0.29, and a non-reducing zone at R_F 0.12. From the R_F 0.36 zone a mixture of at least two steroids was eluted. Rechromatography several times in the benzene-acetone-water system did not resolve the components satisfactorily, but chromatography in a benzene-ethanol-water, 2:1:2, system for 16 hours did resolve the major non-reducing component from a lesser amount of another non-reducing component. The material recovered after elution was concentrated to dryness and the residue crystallized from acetone and ether. The crystalline product was identified as 20β-dihydro-9α-fluorohydrocortisone by comparison of infrared spectra (KBr disk) with spectra of an authentic sample, λ_{max}^{KBr} 2.92 μ, 6.00 μ, 6.05 μ, 9.61 μ, 11.23 μ, 11.45 μ, etc. A second preparation was isolated similarly from papergrams and identified by infrared spectra as 20β-dihydro-9α-fluorohydrocortisone.

The microbiologically produced II was not distinguishable chromatographically from the authentic sample in several solvent systems, and color test behavior of the two samples was identical (negative to tetrazolium blue and the Porter-Silber reagent, positive to isonicotinic acid hydrazide, phosphotungstic acid, phosphomolybdic acid, peach-colored fluorescences with antimony trichloride and 15% phosphoric acid).

Acetylation of a few micrograms of eluted material with acetic anhydride–pyridine gave a non-crystalline material whose infrared spectra in chloroform gave bands at 5.74 and 6.00 μ, identical with 20β - dihydro - 9α - fluorohydrocortisone treated similarly.

Reduction of 9α-Fluorohydrocortisone 21-Aldehyde.—Fermentative reduction experiments were conducted in 250-ml shake flasks with *S. roseochromogenus*, Waksman strain No. 3689 (Rutgers University Collection), grown for 24 hours in 50 ml of the medium of Thoma *et al.* (1957) (185 rpm rotary shaker at 26.5°). The aldehyde IV was added as a 250 μg/ml solution in ethanol (final ethanol concentration less than 1% in each shake flask). After steroid addition the flasks were shaken (aerated) for various lengths of time (0.5 1, 3, 6, 24 hours, etc.), after which times the steroids were extracted with ethyl acetate and the extracts were concentrated in vacuum and analyzed chromatographically and instrumentally (polarograph, colorimetric with alkaline tetrazolium blue). Certain experiments were conducted without shaking after steroid addition.

Identification of 9α-Fluorohydrocortisone Derived from 9α-Fluorohydrocortisone 21-Aldehyde.—The selected fermentation sample obtained without aeration contained at termination of fermentation only one component, recognized as 9α-fluorohydrocortisone on the basis of papergram mobility in systems I, II, III, and IV (Smith *et al.*, 1959) and characteristic color reactions with tetrazolium blue and isonicotinic acid hydrazide. Also, the development of yellow coloration with the Porter-Silber reagent (Birmingham, 1959) was identically that expected of 9α-fluorohydrocortisone.

Acetylation of 20 μg of the steroidal component on Whatman No. 1 filter paper was accomplished by spotting the sample in the usual way, allowing to dry, and then spotting over the steroid zone (at the origin of the chromatogram) with 10 μl of a freshly prepared mixture of acetic anhydride-pyridine (1:3) (Rahandraha and Ratsimamanga, 1956). After a few minutes of drying a second 10 μl of the acetylating mixture was applied. After 10 minutes at room temperature the paper was suspended in the chromatographic tank, equilibrated in the usual manner, and irrigated for chromatography in the selected solvent system. Using systems III, V, and VI a single reducing reaction product was detected, together with unaltered starting material, whose mobility and color

[3] Using the Bate-Smith and Westall relationship (Bate-Smith and Westall, 1950), $R_M = \log [1/R_F - 1]$, ΔR_M values for 16α- and 2β-hydroxy substitution (based on R_M data for several sets of steroids) are +0.56 and +0.67 respectively (in system II). Thus the postulated 16α- and 2β-hydroxylated aldehydes should be sought near R_F 0.27 and 0.22. In most samples examined this region of the chromatogram is devoid of steroids detected by the several reagents used, although very minor traces (<<1%) have been occasionally detected with isonicotinic acid hydrazide.

test behavior was identical with those of 9α-fluorohydrocortisone 21-acetate, either run as the pure standard or prepared from 9α-fluorohydrocortisone by acetylation *in situ* on the paper.

Chromic acid oxidation of 10 μg of product on paper was accomplished by spotting the sample in the usual manner, allowing to dry, and then spotting over the steroid zone 10 μl of the chromic acid reagent (200 mg of chromium trioxide dissolved in 100 ml of water containing 1.0 ml of concentrated sulfuric acid). A second 10 μl of chromic acid reagent was applied, and after 5–10 minutes at room temperature (time to dry) the prepared paper was suspended in the chromatographic tank, equilibrated in the usual manner, and irrigated, etc. Analysis of the oxidation product showed the presence of unaltered steroid reactant plus a single reducing steroidal product with mobility identical with that of 9α-fluorocortisone. Similar treatment of 9α-fluorohydrocortisone at the same time gave an identical distribution of components, as judged by intensity of isonicotinic acid hydrazide fluorescence and diformazan formation from alkaline tetrazolium blue.

Identifieation of 16α-Hydroxy-9α-fluorohydrocortisone from 9α-Fluorohydrocortisone 21-Aldehyde.—A selected fermentation (aerated) sample which contained as a major product the indicated 16α-hydroxy-9α-fluorohydrocortisone (identified by mobility in several papergram systems) was used for chemical identification. Acetonide formation using *ca.* 20 μg of total steroid was accomplished on filter paper (Smith and Foell, 1960), and the reaction products analyzed in systems V and VI. The major reducing component found was recognized as the 16α,17α-acetonide of 16α-hydroxy-9α-fluorohydrocortisone on the basis of mobility. In these systems those steroids present in the sample which would not form acetonides (2β-hydroxy-9α-fluorohydrocortisone, 21-acetylamino-9α-fluoro-11β,17α-dihydroxy-4-pregnene-3,20-dione, etc.) are immobile and are retained at the origin. No other mobile components were detected other than the anticipated acetonide.

Acetylation with use of 20 μg of total steroid by the procedure described above gave a major reducing component at R_F 0.22 (system VI), identical in mobility with 16α-hydroxy-9α-fluorohydrocortisone 16α,21-diacetate, with lesser reducing components at R_F 0.07 and 0.03 (monoacetates?). Chromic acid oxidation on paper of another sample first acetylated in this fashion yielded a major reducing steroid at R_F 0.48, recognized as 16α-hydroxy-9α-fluorocortisone 16α,21-diacetate by mobility. Traces of another component were present at R_F 0.09, and unaltered 16α-hydroxy-9α-fluorohydrocortisone 16α,21-diacetate was present at R_F 0.23.

References

Bate-Smith, E. C., and Westall, R. G. (1950), *Biochim. Biophys. Acta, 4,* 427.

Birmingham, M. K. (1959), *Nature 184,* 67.

Gould, D., Ilavsky, J., Gutekunst, R., and Hershberg, E. B. (1957), *J. Org. Chem. 22,* 829.

McAleer, W. J., Kozlowski, M. A., Stoudt, T. A., and Chemerda, J. M. (1958), *J. Org. Chem. 23,* 508.

Meister, P. D., Peterson, D. H., Eppstein, S. H., Murray, H. C., Reineke, L. M., Weintaub, A., and Leigh Osborn, H. M. (1954), *J. Am. Chem. Soc. 76,* 5679.

Monder, C., and White, A. (1961), *Biochim. Biophys. Acta 46,* 410.

Peterson, D. H. (1956), *Record Chem. Progress 17,* 211.

Rahandraha, T., and Ratsimamanga, A. R. (1956), *Bull. soc. chim. biol. 38,* 897.

Schneider, J. J. (1953), *J. Am. Chem. Soc. 75,* 2024.

Smith, L. L., and Foell, T. (1959), *Anal. Chem. 31,* 102.

Smith, L. L., and Foell, T. (1960), *J. Chromatography 3,* 381.

Smith, L. L., Foell, T., DeMaio, R., and Halwer, M. (1959), *J. Am. Pharm. Assoc., Sci. Ed. 48,* 528.

Smith, L. L., Garbarini, J. J., Goodman, J. J., Marx, M., and Mendelsohn, H. (1960a), *J. Am. Chem. Soc. 82,* 1437.

Smith, L. L., Marx, M., Garbarini, J. J., Foell, T., Origoni, V. E., and Goodman, J. J. (1960b), *J. Am. Chem. Soc. 82,* 4616.

Smith, L. L., Marx, M., Mendelsohn, H., Foell, T., and Goodman, J. J. (1962), *J. Am. Chem. Soc.*, in press.

Smith, L. L., Mendelsohn, H., Foell, T., and Goodman, J. J. (1961), *J. Org. Chem. 26,* 2859.

Thoma, R. W., Fried, J., Bonanno, S., and Grabowich, P. (1957), *J. Am. Chem. Soc. 79,* 4818.