Steroid Hydroxylations. V. Intracellular Location of 16α-Hydroxylase and Its Substrate Specificity in Sow Ovary*

Barney Kadis

ABSTRACT: A sow ovarian steroid 16α-hydroxylase enzyme system which operates on a variety of compounds has been found to function within the microsomal fraction of the tissue. Both C_{21} and C_{19} steroids can serve as substrates for the enzyme with the requirement that an oxygen function (either a ketone or hydroxyl group) be located in the vicinity of the C-16 carbon atom. A very active sulfatase enzyme was also found in this fraction which acted upon both dehydroisoandrosterone and pregnenolone sulfates. Utilizing gas chromatographic techniques, no 16α-hydroxy compounds could be found from the incubation of either estrone or 17β-estradiol. Reaction of 17-methylprogesterone yielded negative results, and a plausible explanation is presented based on nuclear magnetic resonance data.

In part two of this series data were presented which showed that the 17α-hydroxylation of progesterone[1] was dependent upon two factors: the C-20 ketone and the conformation of the C-17 acetyl side chain (Ball and Kadis, 1965). Because of the interest in 16α-hydroxylated steroids, we have extended this same approach to the study of 16α-hydroxylation. These compounds have been isolated from various sources (Dorfman and Ungar, 1965; Bolté *et al.*, 1964; Magendantz and Ryan, 1964; Villee, 1964; Viscelli *et al.*, 1965; Heinrichs *et al.*, 1966; Lawrence, 1966; Nayfeh and Baggett, 1966; Reynolds, 1966), but the function of 16-hydroxylated compounds in biologic systems has not been completely elucidated.

Experimental Section

All tritiated compounds were obtained from New England Nuclear Corp. Reference steroids were obtained from commercial sources except for 17α-methylprogesterone (Ayerst Laboratories) and 16α-hydroxyprogesterone (The Upjohn Co.); 16α-hydroxytestosterone, 16α-hydroxyandrostenedione, 16α-hydroxypregnenolone, and 16α-hydroxyestrone were kindly supplied from the Steroid Reference Collection of Professor W. Klyne, England.

Preparation of the mitochondrial and microsomal fractions, incubation conditions, and isolation and purification techniques are those described by Ball and Kadis (1965). Tritium-measuring and quantitation techniques have been described previously (Kadis, 1966). The soluble fraction was prepared from 15 g of ovary homogenized in 45 ml of buffer. After centrifugation at 105,000*g* for 45 min, the supernatant was removed and spun for an additional 20 min at the same speed. Incubation with [7α-^{3}H]progesterone (7,970,000 dpm) was for 4 hr. The mitochondrial incubation (7,970,000 dpm of progesterone) was carried out identically with that of the microsomal fraction.

Derivative Formation. Acetylations were performed by adding acetic anhydride and pyridine (2:1, v/v) to the steroid at room temperature and the mixture left overnight. Water was added and the compound extracted with ethyl acetate. Acetylation of 17-hydroxyprogesterone was accomplished using the method of Turner (1953).

Acetonide formation was that described by Bernstein *et al.* (1959). 16-Ketoprogesterone was prepared according to the procedure of Bernstein *et al.* (1955). The Oppenauer oxidation reactions were performed as described by Bernstein and co-workers (1954).

Periodic Acid Oxidations. One volume of glacial acetic acid and five volumes of 0.4 N sulfuric acid containing 0.06 M periodic acid were mixed with the material. After 3 hr at room temperature, distilled water was added and the steroid was extracted with ethyl acetate. Sodium borohydride reductions were effected by adding 1 ml of ethanol to the steroid at 0°. Excess sodium borohydride was added and the mixture was kept at 0° for 90 min. A few drops of glacial acetic acid was added to destroy the $NaBH_4$, and the steroid was extracted with ethyl acetate after the ethanol had been removed.

* From the Department of Obstetrics and Gynecology, University of Nebraska College of Medicine, Omaha, Nebraska 68105. *Received August 8, 1966.* Supported by U. S. Public Health Research Grant No. AM 09456 (Arthritis and Metabolic Diseases Branch) and in part by Grant No. TI HD-18 (Child Health and Human Development Branch) and No. FR-5391 (General Medical Branch). Present address: Institute of Hormone Biology, Syntex Research, Palo Alto, Calif.

[1] Trivial names will be used for 3β-hydroxypregn-5-en-20-one, pregnenolone; androst-4-ene-3,17-dione, androstenedione; 3β-hydroxyandrost-5-en-17-one, dehydroisoandrosterone (DHIA); pregn-4-ene-3,20-dione, progesterone; 17β-hydroxyandrost-4-en-3-one, testosterone; 3-hydroxyestra-1,3,5(10)-trien-17-one, estrone; 3,17β-dihydroxyestra-1,3,5(10)-triene, 17β-estradiol; 3,16α,17β-trihydroxyestra-1,3,5(10)-triene, estriol; nuclear magnetic reasonance, nmr.

Paper chromatography of the steroid sulfates was that as described by Schneider and Lewbart (1959).

Results

Table I lists the results of the microsomal incubations with various substrates in which the data clearly indicate that progesterone is the best substrate for the 16α-hydroxylase enzyme system. The results further demonstrate that an oxygen-containing functional group (hydroxyl or ketone) is necessary for enzymatic activity, and furthermore that the position of this group can be located at either position 17 or 20. No C-16 hydroxylated compounds could be detected from either of the pregnenolone sulfate or DHIA sulfate incubations since only one radioactive peak (starting material) could be detected with the paper strip autoscanner. However, once the steroid sulfates were hydrolyzed, reaction did occur. The surprising finding was the large amount of sulfatase activity present, even though this enzyme appears to be quite ubiquitous (Warren and French, 1965). The amount of hydrolysis was greater for DHIA sulfate (76%) as compared to pregnenolone sulfate (58%). Adrenal sulfatase enzyme activity has also been found in the microsomal fraction (Burstein and Dorfman, 1963), as well as in placental microsomes (French and Warren, 1966).

TABLE I: Compounds Which Underwent 16α-Hydroxylation.

Steroid	16-Hydroxy Derivative	% Conversion[a]
Progesterone	+	3
17-Hydroxyprogesterone	+	1
17-Hydroxypregnenolone	+	0.24
Testosterone (2 expt)	+	0.13
Androstenedione (2 expt)	+	0.44
Pregnenolone	+	0.4
Pregnenolone sulfate	−	—
Hydroxylsis (pregnenolone)	+	0.3
Dehydroiosandrosterone sulfate	−	—
Hydrolysis (DHIA)	+	0.2
20α-Hydroxypregn-4-en-3-one	+	0.96
17β-Estradiol (mince)	−	—
Microsomes	−	—
Estrone (mince)	−	—
Microsomes	−	—
17α-Methylprogesterone	−	—
17β-Ethylandrost-4-en-3-one	−	—

[a] Calculated by using the actual amount of substrate converted (starting disintegrations per minute − recovered disintegrations per minute).

The results of the two estrogen incubations were negative in all respects. It appeared, however, that some 16-hydroxylated material had been formed in each case. When the incubation extracts were chromatographed on paper (Kadis *et al.*, 1962), areas corresponding to 16α-hydroxyestrone and estriol were noted. Elution, followed by acetylation, yielded radioactive material which behaved chromatographically like the 16-hydroxylated materials. Upon gas chromatography of these materials, no significant amounts of any 16-hydroxy steroids could be detected.

The nmr spectral data for progesterone and 17-methylprogesterone show that there is a downfield shift of 3 cycles/sec in the C-18 methyl resonances (progesterone, ν 40.02, and 17-methylprogesterone, ν 43.02). The signal associated with the 17-methyl protons is located at ν 67.98.

No significant amount of 16-hydroxylation could be detected when 16α-hydroxyprogesterone was incubated with the mitochondrial fraction. A small amount of hydroxylation did, however, occur with the soluble fraction. The identification of 16α-hydroxyprogesterone was the same as with the microsomal fraction (Table II), but in this case the specific activities did not remain constant. Authentic 16α-hydroxyprogesterone (1 mg) was added to the incubate extract and the material chromatographed in a hexane system for 20 hr to remove the progesterone. After development in a benzene system, the 16α-hydroxyprogesterone was eluted and possessed a specific activity of 24.7 dpm/μg (20,000 dpm). Rechromatography in a benzene–chloroform system afforded steroid of sp act. 11.88 dpm/μg (9300 dpm). Acetylation followed by chromatography in a hexane–benzene system yielded the acetate of sp act. 5.20 dpm/μg (3600 dpm). Recovery of the steroid in each experiment was well within experimental error.

Table II lists the procedures used for structure proof of the isolated 16-hydroxy derivatives. In each case, other than that of progesterone as described above, the extract from the incubate was chromatographed in the chromatography system listed first in the table. The usual procedure was to chromatograph the 16-hydroxylated compound in two different systems, followed by derivative formation. Because the C-5,6-dehydro compounds could not be visualized with an ultraviolet scanner, they were located on the paper by means of the paper strip autoscanner and a compound whose R_F value corresponded to known steroid chromatographed simultaneously on a separate paper strip. The enol system was oxidized to an α,β-unsaturated ketone by means of an Oppenauer oxidation so that the 240-mμ absorbancy peak was present for reading in the ultraviolet region. Authentic 16α-hydroxy steroid (200 μg) was added to the incubate extract before the first chromatography separation.

For each chromatographic separation performed, a strip containing authentic steroid was developed simultaneously. The R_F values of these standards were within a limit of ±0.03.

TABLE II: Structure Proof of Isolated 16α-Hydroxy Compounds.

Steroid (dpm incubd)	R_F of 16α-Hydroxy Compd[a]	Derivative and R_F	Dpm	Sp Act. (dpm/μg)[b]
Progesterone (5,633,872)	16α-Hydroxyprogesterone			
	Bz 0.34		100,000	540
	Bz–Ch 0.65		98,000	533
		Acetate		
		Hx–Bz 0.68	90,000	525
17-Hydroxyprogesterone (8,000,000)	16α,17-Dihydroxyprogesterone			
	Bz 0.24		95,000	505
	Bz–Ch 0.58		94,600	507
		Acetate		
		Bz 0.6	31,000	503
		Acetonide		
		Hx–Bz 0.82	30,000	502
		Periodic acid oxidation		
		Bz–Ch 0.19	29,875	500
17-Hydroxypregnenolone (25,000,000)	3β,16α-17-Trihydroxypregn-5-en-20-one			
	Bz–Ch 0.32		8,500	
		Oppenauer oxidation[c] (16α,17-dihydroxyprogesterone)	8,150	46.5
		Acetate		
		Bz 0.6	8.075	46.0
Testosterone (30,000,000)	16α-Hydroxytestosterone			
	Bz–Ch 0.11		22,200	108
		Acetate		
		Hx–Bz 0.89	22,000	107
Androstenedione (20,000,000)	16α-Hydroxyandrost-4-ene-3,17-dione			
	Bz 0.27		68,800	363
	Bz–Ch 0.5		68,700	362
		Acetate		
		Hx–Bz 0.65	68,550	360
Pregnenolone (25,000,000)	16α-Hydroxypregnenolone			
	Bz–Ch 0.25		86,000	
		Oppenauer oxidation (16α-hydroxyprogesterone)	84,350	465
		Acetate	84,300	464.5
20α-Hydroxypregn-4-en-3-one (6,000,000)	16α,20α-Dihydroxypregn-4-en-3-one			
	Bz–Ch 0.3		50,400	271
		CrO_3 oxidation (16-ketoprogesterone)		
		Hx–Bz 0.17	24,100	270
		Dehydration + CrO_3 oxidation (16-dehydroprogesterone)		
		Hx 0.46	23,775	268
		Hx–Bz 0.88	23,700	266
Pregnenolone sulfate[d] (25,000,000)	16α-Hydroxypregnenolone			
			39,000	
		Oppenauer oxidation (16α-hydroxyprogesterone)	37,450	205
		Acetate	37,330	203.5

TABLE II (*Continued*)

Steroid (dpm incubd)	R_F of 16α-Hydroxy Compd[a]	Derivative and R_F	Dpm	Sp Act. (dpm/μg)[b]
Dehydroisoandrosterone sulfate (20,000,000)	16α-Hydroxy DHIA Bz 0.1		12,600	
		Oppenauer oxidation-(16α-hydroxyandrostenedione)	11,100	61.7
		Acetate	11,000	61.5

[a] Abbreviations used: hexane (Hx), hexane–benzene (Hx–Bz), benzene (Bz), and benzene–chloroform (Bz–Ch). [b] Authentic steroid (200 μg) was added in each instance for the specific activity determinations. [c] For the compounds which underwent the Oppenauer oxidation, the work-up was the same as listed for the oxidized product. [d] The derivatives listed for the sulfates are for the hydrolyzed steroid.

Discussion

It has been previously reported that sow ovary was capable of forming 16α-hydroxyprogesterone from progesterone (Kadis, 1964). The present investigation demonstrates that the 16α-hydroxylase enzyme system is located in the microsomal fraction with only a small amount of activity located in the soluble fraction. Thus, these results are in accord with the fact that the 17α-hydroxylase enzyme system is also found in the microsomal fraction. This finding, however, is in contrast to that of Little *et al.* (1963) who find the 16α-hydroxylase system in the soluble fraction of placenta. On the other hand, a study of mammalian liver shows that the 16α-hydroxylase system is located in the microsomal fraction (Heinrichs *et al.*, 1966). The reason for this difference is not clear.

As in the case of 17α-hydroxylation of progesterone (Ball and Kadis, 1965), an oxygen function is necessary on the steroid molecule in the proximity of the hydroxylation site before any reaction can occur. 16α-Hydroxylation, in contrast to 17α-hydroxylation, can occur if a C-20 alcohol function is present, as illustrated by the reaction of 20α-hydroxypregn-4-en-3-one.

The hydroxylations of testosterone and androstenedione show that C_{19} steroids also depend upon an oxygen function, either as a ketone or hydroxyl group, for 16α-hydroxylase activity. With mince preparations of sow ovary we were unable to demonstrate the 16α-hydroxylation of androstenedione (Kadis, 1964), but in two separate experiments with microsome preparations hydroxylations could be observed.

No hydroxylation was detected for either 17β-ethylandrost-4-en-3-one or 17-methylprogesterone. The lack of any reaction with the androstene compound may be explained by the absence of an oxygen function in the proximity of C_{16}. The reason for no hydroxylation with 17-methylprogesterone may be that the orientation of the C-17 side chain has been changed due to the bulk of the methyl group so that the steroid cannot attach itself to the enzyme. Cross and Beard (1964) have shown utilizing nmr spectra how the orientation of the acetyl side chain and the conformation of ring D of substituted pregnanes are changed by C-16 methyl substitution. Their data are based on shifts of the C-18 methyl resonances and demonstrate a downfield shift of 1–2 cycles/sec for α substitution as compared to β orientation where greater changes are noted (4–20 cycles/sec). In the present case, the shift of 3 cycles/sec is greater than those reported by Cross and Beard for α orientation, and may very well explain the absence of any hydroxylation. Steroid 16α-hydroxylation, then, falls into the general category of microsomal mixed-function oxidations which require reduced pyridine coenzyme and molecular oxygen.

Acknowledgments

The author is grateful to Dr. John Copenhaver, Nebraska Pyschiatric Institute, for his valuable assistance in performing the gas chromatographic analysis. Thanks are also due to Dr. O. L. Champman, Iowa State University, for the nmr spectra.

References

Ball, J. H., and Kadis, B. (1965), *Arch. Biochem. Biophys. 110*, 427.

Bernstein, S., Brown, J. J., Feldman, L. I., and Rigler, N. E. (1959), *J. Am. Chem. Soc. 81*, 4956.

Bernstein, S., Heller, M., Stolar, S. M. (1954), *J. Am. Chem. Soc. 76*, 5674.

Bernstein, S., Heller, M., and Stolar, M. (1955), *J. Am. Chem. Soc. 77*, 5327.

Bolté, E., Mancuso, S., Eriksson, G., Wiqvist, N., and Diczfalusy, E. (1964), *Acta Endocrinol. 45*, 535.

Burstein, S., and Dorfman, R. I. (1963), *J. Biol. Chem. 238*, 1656.

Cross, A. D., and Beard, C. (1964), *J. Am. Chem. Soc. 86*, 5317.

Dorfman, R. I., and Ungar, F. (1965), Metabolism of Steroid Hormones, New York, N. Y., Academic, pp 163–169.

French, A. P., and Warren, J. C. (1966), *Steroids 8*, 79.

Heinrichs, W. L., Feder, H. H., and Colaś, A. (1966), *Steroids 7*, 91.
Kadis, B. (1964), *Biochemistry 3*, 2016.
Kadis, B. (1966), *J. Am. Chem. Soc. 88*, 1846.
Kadis, B., Harris, C., and Salhanick, H. (1962), *J. Chromatog. 7*, 430.
Lawrence, J. R. (1966), *Biochem. J. 99*, 270.
Little, B., Shaw, A., and Purdy, R. (1963), *Acta Endocrinol. 43*, 510.
Magendantz, H., and Ryan, K. (1964), *Federation Proc. 23*, 275.
Nayfeh, S. H., and Baggett, B. (1966), *Endocrinology 78*, 460.
Reynolds, J. W. (1966), *Steroids 7*, 261.
Schneider, J. J., and Lewbart, M. L. (1959), *Recent Progr. Hormone Res. 15*, 201.
Turner, R. B. (1953), *J. Am. Chem. Soc. 75*, 3489.
Villee, D. (1964), *J. Clin. Endocrinol. Metab. 24*, 442.
Viscelli, T. A., Hudson, P. B., and Lombardo, M. E. (1965), *Steroids 5*, 545.
Warren, J. C., and French, A. P. (1965), *J. Clin. Endocrinol. Metab. 25*, 278.

Protein–Nucleic Acid Interaction. I. Nuclease-Resistant Polylysine–Ribonucleic Acid Complexes*

Herbert A. Sober, Stuart F. Schlossman,† Arieh Yaron,‡ Samuel A. Latt, and George W. Rushizky

ABSTRACT: Polylysines react on an equivalent basis with ribonucleic acid (RNA) to form insoluble complexes at low salt concentrations and neutral pH. Soluble complexes are formed, however, at low polylysine:RNA ratios.

Digestion by nonspecific ribonucleases results in the formation of a precipitate with a lysine:nucleotide ratio of 1. The "protected" nucleotide chain has essentially the same chain length as the polylysine of the initial complex and is susceptible to cleavage by the original enzyme. Protection specificity, *i.e.*, high Gp + Cp content of protected fragment, appears to be related to secondary structure and is eliminated by thermal denaturation. Implications of these data in terms of a model of protein–nucleic acid interaction are discussed.

Complexes of proteins and nucleic acids (nucleoproteins), which occur within the cell virtually wherever nucleic acids are found, may play a controlling role in processes of cell growth, differentiation, and replication. While it is known (Katchalsky, 1964) that interaction between oppositely charged polyelectrolytes produces complexes with altered properties, relatively little is known about the specific nature of the interaction, the mechanism by which these processes are effected, or whether sufficient specificity to satisfy the biological requirements can be achieved by such interaction.

This paper is concerned with the interaction between polylysines of relatively well-defined size (the protein model) and ribonucleic acid (RNA), and the effect of this interaction on the hydrolysis of RNA by nucleases. In common with similar studies with deoxyribonucleic acid (DNA) (Spitnik *et al.*, 1955) we have found that, at low salt concentrations (0.10 M) and neutral pH, the addition of polylysine to RNA in solution results in the formation of a precipitate as the two components approach charge equivalence. However, at low polylysine:RNA ratios, complexes of these polyelectrolytes are soluble and amenable to study.

The experimental approach used in our studies is to form these soluble polylysine–RNA complexes and to treat them with nuclease. During the course of digestion, short oligonucleotides and mononucleotides appear and a precipitate is formed which contains both polylysine and RNA material with a nucleotide:lysine ratio of 1. After isolation of the precipitated complex and dissociation and separation into its components, the RNA member of the complex is examined as to chain length, base composition, and susceptibility to nuclease action. The results obtained from these studies establish that the polylysine–RNA complexes contain nuclease-resistant RNA segments, that these protected RNA segments are of essentially the same chain length as the polylysine, and that under certain

* From the Laboratory of Biochemistry, National Cancer Institute, National Institutes of Health, U. S. Public Health Service, Department of Health, Education, and Welfare, Bethesda, Maryland 20014. *Received August 22, 1966.* A preliminary report has appeared (Sober *et al.*, 1965).

† Present address: Department of Medicine, Harvard Medical School, Beth-Israel Hospital, Boston, Mass.

‡ Visiting Scientist. Present address: Department of Biophysics, Weizmann Institute of Science, Rehovoth, Israel.