Journal of Steroid Biochemistry, 1976, Vol. 7, pp. 787–793. Pergamon Press. Printed in Great Britain

STUDIES ON THE MECHANISM OF ESTROGEN BIOSYNTHESIS IN THE RAT OVARY—I*

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(*Received* 24 *November* 1975)

SUMMARY

Methods were evaluated for obtaining a reliable, active estrogen synthetase (aromatase) system from the rat ovary for mechanistic studies. Short term treatment with luteinizing hormone and follicle stimulating hormone in various combinations did not produce appreciable stimulation, whereas long term treatment (8–16 days) with pregnant mare's serum gonadotropin increased activity in homogenates up to nine fold per mg wet wt of tissue. A similar increase per mg protein was noted in the 105,000 *g* microsomal fraction where the bulk of the activity was found. Various conditions for preparing and incubating the aromatase were evaluated to obtain optimal enzyme activity. The potencies of six steroids as aromatase inhibitors were compared in the rat ovarian and human placental microsomal systems. In all cases except one the results were comparable.

INTRODUCTION

While there have been many studies on aromatization (estrogen biosynthesis) in the rat ovary, few have been concerned with the mechanism of the reaction itself. For this, most studies have been carried out in human placenta [1] using the "aromatase" containing microsomal fraction obtained by Ryan [2] or a similar preparation.

In order to study factors related to stimulation of the enzyme and to study the relationship between *in vivo* and *in vitro* properties, a reliable animal source of the aromatase was desired. Since the rat is used extensively for many endocrine studies, including some of our own, we decided to undertake an investigation of the enzyme properties of the aromatase in the ovary of this animal. In this paper we present data on aromatase activity in rat ovary and methods for stimulating activity to make this source more reliable. Also some properties of the induced enzyme are presented.

EXPERIMENTAL

Radioactive steroids. [7-^{3}H]-Testosterone, [4-^{14}C]-estradiol and [4-^{14}C]-estrone were obtained from New England Nuclear Corp., Boston. All were monitored for radiochemical purity by thin-layer and paper chromatography and were found to be over 98% pure.

Gonadotrophins. Human chorionic gonadotrophin (HCG) was obtained from Ayerst Labs., Inc. Pregnant mare's serum gonadotrophin (PMSG) was obtained from Ayerst Labs, Inc. (Equinex), N.V. Organon, Inc. (Gestyl) and the National Institute of Arthritis, Metabolic & Digestive Diseases (NIAMDD), Bethesda, U.S.A. The latter also supplied ovine LH and FSH and rat FSH. Postmenopausal gonadotrophin (Pergonal) was from Cutter Labs., Berkeley, California, U.S.A.

Compounds tested as estrogen synthetase inhibitors. 4-Hydroxytestosterone was prepared by base treatment of 17β-hydroxy-5β-androstan-3-one [3], while the corresponding 17-ketone was prepared by oxidation of the 4-hydroxy-3-ethylene ketal [4]. The corresponding acetates were prepared by stirring the steroids under N_2 in pyridine-acetic anhydride (1:1, v/v) overnight at room temperature. After treating with methanol for 30 min and evaporating the solvents, the residue containing the diacetate of 4-hydroxytestosterone was purified by silica gel t.l.c. in benzene–ethyl acetate (4:1, v/v). The white crystals absorbed in the uv at 245 nm; I.R. (ν) 2930(C–H), 1755, 1725 (C=O acetate), 1675 (3-one). 4-Hydroxy-4-androstene-3,17-dione acetate was purified as above using benzene–ethyl acetate (9:1, v/v.); λ 247 nm; ν 2930 (C–H), 1725, 1755 (C=O, acetate), 1675 (3-one). The propionate of 4-hydroxy-4-androstene-3,17-dione was prepared similarly using propionic anhydride: λ 247 nm; ν 2930 (C–H), 1740 (C=O of ester), 1670 (3-one). The materials were repurified by t.l.c. immediately prior to testing.

1,4,6-Androstatriene-3,17-dione (Steraloids) was purified by dry column chromatography using 100 g of silica gel per g of compound. The column was developed with ether–hexane (3:1 v/v). The material from this column was rechromatographed using benzene–methanol (19:1 v/v). 5α-Androstane (Mann Research Labs.) was used as supplied.

Other materials. All solvents and chemicals employed were analytical grade or were technical grade distilled from glass before use. Liquiflouor

* Portions of this work were presented in abstract form at the 55th Endocrine Society Meeting, 1973.

obtained from New England Nuclear Corp., Boston, USA, was used at 42 ml (4 g PPO and 50 mg POPOP) per liter toluene (spectro quality). Whatman No. 1 chromatography paper was used and t.l.c. plates were prepared with silica gel PF_{254} type 7747 (E. Merck). 0.1% Rhodamine G (Allied Chemical) dye solution (30 g silica gel, 0.3 g of dye, in 84 ml of water) was used as the mixture for coating. Silica gel for dry column chromatography was obtained from ICN Pharmaceuticals, West Germany; NADP was from Calbiochem; glucose 6-phosphate from General Chemicals; nicotinamide from Eastman Kodak and glucose 6-phosphate dehydrogenase was obtained from Boehringer–Mannheim Corp.

Preparation of buffers. Unless otherwise indicated, buffers were used at a concentration of 0.1 M and at a pH of 7.4. Preparations followed procedures or the examples in Methods in Enzymology, Vol. I. Distilled deionized water was used throughout.

Administration of gonadotropins. All gonadotropins were administered by subcutaneous injection. Unless otherwise indicated, long term administration with PMSG consisted of treatment with 100 IU every other day for 15 days. Animals then were sacrificed on the 16th day to obtain the ovaries.

Animals. The female rats of the Sprague–Dawley strain used initially were from Charles River Breeding Labs, Wilmington, Mass., and later from Gofmoor Farms, Westborough, Mass. All weighed not less than 225 g and had vaginal smears taken daily.

Tissue preparation. Human placental microsomes were prepared as previously described [5]. In preparing ovarian microsomes, animals were weighed, sacrificed by decapitation and the excised ovaries were cleaned and placed on ice-cold, buffer-moistened, filter paper. The following operations were carried out at 4–6°C ambient temperature or in refrigerated centrifuges. Weighed tissue was homogenized in ice-cold buffer at 50–100 mg/ml. For cell fractionation, homogenates were pooled and centrifuged at 1200 *g* for 30 min; the supernate then was spun at 12,000 *g* for 30 min. The supernate from this operation was further centrifuged at 105,000 *g* for 1 h in a centrifuge cooled to 0°. Precipitates were resuspended as required using a smooth bore homogenizer fitted with a teflon pestle.

Incubation. Placental incubations were carried out as described previously [5]. In early ovarian incubations, substrate (1 μCi) was added to the flask in ethanol followed by one drop of propylene glycol and then the ethanol was evaporated. [4-C^{14}]-Androstenedione was substrate for placental microsomes and [7-^{3}H]-testosterone for ovarian microsomes. Amounts of non-radioactive substrate were added as indicated in the tables. Later, in order to test inhibitors with ovarian microsomes, [7-^{3}H]-testosterone was added directly in 0.1 ml buffer to the incubation mixture and this practice was continued whether or not inhibitors were present. Homogenate or tissue fraction (equivalent to 50–100 mg wet weight) was incubated together with NADP 9.8 mg, glucose 6-phosphate 5 mg, nicotinamide 2.6 mg and 20 μl glucose 6-phosphate dehydrogenase added in 0.4 ml buffer. The total vol was made to 2.5 ml with buffer. For the routine procedure, phosphate buffer was used and the incubation was carried out in an atmosphere of 95% O_2:5% CO_2 at 37°. When potential inhibitor compounds were tested, incubation time was 30 min; in other experiments the time was 2 h. The incubation was quenched with ethyl acetate and after 1 h at 4°C [4-^{14}C]-estradiol and [4-^{14}C]-estrone (5000 d.p.m. each) were then added as recovery markers (tritiated estrogens were used with placental microsomes) together with 50 μg each of non-radioactive estrone, estradiol, testosterone and androstenedione.

Purification of metabolites. The steroids were extracted with ethyl acetate and were purified by t.l.c. in ether–hexane (3:1 v/v). Routinely, estrone and estradiol zones were eluted with anhydrous ether and a 10% aliquot of each eluate was taken for scintillation counting. The remaining portion of the estradiol eluate was chromatographed in the toluene–propylene glycol paper system for 6 h. An aliquot of the eluted zone containing estradiol was assayed by liquid scintillation counting together with appropriate aliquots of the radioactive substrate and the recovery standards for estradiol and estrone. Many samples were analyzed further by recrystallization to determine if constant specific activity had been achieved. As discussed in the Results section the data show that the paper chromatography step yielded material radiochemically pure. The results on % conversion are reported as the percentage of radioactive substrate converted to estradiol and, unless otherwise noted, the ^{3}H:^{14}C ratios of estradiol used were obtained after the routine paper chromatography.

The protein content of the various tissue fractions was measured by the method of Lowry *et al.* [6].

RESULTS

In our first attempts to stimulate estrogen synthesis in the rat ovary, animals were injected with 20 μg of ovine LH at 5 p.m. on the evening of proestrus and again at 8 a.m. the following morning. Animals were sacrificed at 11 a.m. and ovarian homogenates were prepared in phosphate buffer as detailed in the Experimental section. On incubation, conversion of [7-^{3}H]-testosterone to [7-^{3}H]-estradiol was found to be not significantly different from that obtained using ovaries from untreated rats in proestrus. LH added to the *in vitro* incubation of ovarian homogenates from rats at proestrus was also without effect.

The procedure of Parlow [7] which causes an increase in the weight of the ovaries and brings them to a pseudopregnant state was also tested. Rats 25–27 days old were injected with 50 IU PMSG (Equinex); after $2\frac{1}{2}$ days the animals then were injected with 25 IU HCG and were sacrificed 5 days after the beginning of the experiment. This did not result in stimu-

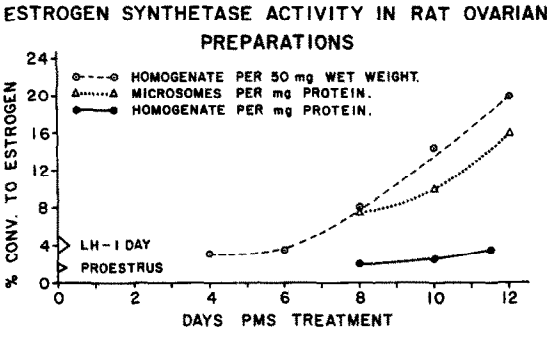

Fig. 1. Rats were treated every other day with 100 IU PMSG. On the days indicated, they were sacrificed and ovarian homogenates and microsomes were prepared. These were incubated for 2 h using 1 μg [7-^{3}H]-testosterone and ovarian material from 50 mg wet weight of ovary. Phosphate buffer was used throughout. After the addition of ^{14}C recovery markers, the estrogens were purified by chromatography to constant S.A. Additional details of incubation conditions and analyses are in the Experimental section.

lation of ovarian aromatase activity as judged by incubation of homogenates as above. If, however, the rats were not sacrificed until 8 days, stimulation (4.6% conversion) occurred as compared to ovaries evaluated after 5 days of treatment (1.5% conversion).

Injections of PMSG (50 IU Equinex) alone on alternate days for 8 days also caused an increase in [7-^{3}H]-testosterone conversion to [7-^{3}H]-estradiol in homogenates of ovaries obtained at the end of this treatment period. Injection of LH on the evening of the 7th day of PMSG treatment did not cause any additional stimulation. However, PMSG injections continued for longer periods further increased ovarian aromatase activity. The increase in activity per 50 mg wet weight of tissue is shown in Fig. 1 (dashed line) for 1 to 12 days stimulation with PMSG.

Equinex PMSG became unavailable commercially for a time and various regimens of other preparations of PMSG were assessed for maximal stimulation of aromatase activity. Low doses of 10 to 25 IU PMSG (NIAMDD or Equinex when available later) had little or no effect in stimulating ovarian aromatase activity whether given twice daily for 8 days or on alternate days for 13 days. Comparison of preparations of PMSG from Ayerst, Organon and NIH did not show any striking differences but there was some variability in the amount of stimulation in several tests with these preparations. NIAMDD-PMSG at 100 IU for 16 days appeared to be consistent in producing large homogeneous ovaries free of cysts. This regimen gave good conversions of [7-^{3}H]-testosterone to [7-^{3}H]-estradiol and was used for all further experiments even with the other brands of PMSG. The mean result of 16 incubations of homogenates was 31 ± 8.1 S.D. ± 2.0 S.E. % per 50 mg wet weight tissue/1 μg substrate/2 h incubation. This is an increase of nine fold from the mean value of 3.7 ± 1.5 S.D. ± 0.41 S.E. % for 14 incubations of homogenates from rats at proestrus. By comparison, there was little stimulation of estrone production with PMSG treatment. The mean conversion for 13 incubations of ovaries from proestrus rats was 0.32 ± 0.1 S.D. ± 0.03 S.E. % and for PMSG stimulated ovaries 0.53 ± 0.2 S.D. ± 0.05 S.E. %. These better values were obtained after the first t.l.c. purification.

The size of the ovaries was also considerably increased from 53.8 ± 19.5 S.D. mg (mean value for proestrus rats) to 386 ± 113 S.D. mg per ovary after 16 days PMSG stimulation.

Other attempts at stimulating ovarian aromatase activity further with injections of 20 μg LH together with PMSG or LH on the last 2 days of the 16 day PMSG (Equinex) treatment gave the same results as using PMSG alone. Also tested was rat FSH at 60 μg, injected twice daily for 9 days or Pergonal (4.7 IU FSH + 4.7 IU LH) injected daily for 11 and 16 days. Both preparations failed to cause any stimulation in ovarian size or conversion of [7-^{3}H]-testosterone.

When homogenates of ovaries from rats treated on alternate days for 16 days with 100 IU of PMSG were fractionated, most of the enzyme activity was associated with the microsomes as shown in Table 1. The homogenate also was treated with a "Polytron" tissue disintegrator (Brinkman Corp.) for 15 s to determine if the percentage activity in the microsomal fraction could be increased. This treatment produced no change in conversion to estradiol by the microsomes nor was there an increase in activity in the supernate following centrifugation at 10,000 *g*.

Table 2 shows data on the conversion of [7-^{3}H]-testosterone to estradiol by whole homogenates and microsomes from the same tissue after several purification steps including crystallization from methanol. The relative activity in the microsomes as compared to homogenate appears to increase with the PMSG treatment period. As regards purity of the estradiol metabolite, no significant change in values occurred after the first t.l.c. However an additional paper chromatography step was always included in the routine procedure for determining conversion to estradiol.

Since the aromatase activity could be influenced

Table 1. % Conversion of [7-^{3}H]-testosterone to [7-^{3}H]-estradiol by ovarian preparations from PMSG-treated rats

Preparation	Experiment (1) Phosphate	(2) Phosphate	(3) Tris–sucrose
Homogenates	55, 43	55, 51	22, 15
1200 *g*	12, 17	12, 17	4.5, 2.9
12,000 *g*	12, 9.3	7, 11	2.9, 2.4
105,000 *g*	45, 37	40, 61	19, 16

Preparation of homogenates and fractions from differential centrifugation are described in the Experimental. Each incubation was carried out for 2 h with 5 μg [7-^{3}H]-testosterone using the routine procedure described in the Experimental. Tris–sucrose buffer was 0.1 M Tris–0.25 M sucrose—pH 7.4.

Table 2. Comparison of aromatase activity in ovarian homogenates and microsomes after PMSG treatment and data on radiochemical purity of the product

Test	Days stimulation with PMSG		Thin-Layer Chrom.	Paper Chrom.	Cryst.
			Percent conversion to [7-^{3}H]-estradiol after each purification stage		
1.	10	Homogenate	20.7	20.9	21.1
		Homogenate	14.9	13.1	12.9
		Microsomes	5.6	5.4	5.7
		Microsomes	4.7	4.9	4.9
2.	12	Homogenate	41.2	41.8	42.1
		Homogenate	27.7	27.4	27.2
		Microsomes	12.3	12.8	12.5
		Microsomes	17.3	17.1	16.9
3.	16	Homogenate	18.5	21.7	21.0
		Microsomes	20.9	19.3	20.7
		Microsomes	16.0	15.6	16.7
			^{3}H dpm/μg estradiol recrystallized after paper chrom.		
				1st	2nd	3rd
4.	16	Microsomes	—	3.62	3.26	3.44
		Microsomes	—	3.40	3.35	3.46
		Microsomes	—	2.72	2.91	2.98
		Microsomes	—	1.71	2.00	1.95

Tissue equivalent to 100 mg wet weight of ovary was incubated with 1 μg [7-^{3}H]-testosterone using the routine conditions given in the Experimental. [^{14}C]-estradiol was added as a recovery marker prior to purification.

by the incubation conditions, a number of experiments were performed with ovarian tissue from rats treated with 100 IU PMSG for 16 days to characterize conditions yielding maximum conversion to estrogen. Incubation for 2 h in a total vol. of 2.5 ml at pH 7.4 was found to be optimal. In a comparison made of buffers used for incubation, aliquots of the microsomal fraction were precipitated at 105,000 *g* from Krebs–Ringer bicarbonate buffer (pH 7.4) and then were taken up in 4 different buffers. The results are shown in Table 3. Tris–sucrose buffer inhibited conversion to estrogen 80–90% as compared to Krebs–Ringer bicarbonate and phosphate buffers with and without the presence of EDTA. Results in Table 1 of separate experiments indicated that Tris–sucrose buffer is inhibitory.

Table 3. The effect of incubation buffers on the conversion of [7-^{3}H]-testosterone to [7-^{3}H]-estradiol by ovarian microsomes from PMSG treated rats

Buffer	Percent conversion to [7-^{3}H]-estradiol Test No. 1	Test No. 2
Krebs–Ringer bicarbonate	32.2, 30.0	30.6, 28.4
Phosphate	47.2, 40.4	—
Phosphate + EDTA	53.1, 42.7	—
Tris–Sucrose	7.4, 5.5	3.9, 3.7

Procedures for the preparation of tissue fractions are given in the text. Ovarian preparations equivalent to 100 mg wet weight of tissue was incubated with 1 μg [7-^{3}H]-testosterone using the routine procedure described in the Experimental. [4-^{14}C]-Estradiol was added as a recovery marker prior to purification. Sucrose concentration, 0.25 M; EDTA, 5 mM; buffers 0.1 M.

The effect of buffers on the preparation of microsomes also was compared. Each excised ovary was quartered and the pieces were randomly divided into two groups. One group was homogenized and fractionated in Krebs–Ringer bicarbonate buffer, the other in 0.25 M sucrose. After centrifugation to obtain the 105,000 *g* precipitates, these were taken up in phosphate buffer for incubation. The results shown in Table 4 suggest that 0.25 M sucrose is superior to Krebs–Ringer buffer for preparing more active microsomes.

Several conditions for the storage of ovarian tissue were also investigated. Homogenates stored in various dilutions of glycerol for 10 days at $-10°$ lost most of their aromatase activity. Microsomal pellets fractionated in Krebs–Ringer bicarbonate, washed with 1.15% KCl and stored for 16 days at $-10°$C also lost most of their original activity. Stored at

Table 4. The effect of homogenization media on aromatase activity of ovarian microsomes from PMSG-treated rats

Homogenization medium	Test No. 1	Test No. 2
	Percent conversion of [7-^{3}H]-testosterone to [7-^{3}H]-estradiol	
Krebs–Ringer bicarbonate	19, 24	16, 19
Sucrose (0.25 M)	31, 27	25, 22

Randomized quartered ovaries were homogenized in the buffers indicated and the mixtures were differentially centrifuged to obtain the microsomal fraction. Microsomes equivalent to 85 mg wet weight of tissue were incubated in phosphate buffer with 1 μg [7-^{3}H]-testosterone using the routine procedure described in the Experimental.

Table 5. Percentage [7-^{3}H]-testosterone substrate remaining after 2 h incubation with ovarian microsomes[1]

Incubation No.	Status of rats at sacrifice	Recovered substrate (%)[2]
1	16 Days PMSG	1.6, 1.7
2	16 Days PMSG	5.7, 5.6
3	Proestrus	24*, 24*
4	Proestrus	41, 42
5	Proestrus	44, 43

[1] Microsomes equivalent to 100 mg wet weight of tissue were incubated in phosphate buffer with 1 μg [7-^{3}H]-testosterone using the routine conditions stated in the Experimental.

[2] Values are from samples purified to constant specific activity by t.l.c. and two recrystallizations after adding [4-^{14}C]-testosterone as a recovery marker. As an example, for the duplicate incubations indicated (*) the ^{3}H/^{14}C ratio after t.l.c. was 40, 41; after the 1st recryst. 39, 42; after the 2nd 39, 40.

−70°C for 16 days, some of the same batch of microsomes retained 30% of their activity. However, when microsomal pellets prepared in sucrose buffer and washed with 1.15% KCl, were stored at −70°C for 16 days under phosphate buffer, i.e. without resuspension, they retained 85% of their original activity (conversions to estradiol using the routine procedure (cf. Experimental) were 17.7% before storage, 15.0% after storage). More recent tests show that the microsomes can be stored by this method for at least six weeks with similar retention of activity.

It is possible that instead of having increased aromatase activity in ovaries from PMSG-stimulated rats, the treatment is instead inhibiting conversion of substrate to other metabolites thus allowing more substrate to be available for aromatization. If this were so, then the amount of substrate remaining in the incubate at a given time should be higher in that from PMSG treated rats than in the incubate of ovaries from rats in proestrus. Table 5 shows that the opposite is occurring. A larger portion (36% of testosterone incubated) remained after incubation of ovaries from proestrus rats than from PMSG stimulated rats (3.8% of testosterone incubated) suggesting that the substrate was not a limiting factor in the incubations with proestrus rat ovaries.

4-Androstenedione appeared to be at least as good as testosterone as substrate for estradiol formation by ovarian microsomes. The percentage conversion after 30 min incubation with microsomes from 100 mg wet weight of ovarian tissue from 16 day PMSG treated rats was 30% for estradiol and 2.6% for estrone estimated in duplicate in one experiment.

After establishing optimum conditions for preparing and incubating the ovarian microsomes, the system was used to test the aromatase inhibiting properties of 6 compounds. The results are shown in Table 6 together with data from placental incubations with some of the compounds. 5α-Androstane, incubated as a control non-inhibitor, caused very little inhibition of the percentage of radioactive substrate converted to estradiol, as compared to incubations without any added compounds. 1,4,6-Androstatriene-3,17-dione caused marked inhibition of the conversion of [7-^{3}H]-testosterone to [7-^{3}H]-estradiol by ovarian microsomes, as well as of [4-^{14}]-androstenedione to [4-^{14}C]-estrogens by placental microsomes. The latter result was reported previously [5]. In addition to the triene, 4-hydroxy-4-androstene-3,17-dione and derivatives also were effective estrogen biosynthesis inhibitors. 4-Hydroxyandrostenedione was tested in both systems and was found to inhibit estrogen production by about 90%. The propionate and acetate were also very effective when tested in the ovarian system while 4-hydroxytestosterone was moderately effective causing 59% inhibition of estrogen biosynthesis in the ovarian system. 4-Hydroxytestosterone diacetate showed somewhat different results in the 2 systems, being less inhibitory in the ovary. However, in neither the ovary nor the placenta was it highly effective.

DISCUSSION

The normal rat ovary weighs 40–50 mg and the aromatase activity present in the microsomal fraction is low and variable, even at proestrus when estrogen production [8] and biosynthesis [9] is known to be maximal. In order to obtain greater amounts of more active tissue preparations for studying various aspects of estrogen biosynthesis we investigated the possibility of stimulating the ovaries by treating the animals

Table 6. Compounds tested for aromatase inhibition

Compound	Mean Percentage Inhibition of Conversion to Estrogens: Placental microsomes[1]	Ovarian microsomes[2]
None	0	0
5α-androstane	0	9
$\Delta^{1,4,6}$-A.	85	90
4-OH-T.	—	59
4-OH-T-diac.	43	19
4-OH-Δ^4-A.	85	91
4-OH-Δ^4A-acet.	—	74
4-OH-Δ^4A-prop.	—	81

[1] Microsomes from 1 g wet weight of tissue incubated with 14 μg [4-^{14}C]-testosterone and 84 μg of inhibitor with an NADPH generating system for 30 min at 37° as described [5]. Products measured were estrone and estradiol.

[2] Microsomes equivalent to 100 mg wet weight of tissue from PMSG-stimulated rats incubated with 6 μg test compound/μg [7-^{3}H]-testosterone under the routine conditions for 30 min. Product measured was estradiol since little estrone is produced in this preparation. Inhibitor wt/substrate wt = 6 in both sets of experiments.

Individual values from at least two incubations were usually within 5% and always within 10% of the mean.

$\Delta^{1,4,6}$-A. 1,4,6-androstatriene-3,17-dione; 4-OH-T. 4-hydroxytestosterone; 4-OH-T-diac. 4-hydroxytestosterone diacetate; 4-OH-Δ^4-A. 4-hydroxy-4-androstene-3,17-dione; 4-OH-Δ^4-A-acet. 4-hydroxy-4-androstene-3,17-dione acetate; 4-OH-Δ^4-A-prop. 4-hydroxy-4-androstene-3,17-dione propionate.

with gonadotrophins. We were able to develop conditions for producing more consistent and highly active preparations of ovarian microsomes, which proved useful in determining the aromatase inhibiting activity of a number of compounds. We were unable to do this using enzyme from ovaries of rats in proestrus due to variable and low aromatase activities.

The most effective treatment for the above purpose was PMSG administration for up to 16 days. With current batches of PMSG (Equinex, Ayerst) we routinely obtain higher aromatase activity with 12 day rather than 16 day PMSG treatment. This also was found occasionally in earlier work (cf. Table 2). After starting PMSG treatment, the daily vaginal smear pattern indicated that the rats ceased cycling in 4 days and became anestrus. Growth of the ovary increased rapidly after about 8 days stimulation with the gonadotropin. After 16 days PMSG treatment, tissue weight had increased 7-fold compared to ovaries of rats in proestrus and there was a nine-fold increase in conversion of testosterone to estradiol. As can be seen in Fig. 1 the percentage conversion in the microsomal fraction per mg protein also increased greatly. However, the conversion per total ovarian protein increased much less markedly. This indicates a specialized stimulation of aromatase activity in the microsomal fraction suggesting the possibility of preferential enzyme synthesis. These observations are in general agreement with studies showing that PMSG is effective in stimulating estrogen production in the ewe [10] and hamster [11] and in enhancing *in vitro* activity in the human ovary when administered to the subject prior to ovariectomy [12]. Papkoff [13] has recently demonstrated that PMSG has both FSH and LH activity in the same molecule. The ratio of LH and FSH activities may be optimal since we noted that no further aromatase stimulation could be achieved with FSH or LH either *in vitro* or *in vivo* in PMSG-treated animals. In untreated animals also, LH *in vivo* or *in vitro* was ineffective in our experiments, although Mills, Davies and Savard [14] stimulated estrogen synthesis from acetate in isolated rabbit follicles incubated with LH *in vitro*.

As mentioned, one of the reasons for developing the ovarian system was to study relationships between *in vivo* and *in vitro* properties of the enzyme. A part of this study is an evaluation of aromatase inhibitors we have developed [5, 15] using the placental system. Some studies of this type also have been presented recently by others [16, 17]. In general the aromatase inhibition in the PMSG-stimulated ovary was similar to the placenta (Table 6). In addition to 1,4,6-androstatriene-3,17-dione, previously reported effective in the placenta [5], 4-hydroxyandrostenedione also was potent in both systems causing 90% inhibition of the ovarian aromatase. One compound, 4-hydroxytestosterone diacetate, did not cause the same extent of inhibition with the ovary as with the placenta, although in neither system was this compound highly effective. Whether this represents a significant difference between the two systems is not known but suggests that the rat ovarian microsomal system is a more appropriate model for studying differences in inhibitory properties of compounds prior to testing these compounds *in vivo* in the same species. At present, we are considering these compounds as antagonists to estrogen sensitive processes, e.g., fertilization and DMBA-induced breast tumor growth [18].

Another attribute of this model system is its use in studies of the mechanism of estrogen biosynthesis. One important exception we have found to date is that while aromatization of 4-androstene-3,17-dione in the placental system is insensitive to carbon monoxide [19], the PMSG-induced rat ovarian system described here is CO sensitive [20] to this substrate, implicating cytochrome P-450 in this aromatization. Conversion to estriol from a 16α-hydroxy precursor is also CO sensitive in the placenta [21] as is estrogen biosynthesis from 19-nor precursors [19]. These results suggest that several aromatases may be operating in nature. Studies with the PMSG-stimulated system may be able to throw additional light on this aspect.

There should also be distinct advantages to being able to stimulate activity over a period of time followed by withdrawal of the stimulus. By so doing we may observe changes in other parameters, e.g. cytochrome P-450 content, activity towards other substrates (e.g. 19-norsteroids) and other enzyme activities such as the possibly related 1β-hydroxylase activity [22, 23]. Studies on these aspects are in progress.

Acknowledgement—This work was supported by NICHD Contract N01-HD-0-2158 and Grants AM-14625 and CA 18595 from the U.S. National Institutes of Health. The skilled technical assistance of Mrs. Catherine McQuain and Mrs. Carol Flynn is also appreciated.

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