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# ACTION OF LH, FSH AND $(Bu)_2$ cAMP ON THE CONVERSION OF [$^3$H]19-HYDROXYANDROSTENEDIONE INTO OESTROGENS BY FOETAL AND INFANTILE RAT OVARIES IN ORGAN CULTURE

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**Summary**—Ovaries from 18–21-day-old foetal as well as from 2–10-day-old infantile rats were cultured *in vitro* in the presence of [$^3$H]19-hydroxyandrostenedione and in the presence or absence of LH, FSH or $(Bu)_2$ cAMP, and oestrone and oestradiol formed were determined by double isotopic dilution and recrystallization to constant specific activity. In foetal ovaries, the stimulation factor with FSH was 0.9–1.3, which was considered insignificant in comparison with the 8–13-fold stimulation obtained with $(Bu)_2$ cAMP. At infantile stages, aromatase activity was stimulated 1.3–3.5-fold, which was close to the 3.9-fold stimulation obtained with $(Bu)_2$cAMP. LH was ineffective at both foetal and infantile stages.

## INTRODUCTION

The biosynthesis of oestrogens from androgens is catalysed by an enzyme complex termed aromatase. It has been shown that FSH, but not LH, stimulates aromatase activity in the immature rat ovary [1]. However, from previous work it is not clear whether FSH also stimulates aromatase activity in the ovary during foetal life. In ovaries explanted on day 22 of gestation and cultured *in vitro* for 3 days either in the presence or absence of FSH, the level of aromatase activity was the same, whereas $(Bu)_2$cAMP enhanced it approximately 10-fold [2]. We obtained similar results at earlier stages [3, 4]. However, positive results with FSH have been obtained by other authors. In 16-day-old foetal rat ovaries, FSH was without effect, but in 20-day-old ones, oestrogen production was doubled [5]. In the foetal mouse ovary, oestrogen production could not be detected at 18 days of gestation in the absence of FSH, but in its presence it could be detected even one day earlier [6].

In our previous study [3, 4], aromatase activity has been assessed by determining the conversion rate of [$^3$H]testosterone into [$^3$H]oestrone. (Oestrogen secretion of the foetal rat ovary consists almost exclusively of oestrone [7]). In the present study, [$^3$H]19-hydroxyandrostenedione has been used as the substrate. The 19-hydroxy derivatives are the first intermediates which form from testosterone or androstenedione during aromatization [8], and it was thought that 19-hydroxyandrostenedione was a better precursor than testosterone. 19-Hydroxyandrostenedione has already been used as a substrate in aromatization studies [9, 10]. So, in the present investigation, we aimed at ascertaining whether or not FSH was capable of stimulating the conversion of [$^3$H]19-hydroxyandrostenedione into oestrone in the 18–21-day-old foetal rat ovary in organ culture. For the purpose of comparison, infantile ovaries were included in this study.

## EXPERIMENTAL

### *General outline of the procedure*

Foetal or infantile rat ovaries were cultured *in vitro* in the presence of [$^3$H]19-hydroxyandrostenedione as substrate for oestrogen biosynthesis and in the presence or absence of FSH, LH or $(Bu)_2$cAMP, and $^3$H-labelled oestrone and oestradiol formed were determined by double isotopic dilution and recrystallization to constant specific activity.

### *Organ culture*

Rats of the Wistar strain were used. Ovaries were removed from 18–21-day-old foetuses and from 2–10-day-old infantile rats. They were cut into two or more pieces, according to their size, and cultured *in vitro* in a plastic Petri dish containing 0.1 ml of Medium 199. The liquid was level with a Millipore cellulose ester filter or a Whatman hardened paper filter on which the explants were put. The Petri dishes were placed in an airtight jar, gassed with $O_2$–$CO_2$ (95:5, v/v) and incubated at 37°C for 24 h. After the 24-h-culture period, the media were inspected for infection, one explant was fixed for histological examination, and the Petri dishes were then stored at −20°C until time of analysis.

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*Radioactive precursor, radioactive tracers and gonadotrophins*

[6,7-$^3$H]19-hydroxyandrostenedione in ethanol was from Du Pont de Nemours (NEN Products); its radiochemical purity was 99%, its specific activity 46 Ci/mmol and its concentration 1 mCi/ml or 0.022 $\mu$mol/ml. 1 $\mu$l of this ethanol solution was added to 100 $\mu$l of culture medium, so that the concentration of the precursor in the culture medium was 0.01 mCi/ml or 0.22 nmol/ml (0.22 $\mu$M). This is about half the concentration used by Ritzen *et al.*[9] and Suárez-Quian *et al.*[10], but is still a saturating concentration, when compared to values found in the literature for testosterone [11, 12].

To make correction for analytical losses possible, tracer amounts (approximately 4000 cpm) of (4-$^{14}$C)-labelled oestrone and oestradiol (Amersham), as well as carrier amounts (approximately 100 $\mu$g) of the radioinert compounds, were added to the culture media at the beginning of the analysis.

Ovine FSH (NIADDK-oFSH-17) was used at a concentration of 0.1–20 $\mu$g/ml, bovine LH (NIH-LH-B9) at a concentration of 0.25–10 $\mu$g/ml and $(Bu)_2$cAMP (Sigma) at a 0.5 mM concentration.

*Extraction and chromatography*

The media were thawed and treated with 1 mol/l NaOH to solubilize the explants. The 1 mol/l NaOH was then made 0.4 mol/l and extracted with benzene–petroleum ether (1:1, v/v) to remove neutral steroids. Oestrogens, which remained in the alkaline solution, were methylated with dimethyl sulphate [13]. The oestrogen methyl ethers were chromatographed on a thin layer of silica gel in chloroform–diethyl ether (9:1, v/v). Carriers were viewed in u.v. light, scraped off and eluted. Oestrone methyl ether was further purified by reducing it to oestradiol methyl ether, which was chromatographed in cyclohexane–ethyl acetate (7:3, v/v). Oestradiol methyl ether was also treated with sodium borohydride to remove contaminants. So, both oestrogens were recrystallized as oestradiol methyl ether.

*Recrystallization and radioactivity counting*

After adding 15 mg of non-radioactive oestradiol methyl ether to the last eluate, repeated crystallizations were carried out in methanol–chloroform–petroleum ether. A 1 mg-sample of crystals was taken off after each crystallization for precise weighing on a microbalance. Mother liquors were dried and weighed. Weighed crystals and mother liquors were then dissolved in toluene containing 4 g Omniflour (Du Pont de Nemours) per l, and their radioactivity was measured in a scintillation spectrometer, with window settings avoiding any passage of $^3$H into the $^{14}$C-channel and keeping the injection of $^{14}$C into the $^3$H-channel around 12%. Under these conditions, $^{14}$C was counted with an efficiency of 60% and $^3$H with an efficiency of 33%. The background values were 14 and 10 cpm, respectively. The net $^3$H-counts were obtained by subtracting 12% of the $^{14}$C-counts from the total counts in the $^3$H-channel.

According to the criterion of Axelrod *et al.*[14], specific activity was considered to be constant whenever 3 successive crops of crystals had values within $\pm$5% of the mean, the specific activity of the last mother liquors being close to this mean. The error of weighing was $\leqslant$1%. Counting time was long enough to afford an error $\leqslant$3%. So, the overall error in specific activity determination was $\leqslant$4%.

*Calculations*

Specific activity was determined relative to both $^{14}$C and $^3$H. Knowing the exact amounts of $^{14}$C-labelled tracer and $^3$H-labelled precursor added, the constant specific activity value relative to $^{14}$C permitted the calculation of the recovery percentage, that relative to $^3$H, corrected for the recovery percentage, the calculation of the conversion percentage. Table 1 gives examples of specific activity values and illustrates the way in which the conversion percentage was calculated.

## RESULTS

A preliminary experiment was aimed at verifying whether 19-hydroxyandrostenedione gave higher

Table 1. Examples of recrystallization data for oestrone and oestradiol

| | Oestrone (Foetal 20 days) | | Oestradiol (Infantile 6 days) | |
|---|---|---|---|---|
| | $^{14}$C | $^3$H | $^{14}$C | $^3$H |
| Crystals 1 | **186** | **448** | **161** | **1070** |
| Mother liquors 1 | 220 | 538 | 165 | 1835 |
| Crystals 2 | **184** | **452** | **163** | **1058** |
| Mother liquors 2 | 259 | 624 | 166 | 1462 |
| Crystals 3 | **182** | **444** | **159** | **1043** |
| Mother liquors 3 | 201 | 469 | 165 | 1112 |
| Constant SA | **184** | **448** | **161** | **1057** |
| Constancy % | 0.01 | 0.01 | 0.01 | 0.01 |
| Activity found ($\times$ 15) | 2760 | 6720 | 2415 | 15,855 |
| Starting activity | 4320 | 640,000 | 3410 | 693,000 |
| Recovery % | 64 | — | 71 | — |
| Activity calculated | — | 10,500 | — | 22,330 |
| Conversion % | — | 1.64 | — | 3.22 |

Activity is expressed in cpm, specific activity (SA) of the crystals and mother liquors in cpm/mg. Constant specific activity values, i.e. values which are within $\pm$5% of the average, are in bold type.

Table 2. Conversion of 19-hydroxyandrostenedione into oestrone by foetal and infantile rat ovaries in the presence of LH, FSH or $(Bu)_2$cAMP or in the absence (−) of these substances.

| Age | Foetal 19 days | | | | Foetal 20 days | | Infantile 4 days | | |
|---|---|---|---|---|---|---|---|---|---|
| Number of ovaries | 5 | | | | 7 | | 1 | | |
| Filter type | Whatman | | | | Millipore | | Whatman | | |
| | — | 3FSH | 3LH | $(Bu)_2$ | — | 3FSH | — | 2FSH | 1LH |
| Conversion % | 2.09 | 2.27 | 2.15 | 27 | 1.94 | 2.07 | 4.71 | 8.17 | 4.89 |
| Stimulation factor ($x$) | — | 1.09 | 1.03 | 12.9 | — | 1.07 | — | 1.73 | 1.04 |

The concentration of $(Bu)_2$cAMP was 0.5 mM, that of LH and FSH is indicated in $\mu$g/ml before the initials.

conversion percentages than testosterone. Ten ovaries from 18-day-old foetuses were cultured in the presence of either testosterone or 19-hydroxyandrostenedione. The conversion percentage of the latter to oestrone was 2.5-fold that of the former (2.2 vs 0.8%). So, planned experiments could be started.

On histological examination, explants cultured on both filter types were in good condition, although on Millipore filters they were judged in slightly better condition than on Whatman filters. On the contrary, with Whatman filters conversion percentages were slightly higher than with Millipore filters.

Examples of conversion percentages and stimulation coefficients are given in Table 2. Table 3 concentrates on the stimulation factor. At foetal stages the stimulation of aromatase activity by $(Bu)_2$cAMP was very intense, whereas with FSH the stimulation factor did not exceed 1.3, which is not considered significantly different from 1.0. LH had no effect. At infantile stages, the stimulation of aromatase activity by FSH was obvious. A statistical analysis of the data was virtually impossible, because FSH was used at widely varying concentrations and because of the small number of cases at each stage. None the less, neglecting this difficulty, if one compares the means of the stimulation coefficient of oestrone synthesis at foetal and infantile stages (1.09 vs 2.05) using Student's $t$-test for small samples, one sees that the difference is highly significant. A concentration of 1–5 $\mu$l/ml elicited maximum action, which came close to that of 0.5 mM $(Bu)_2$cAMP. At 5–10 days, the stimulation of oestradiol synthesis by FSH was greater than that of oestrone synthesis. At 10 days, the action of exogenous FSH seems to vanish, in accord with the results of Pelloux *et al.*[16] probably because there is more endogenous FSH being secreted[17]. LH had no effect.

Table 3. Conversion of 19-hydroxyandrostenedione into oestrone and oestradiol by foetal (F) and infantile (I) rat ovaries. Stimulation factor for oestrone ($E_1$), oestradiol ($E_2$) and their sum ($E_1 + E_2$)

| | FSH | | | | LH | | | | $(Bu)_2$cAMP | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Age | c | $E_1$ | $E_2$ | $E1 + E_2$ | c | $E_1$ | $E_2$ | $E1 + E_2$ | $E_1$ | $E_2$ | $E1 + E_2$ |
| F 18 days | 0.5 | 1.03 | | | | | | | | | |
| | 2.5 | 1.11 | | | | | | | | | |
| | 3 | 1.19 | | | | | | | 8.4 | | |
| F 19 days | 1 | 1.08 | | | | | | | | | |
| | 3 | 1.09 | | | 3 | 1.03 | | | 12.9 | | |
| F 20 days | 1 | 1.29 | | | | | | | | | |
| | 1 | 1.15 | | | 1 | 1.01 | | | | | |
| | 3 | 1.07 | | | | | | | | | |
| | 6 | 1.11 | | | 6 | 0.99 | | | 12.6 | | |
| F 21 days | 0.25 | 1.05 | | | | | | | | | |
| | 2.5 | 0.89 | | | 2.5 | 1.02 | | | | | |
| | 8 | 1.04 | | | | | | | | | |
| I 2 days | 1 | 2.0 | | | | | | | | | |
| | 20 | 2.0 | | | 4 | 0.88 | | | | | |
| I 3 days | 0.1 | 1.32 | | | | | | | | | |
| | 0.5 | 1.56 | | | | | | | | | |
| I 4 days | 2 | 1.73 | | | 1 | 1.04 | | | | | |
| | 5 | 2.2 | | | | | | | | | |
| I 5 days | 0.1 | 1.35 | 1.65 | 1.44 | | | | | | | |
| | 1 | 2.4 | 5.0 | 3.0 | | | | | | | |
| | 1 | 2.2 | 5.6 | 2.9 | | | | | 2.5 | 5.9 | 3.2 |
| | 5 | 3.3 | 4.4 | 3.5 | 10 | 1.02 | 1.32 | 1.11 | 3.5 | 5.9 | 3.9 |
| I 6 days | 0.4 | 1.78 | 2.8 | 2.2 | | | | | | | |
| | 0.8 | 2.7 | 4.8 | 3.6 | | | | | | | |
| | 1.6 | 3.0 | 3.6 | 3.3 | | | | | | | |
| | 8 | 2.9 | 4.1 | 3.4 | | | | | | | |
| I 10 days | 1 | 1.13 | 1.28 | 1.23 | 0.05 | 1.63 | 0.77 | 1.04 | | | |
| | 10 | 1.15 | 1.33 | 1.28 | 0.5 | 1.14 | 0.75 | 0.87 | | | |

At foetal stages, the number of ovaries varied between 5 and 10, at infantile stages between 1 and 2. The concentration of $(Bu)_2$cAMP was throughout 0.5 mM. The concentration of LH and FSH is expressed in $\mu$g/ml and is given under c. Oestradiol was not determined at stages earlier than 5 days after birth, when it represented approximately one third of oestrone. At 10 days, the amount of oestradiol formed was about twice that of oestrone.

## DISCUSSION

The aim of the present study was to ascertain whether FSH stimulated aromatase activity in the foetal rat ovary by measuring the conversion rate of [$^3$H]19-hydroxyandrostenedione into oestrone, which is the main oestrogen formed. The results obtained do not support this hypothesis, as was the case in previous studies using testosterone as the precursor [3, 4]. This negative result is consistent with the absence of FSH receptors in the foetal rat ovary. According to Smith-White and Ojeda[15], these receptors appear at 4–5 days after birth. Maybe that they appear 1 or 2 days earlier, so that the stimulative action of FSH from 2 days after birth can be easily explained. The intense action of cAMP on the foetal ovary proves the presence of a cAMP-responsive steroidogenic apparatus even in the absence of FSH receptors. Finally, LH was devoid of action at all stages studied, as in a previous study performed at the stage of 20 days after birth [1].

To conclude, the stimulation of aromatase activity by FSH in the rat ovary has been demonstrated at infantile stages, but not at foetal ones, although cAMP is highly effective.

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