The effects of serum oestrogen-binding components on the unbound oestradiol-17 β fraction in the serum of developing female rats and on inhibition of [3 H]oestradiol uptake by uterine tissue *in vitro**

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Summary. Total oestradiol concentrations in the serum of young female rats were high and decreased after about 21 days of age. High affinity serum oestradiol-binding components (EBP), however, fell steadily from 5 to 23 days of age while the unbound oestradiol-17 β fraction, which was low early in development, increased between 21 and 28 days of age.

Injection (i.v.) of immature (EBP-rich) oestradiol-free serum into 21-day-old female rats led to a decrease in the unbound oestradiol fraction and an increase in serum FSH concentrations. Incubation of uterine tissue with [3 H]oestradiol, with or without the addition of diethylstilboestrol (DES), showed the rate and degree of total and DES-suppressible uptake of [3 H]oestradiol to be greatest from buffer, less from adult (EBP-poor) serum and negligible from immature (EBP-rich) serum; moreover, there was a positive correlation between the degree of uptake of [3 H]oestradiol and the unbound fraction of [3 H]oestradiol in the incubate. It is concluded that, at least in young rats, oestradiol activity depends more on the availability of free oestradiol than on its total plasma concentrations.

Introduction

There is an apparent paradox in the hormone patterns of developing female rats in that concentrations of total serum oestradiol and follicle-stimulating hormone (FSH) are high until about 21 days of age when the levels of both hormones decrease (Meijs-Roelofs, Uilenbroek, de Jong & Welschen, 1973; Weisz & Gunsalus, 1973; Cheng & Johnson, 1974). This apparent lack of negative steroid feedback on FSH output may, however, be accounted for by the presence of a protein (alpha-fetoprotein) in the serum of young rats which has a high affinity for oestrogen and which decreases from birth to about 23 days of age (Nunez *et al.*, 1971; Raynaud, Mercier-Bodard & Baulieu, 1971; Raynaud, 1973). It has been suggested that only the free, non-protein-bound fraction of steroid hormones is physiologically active (Hoffmann, Forbes & Westphal, 1969; Vermeulen & Verdonck, 1972), and therefore the lack of negative feedback in the young rat may be directly attributable to low levels of free oestradiol in the plasma.

In this study total oestradiol concentrations, high-affinity oestradiol-binding components (EBP) and, in particular, the unbound oestradiol-17 β fraction were measured in the blood of developing female rats. We also investigated the effects of i.v. injection of serum from immature (EBP-rich) and adult (EBP-poor) rats on the unbound oestradiol-17 β fraction and plasma FSH concentrations of young female rats and the extent to which these serum types govern the uptake of [3 H]oestradiol by rat uterine tissue *in vitro*.

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Materials and Methods

Wistar rats were purchased from Charles River (U.K.) Ltd or were bred in the animal house from rats of the same stock. Litters were limited to 8 young and weaned at 21 days. Vaginal opening and first oestrus occurred between 37 and 43 days of age. The animals were maintained under automatic lighting (lights on 05:00–19:00 h) and fed on Dixons 41B diet with water always available. Temperature was maintained between 22 and 24°C. Vaginal smears were taken daily from all adult animals and only those exhibiting regular oestrous cycles were selected for study.

The animals were killed by decapitation and trunk blood was collected and allowed to stand overnight at 4°C before separation of serum. Serum samples for estimation of unbound oestradiol were kept at 4°C and assayed within 48 h of collection; those for radioimmunoassay were stored at –20°C until use.

Radioimmunoassays

Serum oestradiol was measured in 1 ml serum or serum dilutions by the radioimmunoassay described by Hotchkiss, Atkinson & Knobil (1971). The antibody was raised in rabbits using the hapten oestradiol-17 β -6-(O-carboxymethyl) oxime-bovine serum albumin. Antibody cross-reactions were calculated as a ratio of the concentration of steroid with respect to oestradiol-17 β ($\times 100$), measured at the 25% bound level. The antibody cross-reactions were 3.6% for oestrone, 0.14% for oestradiol-17 α , 1.1% for oestriol, and <0.01% for testosterone, progesterone and 5 α -dihydrotestosterone. The sensitivity of the assay was 8 pg/ml. The intra- and inter-assay coefficients of variation were 8% ($n = 10$) and 15.5% ($n = 10$) respectively. Serum FSH concentrations were measured by a radioimmunoassay using NIAMDD-RPL-1 standards (see MacKinnon, Mattock & ter Haar, 1976). The intra-assay coefficient of variation at 512 ng/ml was 22% ($n = 10$); the interassay coefficient of variation measured at 445 ng/ml was 8% ($n = 10$). The sensitivity of the assay was 100 ng/ml.

Steroids and reagents. [1,2,6,7-³H]Oestradiol (sp. act. 90–100 Ci/mmol; Radiochemical Centre, Amersham) was stored in redistilled ethanol at –20°C at a concentration of 2×10^{-7} mol/l (4.5×10^7 d.p.m./ml). The radiochemical purity was checked periodically by thin-layer chromatography (see Greenstein, Puig-Duran & Franklin, 1977), and further purification was effected when necessary by gel filtration (Murphy, 1971). Unlabelled steroids were purchased from Sigma (London) and were not purified further before use. Sephadex was purchased from Pharmacia (U.K.) Ltd. All other reagents were of Analar grade.

Measurement of unbound oestradiol in serum

The percentage of free, non-protein-bound oestradiol was measured by steady-state gel filtration as previously described (Greenstein *et al.*, 1977). Although it was necessary to increase the concentration of [³H]oestradiol to 2.4 nmol/l to measure the unbound fraction, it has already been shown (Greenstein *et al.*, 1977) that this procedure does not influence the percentage of unbound oestradiol even when the oestradiol concentration is increased to 10 nmol/l.

The equilibrium dissociation constant (K_d) and the binding capacity of the reaction between [³H]oestradiol and its binding components in rat serum were measured by equilibrium dialysis as previously described (Pearlman & Crpy, 1967; Pearlman, Fong & Tou, 1969). These parameters were calculated from the binding isotherms which were obtained but not shown here. The intra- and inter-assay coefficients of variation of this assay system were 7.8 and 12.9% respectively. The limit of sensitivity was taken as 2×10^{-7} mol/l.

Preparation and injection of serum

Trunk blood was collected after decapitation from groups of 3–5-day-old and adult dioestrous rats to provide EBP-rich and EBP-poor serum respectively. Endogenous oestradiol was removed from samples (3–5 ml) of pooled sera by a two-step procedure. Each sample was incubated with an equal volume of a suspension of 0.0625% (w/v) Dextran T40 (Pharmacia (U.K.) Ltd) and 0.625% (w/v) Norit A charcoal (Sigma, London) in 0.1 mol/l sodium phosphate buffer, pH 7.2, for 30 min at 4°C. After centrifugation the supernatant was dialysed in Visking tubing (9.36/32 in) against 2000 volumes of sodium phosphate buffer (0.01 mol/l, pH 7.2) at 4°C for 24 h. After dialysis, oestradiol concentrations in samples from the contents of the dialysis sac were undetectable by the radioimmunoassay described above. These serum samples were always used within 24 h of collection.

Groups of 16- or 21-day-old rats were injected via the jugular vein with either adult oestradiol-free (EBP-poor) or immature oestradiol-free (EBP-rich) serum, after various volumes of blood had been withdrawn (see Table 1). The rats were killed 24 h later by decapitation and trunk blood samples were obtained for estimation of the unbound oestradiol-17 β fraction and FSH concentrations. The survival time of 24 h was chosen in order to avoid contamination with any FSH in the administered serum which has a half-life in the circulation of 149 min (Bogdanove & Gay, 1969).

Uptake of [^3H]oestradiol by uterine tissue in vitro

The incubation method was based on that described by Jensen, Brecher, Numata, Smith & De Sombre (1975). Uteri were excised from adult rats on the morning of metoestrus and chilled in 0.9% NaCl. Adhering mesentery was trimmed and 5 mm of uterine tissue adjacent to the utero-ovarian junction and the cervix was discarded. Both uterine horns were slit longitudinally and cut into small fragments of approximately 10 mg each. The mean $\pm$ s.e.m. uterine fragment weight was 9.98 ± 0.1 mg ($n = 150$). Fragments of comparable size were prepared from the diaphragm.

Sera from either immature or adult animals were diluted with Krebs–Ringer–Henseleit (KRH) buffer, pH 7.3 (see Jensen *et al.*, 1975), and 10 ml aliquots of the dilute serum or the KRH buffer were placed in glass conical flasks with 5 μl of a solution of [^3H]oestradiol in ethanol to give a final concentration of the hormone of 0.1 nmol/l. Parallel sets of flasks were prepared to which were added 10 μg diethylstilboestrol (Sigma, London) in ethanol to give a final concentration of 10^{-6} mol/l. Ethanol alone (10 μl) was added to the incubates containing only [^3H]oestradiol. One fragment of uterine or diaphragmatic tissue was added to each flask and incubation was allowed to proceed for the required time at 37°C with continuous agitation. Incubation was terminated by removing the tissues, rinsing the fragments three times in ice-cold KRH buffer and blotting on Whatman No. 1 filter paper. The tissues were then placed in pre-weighed polyethylene scintillation vials and the vials were re-weighed. Tissues were solubilized by the addition of 0.2 ml Soluene (Packard) and 0.5 ml distilled water. After heating in a dry-air oven at 60°C for 24 h, the digests were neutralized with 0.1 N-HCl and 10 ml scintillation fluid were added.

Total protein was measured by the method of Lowry, Rosebrough, Farr & Randall (1951). Radioactivity was measured in a Beckman Liquid Scintillation system using a scintillation fluid consisting of 2,5-diphenyloxazole (PPO; 0.3%) and 1,4-bis-2-(1-methyl-5-phenyloxazolyl)-benzene (dimethyl POPOP; 0.03%) in toluene. The efficiency of counting was 46% as determined by using a reference preparation of [^3H]toluene (Radiochemical Centre, Amersham) as internal standard. Extraction of radioactivity into the scintillation fluid was maximal after 6 h.

Differences within groups were analysed by a one-way analysis of variance followed by Duncan's multiple range test (Duncan, 1955). Differences between groups were analysed using a one-tailed Student's *t* test.

Results

Serum oestradiol concentrations during development

The serum concentrations of oestradiol were high during the first 3 weeks of life, especially between Days 10 and 21, but they then decreased (Text-fig. 1). Between 5 and 18 days of age less than 1% of the total oestradiol in the serum was unbound (Text-fig. 1). At about 21 days of age the unbound fraction began to rise and was significantly higher by 23 days ($P < 0.001$). By 28 days of age the values were indistinguishable from those measured in serum from adult animals. The mean concentration of free oestradiol ranged from 0.46 pg/ml in 5-day-old rats to 1.23 pg/ml in 28-day-old rats, but in view of the wide range of total oestradiol concentrations encountered within any one age group, the validity of the estimation is questionable.

Binding affinity and capacity

Scatchard plots obtained after measurement of oestradiol-binding components in rat serum from a 21-day-old rat were consistent with the presence of at least two classes of binding sites (not shown). One component bound oestradiol with high affinity and the dissociation constant (K_d) of the binding reaction was approximately 1.5×10^{-9} mol/l. An apparently unsaturable component of much lower affinity with a K_d of $> 10^{-6}$ mol/l was also present. The high affinity

Text-fig. 1. Mean $\pm$ s.e.m. (4–8 observations) concentrations of high-affinity oestradiol-binding components ($\blacktriangle$) measured by equilibrium dialysis and the total concentrations ($\bullet$) and the % unbound fraction ($\circ$) (mean $\pm$ s.e.m., 4–6 observations) of oestradiol during development of the female rat.

component decreased in abundance from Day 5 after birth to Day 23, and was undetectable in serum from 25-day-old rats (Text-fig. 1), when only the low affinity component was present.

Administration of immature or adult rat serum

Administration of oestradiol-free immature (EBP-rich) serum to 21-day-old females caused a significant reduction in the unbound fraction of oestradiol and a significant rise in serum FSH concentrations, an effect which appeared to be dose dependent (Table 1). Adult (EBP-poor) serum, however, failed to depress the unbound fraction or to elevate serum FSH concentrations in 22-day-old rats (Table 1). In the 16-day-old animals the unbound oestradiol fraction remained low (1%) and serum FSH concentrations were apparently unaffected after administration of adult (EBP-poor) or immature (EBP-rich) serum.

Table 1. Effect of oestradiol-free, immature (EBP-rich) or adult (EBP-poor) serum on the percentage unbound oestradiol and serum FSH concentrations in 16- and 22-day-old female rats

Age of rat (days)	Serum injected (/100 g body wt)	% unbound oestradiol	Conc. FSH (ng/ml)
16	Immature, 0.2 ml	1.05 ± 0.04 (5)	1001 ± 80 (6)
	Adult, 0.2 ml	1.01 ± 0.05 (4)	1063 ± 96 (6)
21	Immature, 0.062 ml	1.88 ± 0.12 (5) ^a	762 ± 71 (6) ^e
	Immature, 0.125 ml	1.44 ± 0.14 (5) ^b	897 ± 62 (6) ^f
	Immature, 0.2 ml	1.03 ± 0.04 (6) ^c	1111 ± 89 (6) ^g
	Adult, 0.2 ml	2.92 ± 0.07 (5) ^d	679 ± 81 (6) ^h

Values are mean ± s.e.m., no. of rats in parentheses.

Significance levels: ^{e,f} $P < 0.05$; ^{a,b}, ^{g,c} $P < 0.025$; ^{b,c}, ^{g,h} $P < 0.01$; ^{a,d}, ^{c,d} $P < 0.001$.

Uterine uptake of [³H]oestradiol

The total uptake of [³H]oestradiol into uterine tissue (Text-fig. 2a) increased significantly ($P < 0.001$) from 2 to 6 h in all incubates, and apparently continued to increase in incubates containing buffer, although not in those containing serum. Total radioactive uptake from buffer was significantly higher than that from adult (EBP-poor) serum ($P < 0.01$) or immature (EBP-rich) serum ($P < 0.001$) at all times tested.

The inclusion of unlabelled diethylstilboestrol in the incubations significantly reduced the uptake of radioactivity by uterine fragments but only after 2 h of incubation (Text-fig. 2b). The uptake of oestradiol which was suppressed by diethylstilboestrol was at all times significantly higher from buffer than from the diluted sera ($P < 0.001$). The suppressible uptake from adult (EBP-poor) serum was, after 2 h incubation, significantly higher ($P < 0.001$) at all times thereafter than that from immature (EBP-rich) serum. The suppressible uptake of radioactivity from the immature (EBP-rich) serum increased from 2 to 6 h ($P < 0.05$) with no further increase thereafter.

The uptake of [³H]oestradiol from buffer into diaphragmatic tissue was tested and apparently only non-suppressible uptake occurred. In the absence and presence of diethylstilboestrol the tissue took up 780 ± 60 and 750 ± 45 (s.e.m.) d.p.m./mg tissue weight respectively.

There was a positive correlation between the mean values of the diethylstilboestrol-suppressible uptake from immature (EBP-rich) and adult (EBP-poor) serum and the corresponding values of the percentage of unbound oestradiol (Text-fig. 2c). The regression equation of the line was: uptake (d.p.m./mg) = $(4.8 \times \% \text{ unbound}) + 50$. This correlation suggests that the [³H]oestradiol uptake was directly proportional to the fraction of unbound oestradiol present in the serum.

Text-fig. 2. Time course of (a) total $[^3\text{H}]$ oestradiol uptake and (b) DES-suppressible uptake into uterine fragments from incubation media containing 2.25% adult or immature female rat serum in KRH buffer, or buffer only ($n = 3$). (c) Correlation between the mean values of DES-suppressible uptake and mean values of percentage unbound oestradiol obtained from dilute sera from adult (O) and immature (●) female rats at comparable dilution levels ($r = 0.97$, $n = 10$, d.f. = 8, $P < 0.001$).

Discussion

The profile of total plasma oestradiol concentrations which were high in the immature rat and decreased markedly at about 21 days is in accord with earlier reports (Meijs-Roelofs *et al.*, 1973; Weisz & Gunsalus, 1973; Cheng & Johnson, 1974) as is the decrease in serum high-affinity oestrogen-binding protein (EBP) concentrations from 5 to 20 days of age and its absence thereafter (Nunez *et al.*, 1971; Raynaud *et al.*, 1971; Raynaud, 1973). Measurement of the unbound fraction of oestradiol, however, has shown that it begins to rise at about 18 days of age when the falling levels of high-affinity EBP appear to have reached a critical concentration. That serum EBP concentrations did not continue to decrease after about 20 days of age, concomitantly with the increase in the unbound oestradiol fraction, may possibly be accounted for by the limit of sensitivity within the EBP assay system. Despite this apparent anomaly it is tempting to suppose that the fall in EBP concentrations and the increase in the free oestradiol fraction are functionally related and this suggestion is reinforced by the observation that injection of oestrogen-free, EBP-rich serum from immature rats into 21-day-old female rats resulted in a reduction of the unbound fraction of serum oestradiol 24 h later. Furthermore, the physiological significance of the unbound oestradiol fraction is indicated by the negative feedback effect that the injection of serum from immature rats had on serum FSH concentrations 24 h later in 22-day-old females. The appearance of unbound oestradiol in the plasma of developing female rats after 21 days of age may likewise be responsible for the fall in serum FSH concentrations which occurs at about this time (MacKinnon *et al.*, 1976). A further indication of the increasing concentrations of free and physiologically active oestradiol in the blood is the increase in weight of the uterus from about 26 days of age which has been reported by Price (1947) and Donovan & O'Keefe (1966).

From our in-vitro studies it is clear that although the presence of adult (EBP-poor) serum or immature (EBP-rich) serum inhibits the uptake of [^3H]oestradiol into uterine tissue when compared with uptake from media containing only buffer, serum from immature rats exerts a more profound inhibition in terms of both degree and rate of uptake. This difference is presumed to be due to the smaller percentage of free oestradiol in immature (EBP-rich) serum because the degree of diethylstilboestrol-suppressible oestradiol uptake can be positively correlated with the fraction of unbound oestradiol in the medium.

Lasnitzki & Franklin (1972) observed a similar inhibition of uptake of [^3H]dihydrotestosterone into prostate slices when adult male serum was included in the incubation medium, and the inhibition was directly proportional to an increase in serum concentration. Burke & Anderson (1972), using purified human sex hormone-binding globulin (SHBG), have likewise observed that the percentage of free, non-protein-bound oestradiol is inversely related to the concentration of SHBG. These findings suggest that a quantitative relationship exists between the plasma level of steroid-binding proteins and the free plasma level of the hormone. Using a constant-flow organ culture system with human prostate explants, purified human serum albumin and SHBG, Mercier-Bodard *et al.* (1976) have shown that both albumin and SHBG decreased the uptake of testosterone by the prostate explants in direct proportion to the fraction of unbound testosterone in the superfusion medium. However, SHBG, but not albumin, altered the metabolism of testosterone in the tissue explants in a manner that was critically dependent on the SHBG concentration in the medium.

The level of binding of steroid hormones in the blood may dictate the amount of steroid available for entry into the brain; a study of the transfer of progesterone, dihydrotestosterone, testosterone, oestradiol, 17-hydroxyprogesterone and cortisol across the blood-CSF barrier in the rhesus monkey has indicated a positive correlation between the levels of unbound hormone in the plasma and the CSF: plasma ratio of that hormone (Marynick, Smith, Ebert & Loriaux, 1977).

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