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Nuclear Binding of Oestradiol-17β and Induction of Protein Synthesis in the Rat Uterus during Postnatal Development

By DALIA SÖMJEN, G. SÖMJEN, R. J. B. KING*, A. M. KAYE and H. R. LINDNER
Department of Biodynamics, Weizmann Institute of Science, Rehovot, Israel

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1. The uterine response to a single injection of oestradiol-17β during postnatal development of the rat was studied with respect to (i) nuclear binding of oestradiol-17β; (ii) induction of the synthesis of a specific cytoplasmic protein ('induced protein' of Gorski); (iii) rate of incorporation of ^{3}H-labelled amino acids into total protein and into nuclear acid-soluble and acid-insoluble protein; and (iv) rate of [^{3}H]thymidine incorporation into DNA. 2. Specific nuclear binding of oestradiol-17β could be demonstrated even at birth. Administration of oestradiol-17β *in vivo* caused a significant increase in the number of nuclear binding sites in rats aged 10 days or older. 3. A rapid method is described for the detection of the 'induced protein', based on cellulose acetate electrophoresis. Induction of this protein could be demonstrated at the age of 10, 15 and 20 days, but not in 5-day-old rats. 4. In 20-day-old rats the rate of ^{3}H-labelled amino acid incorporation into protein increased by 3h after oestradiol administration. Incorporation into the different protein fractions reached peak values asynchronously: at 3–4h for acid-insoluble nuclear protein, at 6h for total protein and at about 12h for acid-soluble protein. 5. Treatment with oestradiol failed to stimulate amino acid incorporation into protein in 5- or 10-day-old rats; at the age of 15 to 30 days the hormone caused a significant increase in incorporation into total protein and into both types of nuclear protein. 6. Since the capacity for nuclear binding of oestradiol and for synthesis of the induced protein is demonstrable in the rat uterus before it acquires the ability to respond to the hormone with enhanced general protein synthesis and DNA synthesis, it appears that nuclear binding and the synthesis of the induced protein may be necessary but not sufficient conditions for the trophic action of oestradiol.

The induction of uterine protein synthesis by oestradiol-17β (for reviews see Gorski *et al.*, 1968; Hamilton, 1968; Mueller *et al.*, 1972) is thought to be mediated by the association of oestradiol-17β with a specific cytoplasmic receptor protein, followed by migration of an oestradiol-17β–protein complex into the nucleus where it combines with an acceptor in the chromatin (Jensen *et al.*, 1969; Shyamala & Gorski, 1969; King & Mainwaring, 1973). Although the molecular events occurring between the chromatin binding of the steroid–receptor complex and the general stimulation of protein synthesis are not yet clear (O'Malley *et al.*, 1972; Baulieu *et al.*, 1972; King & Gordon, 1972), two observations may provide a clue. The earliest effect of oestradiol-17β on protein synthesis is the induction of a specific protein ('induced protein') which can be demonstrated both *in vivo* (Notides & Gorski, 1966) and *in vitro* (Katzenellenbogen & Gorski, 1972) within 1 h after treatment with oestradiol-17β. A correlation between the amount of oestradiol-17β that is bound to receptor and the rate of synthesis of induced protein indicates that the two parameters might be related (Katzenellenbogen & Gorski, 1972). The second observation concerns the stimulation of nuclear non-histone protein synthesis in the uterus, which occurs about 4h after injection of oestradiol-17β in the mouse (Smith *et al.*, 1970) and rat (Sömjen *et al.*, 1972).

Thus in 20-day-old and older rats part of the sequence of oestradiol-17β-induced events appears to be binding of the hormone to receptors, induced protein synthesis, non-histone nuclear protein synthesis and general cellular protein synthesis, followed by DNA synthesis and cell division. We decided to examine whether these responses to the hormone are acquired in similar sequence during the course of postnatal development. Previous studies (Sömjen *et al.*, 1973) have shown that 5- and 10-day-old rats do not respond to oestradiol-17β with an increase in uterine protein content, whereas they do so at the age of 15 days or older. On the other hand, the concentration of the uterine cytoplasmic receptor reaches its peak at the age of 10 days (Clark & Gorski, 1970).

No information has been published on the ontogeny of the nuclear receptor, so it is possible that the above results might be explained by an inability of the

* On leave from the Department of Hormone Biochemistry, Imperial Cancer Research Fund, Lincoln's Inn Fields, London W.C.1, U.K.

cytoplasmic receptor to enter the nucleus. This has been suggested to occur in certain hormone-insensitive mouse mammary tumours (Shyamala, 1972) and in the hypothalamus of the male rat (Clark *et al.*, 1972).

The present paper describes the changes during postnatal development in nuclear oestradiol-17β binding capacity, in induced protein synthesis, and in the extent of stimulation of nuclear and general protein synthesis in the rat uterus. Part of this work has been presented to the 12th annual meeting of the American Society for Cell Biology (Sömjen *et al.*, 1972).

Materials and Methods

Animals

Female rats of the Biodynamics Department colony, derived from Wistar Stock, were used at ages of up to 1 month. The colony was housed in air-conditioned quarters lit from 05.00 to 19.00h and had free access to pelleted food (Purina Laboratory Chow, Ralston Purina Co., St. Louis, Mo., U.S.A.) and water. The day on which pups were found with their mother was designated day 1.

Materials

Oestradiol-17β was a product of Organon, Oss, The Netherlands. For binding experiments, unlabelled oestradiol-17β was obtained from Ikapharm, Ramat Gan, Israel. Diethylstilboestrol was obtained from British Drug Houses Ltd., Poole, Dorset, U.K. ^{3}H-labelled amino acid mixture (reconstituted protein hydrolysate in 0.01M-HCl) at a specific radioactivity of 1mCi/mg was obtained from International Chemical and Nuclear Corp., Irvine, Calif., U.S.A. [^{3}H]Leucine (36Ci/mmol), [^{14}C]leucine (331mCi/mmol), [^{3}H]oestradiol-17β (100Ci/mmol) and [*Me*-^{3}H]thymidine (21.9Ci/mmol) were purchased from The Radiochemical Centre, Amersham, Bucks., U.K.

Dithiothreitol (Cleland's Reagent) was obtained from Calbiochem, Los Angeles, Calif., U.S.A. Cellogel strips of cellulose acetate gel were obtained from Chemetron, Milan, Italy and Ponceau S from Edward Gurr Ltd., London S.W.14, U.K. Enzyme-grade $(NH_4)_2SO_4$ was purchased from Schwartz/Mann, Orangeburg, N.Y., U.S.A. 'Soluene' sample solubilizer and scintillation reagents were products of Packard Instrument Co., Downers Grove, Ill., U.S.A. Bovine serum albumin was obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. and calf thymus DNA was purchased from Worthington Biochemical Corp., Freehold, N.J., U.S.A. Medium 199 in powder form was a product of Grand Island Biochemical Corp., Madison, Wis., U.S.A.

Administration of oestradiol-17β

A stock solution of oestradiol-17β in ethanol at a concentration of 1mg/ml was stored at 4°C and diluted with double-distilled water before use. Control animals were given injections of dilute ethanol. Volumes of 0.5ml or less were injected intraperitoneally with a number 27 needle.

Incorporation of amino acids and of thymidine

For measurement of the incorporation of ^{3}H-labelled amino acids into surviving uteri, groups of three whole uteri of 20-day-old rats, or the equivalent weight of uteri from younger or older animals, were incubated in 1ml of phosphate-buffered saline medium (Dulbecco & Vogt, 1954) with 5μCi of ^{3}H-labelled amino acid mixture for 1h at 37°C. The samples in 50ml polypropylene centrifuge tubes under an atmosphere of O_2+CO_2 (95:5) were agitated in a Dubnoff shaker at a shaking speed of about one stroke/s. [^{3}H]Thymidine was used at a concentration of 3μCi/ml in medium 199. The times given in rate measurements are in all cases the time at which the uteri were removed from the rats. Ages refer always to the day on which oestradiol-17β or control injections were given and therefore not necessarily to the day on which the measurement was made.

Fractionation and analysis of uteri

The organs from each incubation tube were placed in 1ml of buffer (10mM-Tris–HCl, pH7.5, 250mM-sucrose, 5mM-dithiothreitol and 5mM-$MgCl_2$) and homogenized for a few seconds at 1700rev./min in all-glass motor-driven Potter–Elvehjem homogenizers. The homogenates were centrifuged at 700$g_{max.}$ for 5min. The crude nuclear pellet was washed and resuspended in 1ml of the buffer. Samples of the total homogenate and the nuclear fraction were used to measure the amount of radioactivity in material precipitated by 5% (w/v) trichloroacetic acid and the concentration of protein.

For extraction of acid-soluble proteins, the nuclear fraction was made up to 0.25M-HCl, stored overnight at 4°C and centrifuged at 37000$g_{max.}$ for 5min. The precipitate was washed with 0.25M-HCl and the supernatant solution and washings were combined to form the acid-soluble fraction. The acid-insoluble nuclear proteins were suspended in 0.3M-NaOH.

Determination of induced protein

Uteri from control (ethanol-treated) rats were incubated in phosphate-buffered saline medium containing 5μCi of [^{14}C]leucine/ml; uteri from oestradiol-17β-treated rats were incubated in phosphate-buffered saline in the presence of 25μCi of [^{3}H]-leucine/ml.

After incubation for 1h at 37°C, 3–16 uteri (depending on age) were homogenized in 0.5–0.6 ml of 0.05% EDTA (disodium salt) (Barnea & Gorski, 1970) or in 0.25M-sucrose in 50mM-Tris–HCl (pH7.5)–25mM-KCl–5mM-$MgCl_2$ (TKM) medium (Blobel & Potter, 1966). The supernatant solutions of control and oestrogen-treated uteri obtained by centrifugation at 150000$g_{max.}$ in a Spinco ultracentrifuge for 1h at 4°C were mixed and analysed by polyacrylamide-gel electrophoresis as described by DeAngelo & Gorski (1970) or by electrophoresis on Cellogel strips (12cm×2.5cm). Strips were presoaked in the electrophoresis buffer (40mM-sodium barbitone, pH8.6) for at least 10min. The samples (20μl) were applied to the strips and run for 75min at 20V/cm. They were fixed for 5min in 5% (w/v) trichloroacetic acid and then cut into 2mm wide pieces for counting of radioactivity. The ratio of ^3H to ^{14}C radioactivity on each segment was corrected for crossover of ^{14}C counts into the ^3H channel (12–14%). The relative amounts of induced protein were calculated from the ratio of ^3H to ^{14}C as described by Katzenellenbogen & Gorski (1972). When necessary, the cytosol sample was concentrated before electrophoresis, so that the radioactivity of either isotope in the region of the induced protein was more than five times the background radioactivity. Bovine serum albumin was used as a reference marker; strips were fixed and stained with Ponceau S in 5% (w/v) trichloroacetic acid and destained with 5% (v/v) acetic acid.

For protein fractionation, solid $(NH_4)_2SO_4$ (291mg/ml, to reach 50% saturation at 0°C) was added to the combined 150000$g_{max.}$ supernatant fractions. The suspension was centrifuged at 37000$g_{max.}$ for 5min at 2°C and $(NH_4)_2SO_4$ was added to the supernatant solution (194mg/ml, to reach 80% saturation). The precipitates were dissolved in 0.01M-Tris–HCl buffer, pH7.4, and dialysed overnight at 4°C against the same buffer.

Determination of nuclear binding sites for oestradiol-17β

The radiochemical purity of [^3H]oestradiol-17β was tested periodically by paper chromatography and, when found to be less than 95%, the steroid was purified by descending paper chromatography on methanol-washed paper (Schleicher & Schuell no. 2045a) in the system toluene–light petroleum (b.p. 80–100°C)–methanol–water (5:5:7:3, by vol.; Bush, 1952). The exchange-binding method of Anderson *et al.* (1972*b*) was adapted to use with samples of 2mg wet weight.

Half the animals of each age group were given an intraperitoneal injection of oestradiol-17β (approx. 80ng/g body wt.) to cause transfer of the cytoplasmic receptor into the nucleus, and killed 1h later; the other half were killed without this priming injection. Uteri were homogenized in cold 0.25M-sucrose–0.01M-Tris–HCl (pH7.4)–1.5mM-EDTA (disodium salt) (STE buffer) in a Teflon–glass homogenizer at 1700rev./min. For rats of 5–20 days of age, two equal portions of the homogenate of a single uterus were taken, whereas for 1-day-old rats two uteri were necessary for one determination. The homogenates were centrifuged at approx. 14000$g_{max.}$ in a Beckman/Spinco microcentrifuge for 2min (total time). The nuclear–myofibrillar pellets were resuspended and washed twice with 0.4ml of STE buffer. Each time, the pellets were resuspended by shaking in a Beckman/Spinco microshaker for 30s. The washed pellets were incubated for 1h at 37°C in STE buffer containing 13nM-[^3H]oestradiol-17β (10Ci/mmol), with or without the addition of 1μM-diethylstilboestrol.

After incubation, the suspensions were centrifuged at 14000$g_{max.}$ for 2min; the nuclear–myofibrillar pellets were washed twice with 0.4ml of cold STE buffer and then dissolved by addition of 100μl of 0.3M-NaOH. Duplicate portions (25μl each) were taken for both DNA determination (Burton, 1956) and for scintillation counting after collection on glass-fibre filters. The values stated represent the difference in radioactivity between paired samples incubated in the presence and in the absence of diethylstilboestrol.

Anderson *et al.* (1972*b*) presented evidence that this method measures oestradiol-17β-specific nuclear receptor sites. Although they used 700*g* pellets rather than 14000*g* pellets as in our experiments, we obtained identical results with the two types of pellets; washing 14000*g* pellets with 0.1% Triton X-100 did not change the values for nuclear binding sites.

Sucrose-gradient analysis of nuclear oestradiol-17β receptor

Nuclear–myofibrillar pellets were prepared (as described above) from rat uteri and incubated for 1h at 37°C in STE buffer containing 10nM-[^3H]oestradiol (100Ci/mmol), in the presence or absence of 1μM-diethylstilboestrol. The pellets were washed once with 0.4ml of cold STE buffer, resuspended and left in 0.4M-KCl, 10mM-Tris–HCl, pH7.4, 1mM-EDTA (disodium salt) for 30min at 4°C, and centrifuged for 2min at 14000$g_{max.}$. Dextran-coated charcoal [the pellet obtained from the centrifugation for 2min at 14000$g_{max.}$ of 0.4ml of 0.25% charcoal plus 0.0025% dextran in 10mM-Tris–HCl, pH7.4, 1mM-EDTA (disodium salt)] was mixed with the KCl extract to remove unbound oestradiol-17β. After 15min at 4°C, the dextran-coated charcoal was removed by centrifugation for 2min at 14000$g_{max.}$; the supernatant solutions were centrifuged for a further 30min at 150000$g_{max.}$. The resulting high-speed supernatant solutions were applied to 5–20% sucrose gradients

containing 10mM-Tris–HCl, pH7.4, 1mM-EDTA (disodium salt) and 0.4M-KCl and centrifuged at 46000rev./min for approx. 18h at 0°C in the SW56 rotor of a Beckman L2-65B ultracentrifuge.

Determination of protein and DNA

Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as a standard. DNA was determined either by the method of Burton (1956) or by u.v. absorption (Layne, 1957) with calf thymus DNA as a standard.

Liquid-scintillation counting

^{3}H- and ^{14}C-labelled samples collected on Whatman GF/C glass-fibre filters were dried under i.r. lamps and counted for radioactivity in vials containing 5ml of 0.5% 2,5-diphenyloxazole and 0.03% of 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene in toluene. For counting of radioactivity of [^{3}H]-oestradiol or of Cellogel strips, 0.5ml of Soluene was added to each vial. Sufficient counting time was employed to minimize the statistical error to less than 3%.

Results

Rate of incorporation of ^{3}H-labelled amino acids into uterine proteins

Kinetics of response to oestradiol-17β in 20-day-old rats. The time-course of the increase in the incorporation of ^{3}H-labelled amino acids into total uterine protein and into nuclear proteins, in 20-day-old rats killed at different times after injection of 0.5μg of oestradiol-17β, showed an asynchronous response (Fig. 1). The maximum rate of incorporation of amino acids into total proteins was reached between 4 and 6h after oestradiol-17β administration. Incorporation of label into acid-soluble nuclear proteins had increased significantly by 3h after oestradiol-17β administration, reached a maximum at 12h and then decreased. By contrast, incorporation into acid-insoluble nuclear protein increased to a peak at 3–4h and subsequently declined.

The highest rate of DNA synthesis occurred 24h after oestradiol-17β injection (Fig. 1; see Kaye *et al.*, 1972).

Since the maximum change in incorporation into acid-insoluble proteins, and a significant change in incorporation into acid-soluble proteins, occurred 4h after injection of the hormone, this early time was selected for investigating the effect of oestradiol-17β on the rate of protein synthesis in immature rats of different ages.

Response to oestradiol-17β during postnatal development. A single intraperitoneal injection of 0.5μg of oestradiol-17β into rats between the ages of 15 and 30 days resulted within 4h in significant increases in incorporation of ^{3}H-labelled amino acids into total protein and into acid-soluble and acid-insoluble nuclear protein (Fig. 2). No significant stimulation by oestradiol-17β of the incorporation of ^{3}H-labelled amino acids into these proteins was observed in animals aged 5 or 10 days. Likewise, an increase in the total protein content of uteri measured 24h after oestradiol stimulation was previously shown to occur in 15-day-old but not 10-day-old rats (Sömjen *et al.*, 1973).

The maximal response in incorporation into total

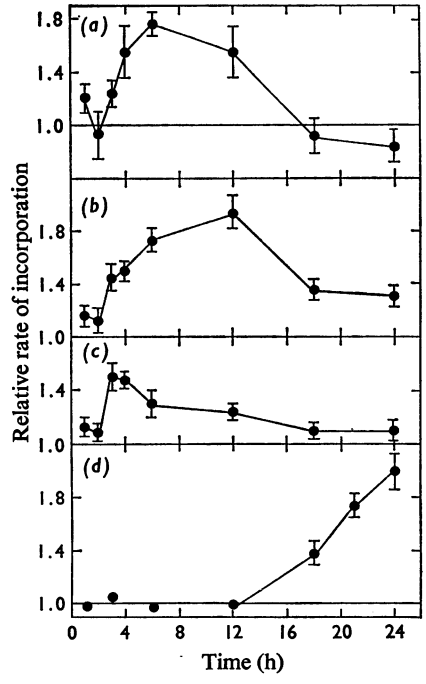

Fig. 1. *Time-course of stimulation of incorporation of ^{3}H-labelled amino acids into protein and of incorporation of [^{3}H]thymidine into DNA in uteri of 20-day-old rats after injection of 0.5μg of oestradiol-17β*

The results are expressed as the ratio between specific radioactivity (c.p.m./μg of DNA or mg of protein) in the uteri of treated animals and of rats given control injections of 0.1% ethanol. The incubation and assay techniques are described in the Materials and Methods section. Vertical bars indicate S.E.M. derived from four to six determinations (protein fractions), each on pooled uteri from three rats, or three to fifteen determinations (DNA), each on one uterus. (*a*) Total proteins; (*b*) acid-soluble proteins; (*c*) acid-insoluble proteins; (*d*) DNA.

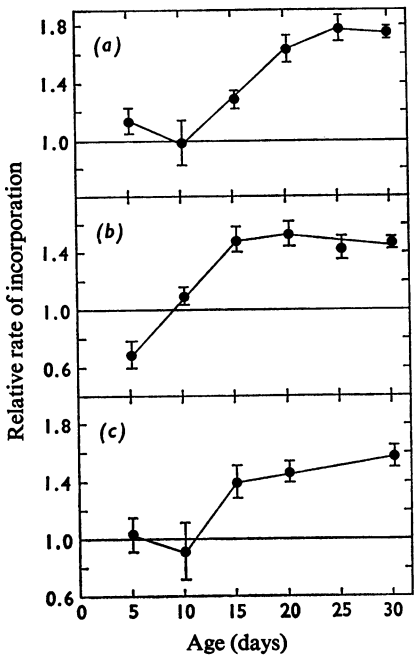

Fig. 2. *Development of uterine responsiveness to stimulation of protein synthesis by oestradiol-17β*

The incorporation of a ^{3}H-labelled amino acid mixture into total protein (*a*), acid-soluble nuclear proteins (*b*) and acid-insoluble nuclear proteins (*c*) was measured 4h after an intraperitoneal injection of oestradiol-17β. Rats 15 days old or older received 0.5μg of oestradiol-17β, 10-day-old animals received 0.4μg and 5-day-old rats received 0.2μg. The results are expressed as the ratio of specific radioactivity (c.p.m./mg of protein) in the uteri of treated animals and of rats given injections of 0.1% ethanol. Vertical bars indicate the S.E.M. of the results from six or more incubations.

protein was found in 20-day-old and older animals, whereas the maximal stimulation by oestrogen of incorporation into the nuclear proteins occurred already in 15-day-old rats. In 5-day-old rats, oestradiol-17β appeared to cause a decrease in the rate of incorporation into acid-soluble nuclear proteins.

Synthesis of induced protein in uteri of immature rats

The Cellogel technique for analysing induced protein synthesis, devised in the course of the present work, afforded a simpler method than the previous use of polyacrylamide gels. It is quicker; the strips are easier to slice than the gels, the proteins are completely solubilized from the strips and more reproducible ^{3}H/^{14}C ratios are obtained; in four determinations of the ratio of ^{3}H/^{14}C of the induced protein peak in 20-day-old rats determined by the Cellogel technique, a value of 2.01±0.12 (S.E.M.) was obtained. It appears to have the additional advantage that fewer other proteins migrate with induced protein in this system, so that the ^{3}H/^{14}C ratio in the induced-protein-bearing segment is higher when the proteins are analysed by Cellogel than by polyacrylamide-gel electrophoresis (Fig. 3). The mean ^{3}H/^{14}C ratio of all the other areas of the electrophoretogram is the same in both methods. Although some ^{14}C-labelled protein is found in the induced-protein region of the electrophoretogram, we do not know whether or not any induced protein is made in the absence of oestradiol-17β. Reversal of the isotopes revealed the same induced-protein peak.

The Cellogel technique permits reproducible and unequivocal demonstration of induction *in vitro* of induced protein in surviving uteri incubated in the presence of 10nM-oestradiol-17β for 1h before incubation with labelled leucine (Fig. 4*a*).

Ontogeny of induced protein

Three experiments with polyacrylamide-gel and two with Cellogel electrophoresis revealed the presence of the induced protein in uteri from 20-, 15- and 10-day-old rats (Figs. 4*a*, *c* and *d*) but failed to reveal any induced protein in uteri from 5-day-old rats (Fig. 4*e*). The rate of synthesis of induced protein at 10 days was about two-thirds of that observed at 20 days.

Induced protein was precipitated by $(NH_4)_2SO_4$ at 50–80% saturation; the precipitate can be redissolved in 0.05% EDTA (disodium salt) and applied to the Cellogel strips. This purification step results in higher ^{3}H/^{14}C ratios in the induced protein region (Fig. 4*b*, ratio of 2.89) than obtained with unfractionated extracts (ratio of 2.01, see the preceding section). Even by using this refinement induced protein could not be detected in uteri from 5-day-old rats (Fig. 4*f*). Although it is not possible to conclude that induced protein cannot be induced in such uteri, we estimate that if made at all it would be at less than 20% of the rate found in 20-day-old animals.

Uterine nuclear binding of oestradiol-17β during postnatal development

There was no significant variation with age between the first and twentieth day after birth (Fig. 5) in the concentration of oestradiol-17β-binding sites in the absence of a priming injection of oestradiol-17β *in vivo*, as determined by the exchange binding technique of Anderson *et al.* (1972*b*). Injection of unlabelled oestradiol-17β, 1h before killing, increased the mean value of nuclear receptor sites in 5-day-old

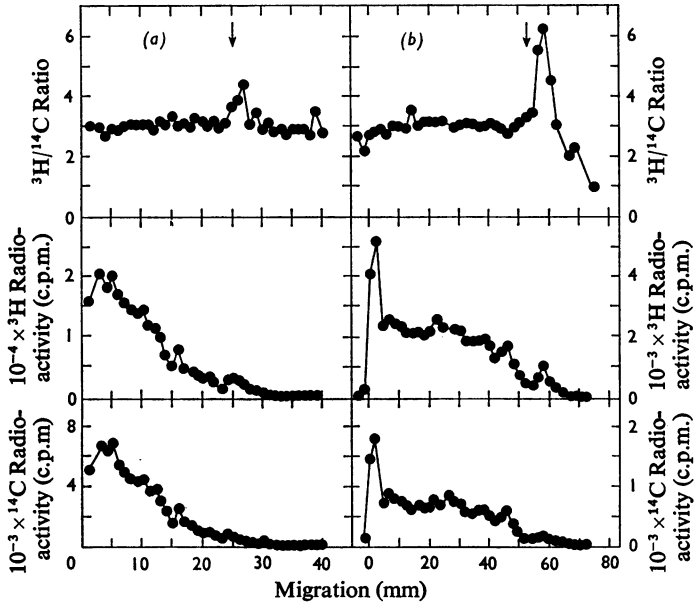

Fig. 3. *Comparison of the electrophoretic migration of uterine soluble proteins in polyacrylamide gels and in Cellogel strips*

Soluble proteins from uteri collected 2h after an injection of 5 μg of oestradiol-17β and incubated for 1 h in either [^{3}H]leucine (oestradiol-treated) or [^{14}C]leucine (controls) were analysed either on polyacrylamide gels (*a*) or on Cellogel strips (*b*), as described in the Materials and Methods section. Arrows indicate the position of bovine serum albumin. The direction of migration was from left to right, towards the positive pole.

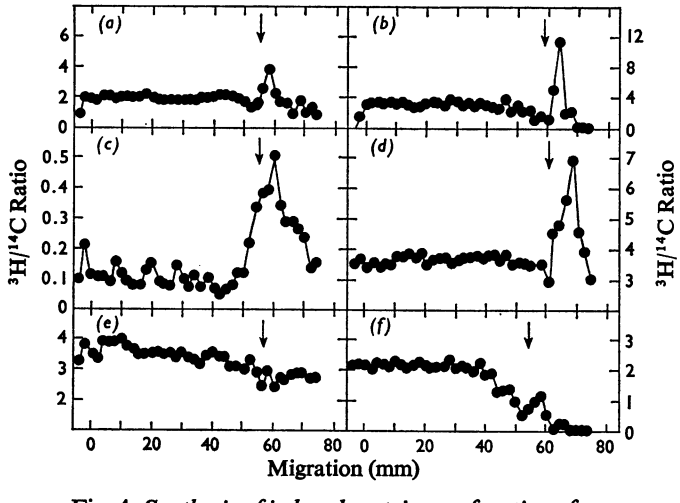

Fig. 4. *Synthesis of induced protein as a function of age*

The electrophoretic distribution on Cellogel strips is shown in uterine protein fractions prepared as described in the Materials and Methods section. (*a*) Soluble proteins from surviving uteri of 20-day-old rats after 1 h incubation in 30 nM-oestradiol-17β in phosphate-buffered saline. (*b*) $(NH_4)_2SO_4$ fraction (50–80% saturation) of soluble proteins from uteri of 20-day-old rats 1 h after injection of 5 μg of oestradiol. Soluble proteins from uteri removed 1 h after injection of (*c*) 5 μg of oestradiol into 15-day-old rats, (*d*) 4 μg of oestradiol into 10-day-old rats and (*e*) 2 μg of oestradiol into 5-day-old rats. (*f*) $(NH_4)_2SO_4$ fraction (50–80% saturation) of soluble proteins from uteri of 5-day-old rats removed 1 h after injection of 2 μg of oestradiol. Arrows indicate the position of bovine serum albumin.

animals; maximal values (10.7fmol of oestradiol-17β/μg of DNA) were reached at 10 days of age.

Sucrose gradient analysis of 0.4M-KCl extracts of uterine nuclei from 1-day-old and from 15-day-old rats treated with oestradiol 1h before killing revealed binding of radioactivity to a component with a sedimentation coefficient of approx. 5S. This binding was abolished in the presence of excess of diethylstilboestrol, confirming the specificity of this assay procedure (Fig. 6).

The results obtained by these two independent methods, namely 'exchange binding' and sucrose-gradient centrifugation, strongly suggest that nuclear receptor is present in the uterus as early as the first day of life. The significance of the limited nuclear-binding capacity observed in unprimed rats requires further investigation.

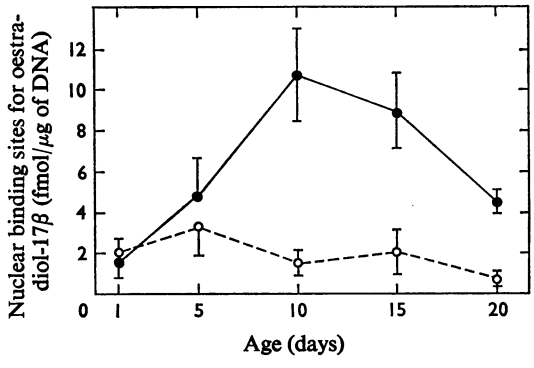

Fig. 5. *Uterine nuclear-binding capacity for oestradiol-17β as a function of age*

Immature rats were given an intraperitoneal injection of oestradiol-17β (approx. 80ng/g body wt.) (●) or 0.15M-NaCl (○) 1h before they were killed. The number of binding sites for oestradiol-17β was calculated after subtraction of the corresponding value for a control incubated with an excess of diethylstilboestrol (as described in the Materials and Methods section). The bars indicate the S.E.M. of five to eight determinations.

Discussion

During the postnatal development of the rat, it was possible to distinguish a series of stages in the response of the uterus to a single intraperitoneal injection of oestradiol-17β. Nuclear binding of [^{3}H]oestradiol is present even at birth. The response to oestradiol-17β by synthesis of a specific 'induced protein' develops between days 5 and 10 after birth, whereas oestradiol stimulation of general protein synthesis and synthesis of nuclear proteins is first demonstrable between days 10 and 15. The capacity to respond to oestradiol with increased DNA synthesis only develops between days 15 and 20 (Kaye *et al.*, 1972). Thus by the age of 20 days the rat uterus has acquired full competence to respond to oestradiol-17β administration by increased growth and cell division.

A similar temporal sequence in the appearance of

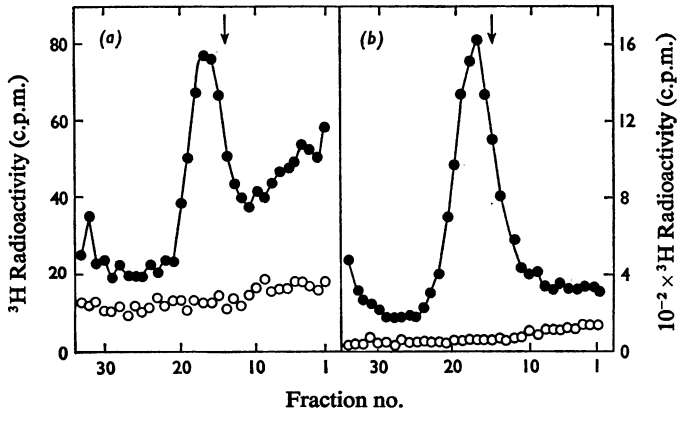

Fig. 6. *Sucrose-gradient analysis of nuclear oestradiol-17β receptor*

Extracts of the nuclear–myofibrillar pellet from uteri of oestradiol-17β-primed 1-day-old rats (eight) (*a*) and 15-day-old rats (two) (*b*), were prepared, incubated in the presence of 10nM-[^{3}H]oestradiol (●) or 10nM-[^{3}H]oestradiol plus 1μM-diethylstilboestrol (○) and analysed on 5–20% sucrose gradients as described in the Materials and Methods section. Fractions of six drops each were collected. The arrows indicate the position of the bovine serum albumin marker. The direction of sedimentation is from right to left.

the various components of the cellular response to the hormone, though on a shorter time-scale, is seen after a single injection of oestradiol into the 20-day-old rat; association of oestradiol-17β with cytoplasmic receptor and transport of this hormone–receptor complex into the nucleus takes place within minutes (Williams & Gorski, 1972). The first protein whose rate of synthesis is stimulated by oestradiol-17β is induced protein, which appears within 1h after injection of the hormone (Barnea & Gorski, 1970). Stimulation of the rate of incorporation of ^{3}H-labelled amino acids into total and nuclear proteins was maximal 4–10h after oestradiol injection in the mouse (Smith *et al.*, 1970) and between 4 and 12h in the rat (Fig. 1). DNA synthesis was first detectable at 18h and reached its maximum at 24h (Fig. 1 and Kaye *et al.*, 1972). Thus there is an apparent physiological recapitulation in the fully competent (20-day-old) rat of the sequence of hormonal effects observed during postnatal acquisition of responsiveness to oestradiol.

Although the present experiments show that amino acid incorporation into total uterine proteins, as well as into acid-soluble and acid-insoluble nuclear proteins, can be stimulated by oestradiol on the 15th but not the 10th day after birth (Fig. 2), the results do not indicate which class of proteins is first to respond. The stimulation of acid-soluble protein (predominantly histone) synthesis in 20-day-old rats preceded that of DNA synthesis by at least 9h (Fig. 1). Such an asynchrony of DNA and histone synthesis has been noted in other experimental systems (Stein & Borun, 1972; Smith *et al.*, 1970; reviewed by Stellwagen & Cole, 1969).

Sequence of responses and mechanism of oestrogen action

The uterine content of cytoplasmic oestradiol-17β receptor rises between days 2 and 5 of life (Clark & Gorski, 1970) and the present results show that the full machinery for transporting the receptor into the nucleus is present at this age. The close parallel between the changes with age in the cellular content of cytoplasmic receptors (Clark & Gorski, 1970) and the nuclear receptor (the present results) suggests that synthesis of receptor and the nuclear transport machinery develop in unison. Although synthesis of induced protein could not be detected until the 10th day of life in spite of the presence of receptor, in older animals (22–25 days) a close correlation exists between induced protein synthesis (Katzenellenbogen & Gorski, 1972), the uterine weight response to oestradiol and receptor occupation (Anderson *et al.*, 1972*a*). Whereas in 5-day-old rats we found the same nuclear binding capacity for oestradiol as in 20-day-old animals, no induced protein was found, although 20% of the amount present at 20 days would have been readily detectable by our assay. It thus appears that in the uterus of the immature rat, the presence of oestradiol as well as of its nuclear receptor is not sufficient to elicit this biosynthetic response. Hence we favour the view that further developmental changes are necessary after the 5th day of life for the uterus to attain complete responsiveness to oestradiol-17β.

An exception to the resemblance between the sequence of acquisition of responsiveness to oestradiol during postnatal development and of its expression in fully competent rats is oestradiol-17β-induced ornithine decarboxylase synthesis. This induction occurs in uteri from 2- and 5-day-old rats (Kaye *et al.*, 1971, 1973), in which no other biosynthetic response to oestrogen is yet observed. This difference may indicate the existence of multiple acceptor sites for the oestradiol–receptor complex on the chromosome (King & Gordon, 1972) or multiple oestradiol-binding receptor molecules (Mueller *et al.*, 1972).

The age-related changes in uterine responsiveness described in the present paper may provide another approach to the understanding of the mechanism of action of oestradiol-17β, namely the attempt to identify the factors essential for a response to the hormone that are lacking in the rat uterus before a particular stage of development.

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