Journal of Steroid Biochemistry, 1971, Vol. 2, pp. 1–10. Pergamon Press. Printed in Great Britain

ASSAY OF PLASMA OESTRADIOL BY COMPETITIVE-BINDING TO UTERINE SUPERNATANT PROTEIN

J. MEŠTER, D. M. ROBERTSON and A. E. KELLIE
The Courtauld Institute of Biochemistry, The Middlesex Hospital Medical School, London W1P 5PR, U.K.

(*Received* 15 *July* 1970)

SUMMARY

A sensitive procedure for the determination of oestradiol in small plasma samples by competitive-binding to uterine supernatant preparations has been described. Although preparations from other species were examined, uterine supernatants from New Zealand White rabbits (2–2·5 kg) were preferred because of the low dissociation constant (K_d) of the equilibrium. The quality of these preparations was dependent on the state of maturity of the animal and was not affected by previous mating. The assay, which was carried out at 30°C, reached equilibrium within 30 min yet gave high precision and low blanks. The method has been applied to human peripheral plasma throughout the menstrual cycle and to rat ovarian plasma throughout the oestrous cycle.

RECOGNITION of the presence of a high-affinity oestrogen receptor in uterine supernatant preparations has made it possible to apply the technique of competitive-binding for the measurement of picogramme amounts of the principal oestrogens. Several groups have applied this principle for the determination of plasma oestradiol levels. The available methods have in common a preliminary extraction of the oestrogens into an organic phase, but they differ in the preparation of the sample for assay. The separation of the oestrogens is an essential step in the purification of the extract and this has been achieved by chromatography on paper [1], on thin-layer alumina [2], on columns of LH 20 Sephadex [3] or Celite [4].

In the competitive assay, the purified non-radioactive oestradiol is made to compete with trace amounts of [^{3}H] oestradiol of high specific activity for limited receptor sites in the uterine supernatant preparation and it is clear from a study of the equilibrium reaction [5, 6] that the principal factors affecting the sensitivity of the assay are the dissociation constant of the equilibrium (K_d), the specific radioactivity of the oestradiol, the volume of the solution in which binding takes place and the proportion of the plasma oestradiol present in the equilibrium solution.

In existing methods, the necessary sensitivity has been achieved by carrying out the assay at 4°C, at which temperature equilibrium is reached in approx. 15 h. The present paper describes a sensitive assay procedure suitable for the determination of oestradiol levels in small plasma samples. The assay, which is carried out at 30°C achieves equilibrium within 30 min yet has good precision and gives low blank values.

MATERIALS

Solvents. Ether, cyclohexane and ethyl acetate, solvents used in the extraction and chromatography of the oestrogens, were of analytical grade and were distilled

before use. Ether was redistilled daily and stored in an opaque bottle. Each solvent was checked for the absence of materials which competed with oestradiol for the binding sites or which otherwise affected the assay.

Buffer solution contained 10 mM-tris-HCl pH 8·0, 1 mM-EDTA and 0·25 M-sucrose. It was also used as a homogenizing medium [7].

Radioactive [2,4,6,7-^3H] oestradiol, S.A. 100 ci/mmol, obtained from The Radiochemical Centre, Amersham, Bucks., U.K., was diluted with benzene to a concentration of 20 ng/ml (stock solution) and was stored at 6°C. Purity was confirmed routinely by t.l.c. on silica gel in two solvent systems, cyclohexane–ethyl acetate (6 : 4, v/v) and chloroform-acetone (9 : 1, v/v); no significant deterioration (< 5%) was observed over a period of 7 months. Standard solutions were prepared by evaporating the appropriate volume of stock solution and redissolving the residue in buffer. They were stable at 6°C for at least 2 weeks. The concentration of oestradiol in the [^{3}H] oestradiol solution was based on the stated specific radioactivity of the [2,4,6,7-^{3}H] oestradiol supplied and to confirm the concentration of this solution, Scatchard plots were obtained using 10 pg [^{3}H] oestradiol plus 0–100 pg oestradiol or 10–100 pg [^{3}H] oestradiol under standard conditions [6]. The two Scatchard plots were identical confirming that the concentrations of oestradiol in the radioactive and non-radioactive solutions were the same and that the dissociation constants of the receptor-[^{3}H] oestradiol and the receptor-oestradiol complexes were identical.

Non-radioactive oestradiol, obtained from Sigma (London) Chemical Co., London S.W. 6, U.K. was chromatographically pure and was stored in stock solution in ethanol (0·4 mg/ml). A series of standard solutions containing 10–100 pg/0·1 ml were prepared by diluting the stock solution with buffer. These standard solutions were stable for at least 2 weeks at 6°C.

Dextran–charcoal suspension, used to remove unbound oestradiol, contained 0·25% Norit A (Hopkin and Williams Ltd., Chadwell Heath, Essex, England) and 0·0025% dextran (The British Drug House Ltd., Poole, England; mol. wt. 60,000–100,000) in buffer.

Scintillation liquids. Samples in aqueous phase (0·05–0·5 ml) were counted in a Packard Model 3375 spectrometer in 10 ml of toluene scintillation liquid (0·01% POPOP, 0·4% PPO); the contents of the vial were mixed on a Whirlimix agitator (Fisons Scientific Co. Ltd., Loughborough, Leics., U.K.) and allowed to stand in the dark for 2 h before counting (efficiency ~ 35%). Alternatively, counting was done in 10 ml of dioxan scintillation liquid (0·025% POPOP, 1% PPO, 10% naphthalene) (efficiency ~ 25%). Both counting procedures gave comparable results.

METHODS

Preparation of the uterine supernatant. Supernatant preparations from rabbit uterus and lamb uterine endometrium have been examined as sources of high-affinity oestradiol receptors. While the rabbit uterus can provide an excellent source of oestrogen-binding sites the quality of the supernatant preparations from this source appeared to be dependent on the state of maturity of the animal. Analysis of data from some 70 rabbits in terms of the concentration of receptor sites per mg wet weight of uterus has shown that a high concentration of oestrogen receptors is found in the uteri of immature rabbits but that the organs are too small for practical purposes (Figs. 1,2). As a compromise, rabbits at puberty (animal weight 2–2·5 kg, uterine wt. 1·5 g) were selected (Figs. 2,3). Mating the animals

Fig. 1. The variation in total uterine content of high-affinity oestradiol receptors in relation to rabbit weight. The content of high-affinity receptors was determined using a method based on Scatchard plot analysis [6]. Rabbit weights 1–2 kg correspond to ages 7–11 weeks.

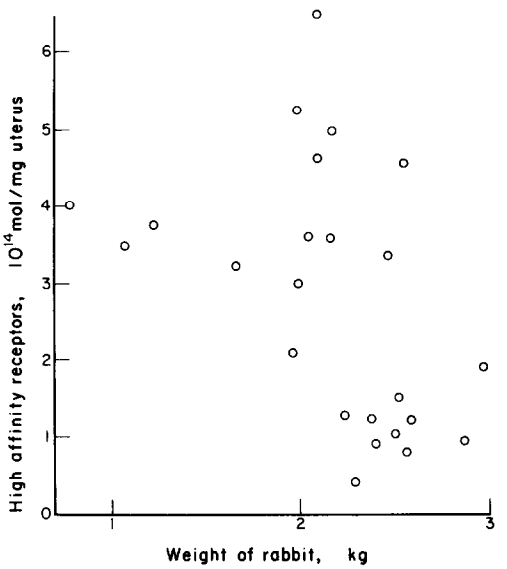

Fig. 2. The variation in the uterine concentration of high-affinity oestradiol receptors in relation to rabbit weight. The concentration was determined as in Fig. 1 on the basis of wet uterine weight.

did not appear to improve the product. The dissociation constant (K_d) of the equilibrium between oestradiol and the high-affinity receptors was of the order of $0{\cdot}8–1{\cdot}3 \times 10^{-10}$M at 30°C and $0{\cdot}2–0{\cdot}9 \times 10^{-10}$M at 4°C. By using older rabbits (2·5–3·5 kg; uterine wt. 1·5–7·5 g) the total yield of high-affinity sites per animal was higher but increasing adulteration of the preparation by low-affinity binding sites increased the apparent K_d and greatly reduced the sensitivity.

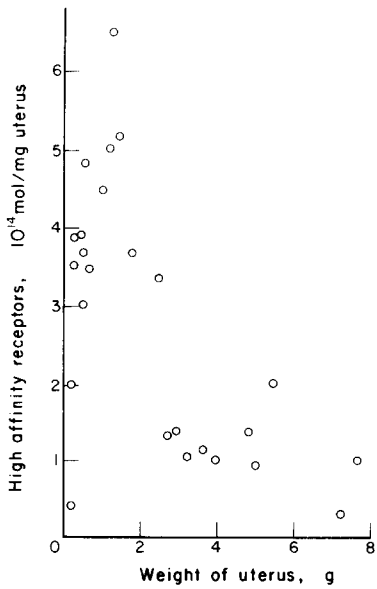

Fig. 3. The variation in uterine concentration of high-affinity oestradiol receptors in relation to uterine weight. The concentration was determined as in Fig. 1 on the basis of wet uterine weight.

Because of their greater size and availability uteri from calf and lamb provide potentially a better source of high-affinity binding protein. Nevertheless because of the higher K_d values obtained from these tissues (Table 1) they have not been extensively used.

The experimental work described in this paper was carried out with uterine supernatant preparations prepared from virgin, pubertal, New Zealand White rabbits (2–2·5 kg). The uteri were excised immediately after sacrificing the animal, were finely chopped with scissors and homogenized at 0°C in 3–10 vol. of buffer (v/w) using a Silverson micro tissue disintegrator (Silverson Machine Ltd., Waterside, Bucks., U.K.) under the precise conditions of 3 successive bursts of 10 sec duration interspersed by cooling periods of 20 sec. The homogenate was

Table 1. Dissociation constants (K_d) of the high-affinity oestradiol-receptor complexes for rabbit and lamb uteri. The values of K_d were obtained by analysis of the Scatchard plot (Mester, Robertson, Feherty and Kellie)[6]. The equilibrium of the binding reaction was reached after incubation for 30 min at 30°C or for 22 h at 4°C

	$K_d \times 10^{10}$ [M]	
	30°C	4°C
Rabbit (1–2 kg body wt.)	0·6–0·8	0·2
Rabbit (2–3 kg body wt.)	1·2–1·5	0·8–0·9
Lamb	1·5	0·8

centrifuged at 105,000 $g_{av.}$ for 1 h at 4°C. The supernatant was stored, after dilution, at −10°C in the presence of 0·1 M-mercaptoethanol and under these conditions no significant loss of binding capacity was observed over at least 2 months. A lower concentration of mercaptoethanol (0·01 M) did not prevent loss of binding capacity over this period of time.

Establishment of the optimum binding protein concentration in the assay. Increasing volumes of uterine supernatant fluid (0–50 μl) were transferred to a series of tubes containing 10 pg of [^{3}H] oestradiol in a final volume of 0·2 ml of buffer. A parallel series of tubes containing 10 pg [^{3}H] oestradiol and 100 pg of non-radioactive oestradiol was similarly treated and all tubes were incubated at 30°C for 30 min. Determinations at all levels were made in triplicate. At the end of the incubation period the tubes were cooled in ice and dextran-charcoal suspension (0·5 ml) was added to each to remove unbound oestradiol. The contents of the tubes were mixed and centrifuged at 800 $g_{av.}$ for 5 min. The radioactivity in the centrifuge supernatant (bound oestradiol) was measured by scintillation counting. A portion of the original solution containing 10 pg [^{3}H] oestradiol was similarly counted. The results are expressed graphically in Fig. 4 in terms of percentage

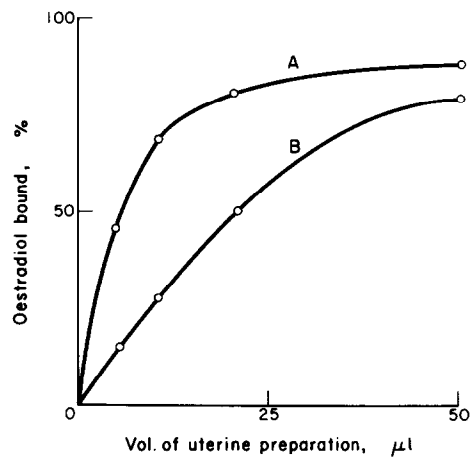

Fig. 4. The percentage binding of oestradiol in relation to increasing amounts of rabbit uterine supernatant. Concentrations of oestradiol in the reaction solution: Curve A. 10 pg [^{3}H]oestradiol/0·3 ml; Curve B. 10 pg [^{3}H]oestradiol plus 100 pg oestradiol/0·3 ml. Assay conditions as given in text. The rabbit uterine preparation (10μl) contained 7×10^{-14}mol of high-affinity receptor sites.

oestradiol bound by increasing amounts of uterine supernatant preparation. The change in percentage binding produced by 100 pg of non-radioactive oestradiol is represented by the vertical distance between the two curves and this graph indicates the optimum conditions of assay in the chosen range of oestradiol concentration. Thus using 10 μl of supernatant preparation in a total volume of 200 μl, 100 pg of non-radioactive oestradiol will produce a change in percentage binding from 66–27%. This information may be used to calculate what dilution of the original supernatant preparation with buffer-mercaptoethanol solution will provide the optimum amount of receptor protein in 100 μl. The amount of uterine super-

natant preparation used in routine assays contained approximately $5–7 \times 10^{-14}$mol of high-affinity receptors per 100 μl. The concentration of high-affinity receptors was determined as described previously [6].

Assay of plasma oestradiol levels

Blood samples collected in heparin were centrifuged immediately after collection and the plasma was stored for short periods at − 10°C. For the assay, the plasma (1 ml in duplicate) was transferred to conical tubes containing 20 pg [^{3}H] oestradiol (16,000 d.p.m.) and was allowed to equilibrate at room temperature for 10 min. An ether extract (9 ml) was prepared and evaporated to dryness under nitrogen.

Thin-layer chromatography. Silica gel, without binder, Merck HR puriss. (E. Merck A.-G., Darmstadt, Germany) was washed extensively with ether (10 vol. v/w), with ethanol (1 vol. v/w) and with water (2 × 10 vol. glass-distilled water) before preparation of the plates. Thin-layer plates (20 × 10 cm) prepared at 0·25 mm thickness were dried at 110°C for 1 h and were stored in an air-tight desiccator. The samples were transferred to tracks 1·15 cm wide cut out on the plates leaving at least 0·1 cm between parallel tracks and at least 0·5 cm at the parallel edges of the plate. Transfer of the extracts was made in ether (4 drops) applied 4 cm from one end of each track and using a further 2 drops of ether to transfer any residue. A marker dye (a slow-moving component of 4-amino-1-methylaminoanthroquinone, F 11 [8], supplied by Imperial Chemical Ind., Ltd., U.K.) was applied to the edge of the tracks and to the central track of each plate. In this system oestrone, oestradiol and oestriol were separated from each other and the mauve marker dye moved in close proximity to oestradiol (R_F values: oestrone 0·60; oestradiol 0·44; oestriol 0·08; mauve marker dye 0·37). On the basis of the position of the marker dye the position of oestradiol on the tracks of the sample plates was determined and sections of track 2·5 cm long were isolated. The silica from each section was scraped into a tube and extracted with ether (2–2·5 ml); the extracts were evaporated to dryness at 45°C and the residues were dissolved in buffer (0·1 ml) by agitation. The recovery of oestradiol at this stage was 40–50%.

Assay of oestradiol by competitive binding. Unknown amounts of oestradiol were determined by comparison with a calibration graph prepared simultaneously from standards containing 10 pg [^{3}H]oestradiol (= 50% of 20 pg) and 10–100 pg oestradiol in triplicate. Tubes containing purified plasma extracts or standards, dissolved in buffer (0·1 ml), were cooled in an ice-bath and to each diluted rabbit uterine supernatant solution (0·1 ml) was added. The tubes were incubated at 30°C for 30 min and were again cooled in an ice-bath. One quarter of the contents of each tube (50 μl) was transferred to a counting vial to determine the recovery of the internal standard and to calculate the total radioactivity in the equilibrium mixture (r d.p.m.). To the remaining three-quarters (0·15 ml) dextran-charcoal suspension (0·5 ml) was added to remove unbound oestradiol; the contents of the tubes were mixed and centrifuged at 80 $\mathbf{g}_{av.}$ for 5 min. Counting of the centrifuge supernatant layer (0·5 ml) enabled the bound radioactivity to be determined (b d.p.m.) and made it possible to calculate the percentage of the radioactivity bound in each tube. The total number of counts added to each tube (t d.p.m.) was determined by counting 20 pg of [^{3}H]oestradiol.

$$\% \text{ Recovery of oestradiol} = \frac{4r}{t} \times 100 \ (\sim 50\%)$$

$$\% \text{ Bound} = \frac{b \times 0{\cdot}65 \times 4}{4r \times 0{\cdot}5 \times 3} \times 100 = 43{\cdot}3 \times \frac{b}{r}.$$

The calibration curve was constructed by plotting the percentage of radioactivity bound against the standard amounts of oestradiol, and the unknown values were determined by reference to this curve (Fig. 5). By this method the total amount

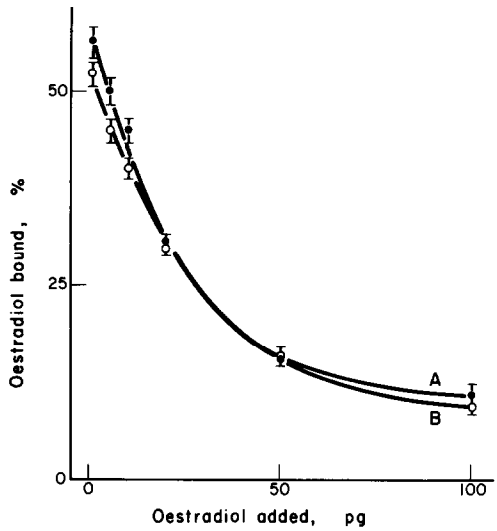

Fig. 5. Calibration curve for oestradiol assay. Curve A(●): Incubation at 4°C for 22 h. Curve B(○): Incubation at 30°C for 0·5 h. The range of experimental points shown indicates the standard deviation of the results of 5 replicate determinations. Assay conditions as described in the text.

of oestradiol in the assay sample was measured and the [^{3}H] oestradiol internal standard was subtracted to give the value of the unknown. This was possible as the concentration of oestradiol in the radioactive oestradiol solution was accurately known and the binding characteristics (K_d) of the radioactive oestradiol and non-radioactive oestradiol were the same.

To ensure that the silica gel did not contain significant amounts of contaminants to inhibit the binding reaction, silica gel (2·5 cm) in the oestradiol position from a track with no sample applied was extracted with ether, the ether extract was evaporated to dryness and the residue taken up in buffer (0·1 ml) containing 10 pg [^{3}H]oestradiol (= 50% of 20 pg). Two of 7 tracks on each plate were used in this way for the measurement of blank values arising from trace contaminants on the silica. The extracts were treated in the same manner as the unknown and standard samples. The accuracy and inherent blank value of the assay were studied by measuring 20 pg [^{3}H]oestradiol and 0–100 pg oestradiol added to buffer and to plasma from ovariectomized rats (Fig. 6).

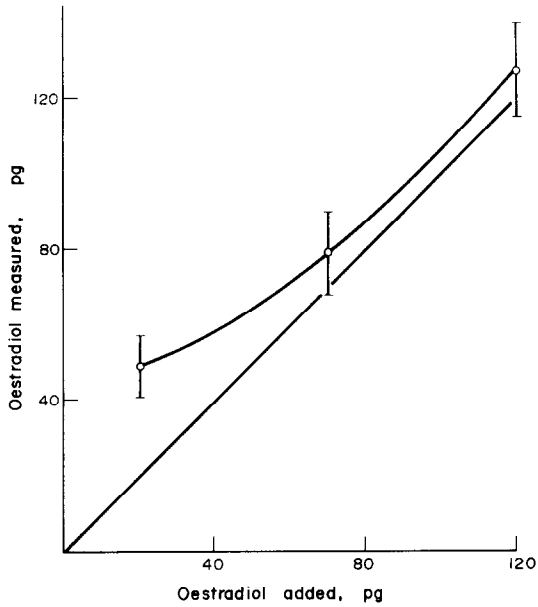

Fig. 6. Determination of standard amounts of oestradiol added to ovariectomized rat plasma or to buffer. The figure illustrates the loss of accuracy at concentrations below 70 pg/ml (equivalent to 5 ng/100 ml). The determination of oestradiol in samples of plasma or buffer was performed as described in the text. The values shown include 20 pg of [^{3}H] oestradiol added as internal standard. Each value is the mean of 5 replicate determinations ± 1 S.D.

RESULTS AND DISCUSSION

The calibration curve obtained at 30°C differed only slightly from that at 4°C and 22 h (Fig. 5). Amounts greater than 10 pg oestradiol in the reaction mixture could be determined by using the calibration curve at 30°C and as the recovery of oestradiol through the procedure was 40–50%, plasma concentrations of the order of 5 ng/100 ml or more could be determined using 1 ml plasma samples. When plasma oestradiol levels exceeded 25 ng/100 ml a proportionately smaller sample was used.

The precision of the method has been examined by carrying out replicate determinations on samples prepared by adding 20 pg[^{3}H]oestradiol alone and with 50 and 100 pg oestradiol to buffer and to ovariectomized rat plasma (1 ml). The results of these determinations (Fig. 6) show that the method was accurate at concentrations of 70 pg per sample (corresponding to 5 ng oestradiol/100 ml plasma) or more.

The blank values observed at lower oestradiol levels are due either to small amounts of competing contaminants in the solvents or silica gel or to environmental factors causing decomposition of oestradiol during thin-layer chromatography on silica gel [9]. The contribution to the blank value introduced by the contaminants was not greater than the equivalent of 10 pg of oestradiol per sample (corresponding to 1 ng/100 ml plasma). During thin-layer chromatography approximately 30% of the [^{3}H]oestradiol was consistently lost. This is in agreement with Coyutupa

et al.[9] who found that a considerable proportion of oestradiol applied to thin-layer plates was decomposed during chromatography into non-extractable products. It appears that a portion of these decomposition products is extracted by ether but does not bind to the receptor sites in the final assay solution, thus leading to overestimation of the oestradiol in the sample. It is likely that the major part of the blank value at low oestradiol levels is due to the decomposition of oestradiol rather than to the presence of contaminants in the solvents or silica gel.

Application of the assay method

The method has been employed to measure changes in peripheral plasma oestradiol concentration throughout the menstrual cycle (Fig. 7). These values agree well with concentrations reported by Korenman, Perrin and McCallum[10] using a similar competitive-binding assay and by Mikhail, Wu, Ferin and Vande Wiele [3] using a radio-immunological assay.

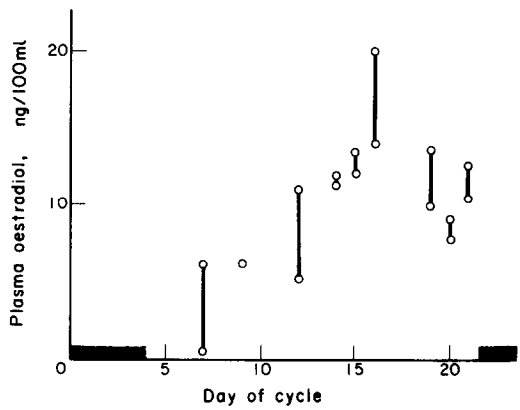

Fig. 7. The variation of plasma oestradiol concentration through the menstrual cycle. Each experimental point was obtained from a single processed plasma sample. Duplicates are joined by vertical lines. ■ indicates menstruation.

The method has also been applied to measure the ovarian secretion of oestradiol at various stages throughout the oestrous cycle of the rat (Fig. 8). Blood samples were collected over 30 min from the left renal vein by a modification of a method described by Telegdy and Endroczi[11]. The amounts found show correspondence with those measured by biological assay[12] and the pattern of secretion is similar to that observed by Hori, Ide and Miyake[13] who measured the total oestrogenic activity of rat ovarian blood.

This method for the determination of plasma oestradiol concentrations has several advantages. The use of the internal standard [^{3}H]oestradiol as the tracer in the final reaction mixture eliminates a second addition of radioactive material, simplifies the calculation of percentage oestradiol bound by the uterine supernatant receptor protein and enables the total amount of purified oestradiol to be included in the final assay solution. By inclusion of the tracer [^{3}H]oestradiol in the whole purification procedure the blank value of the method allowed for possible decomposition of oestradiol. No correction for blank values was necessary at oestradiol levels above 5 ng/100 ml plasma.

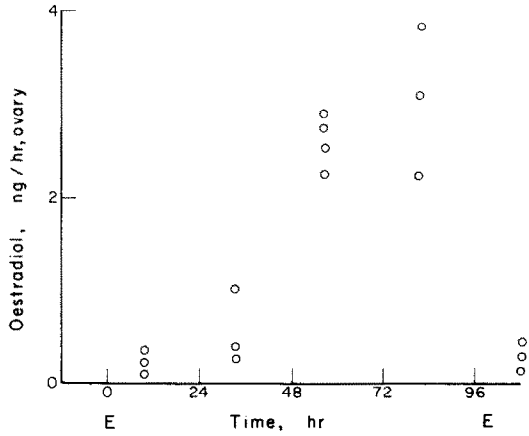

Fig. 8. The ovarian secretion of oestradiol during the rat oestrous cycle. Each experimental point was obtained from an individual rat plasma as described in the text. E = oestrus.

ACKNOWLEDGMENTS

The authors J.M. and D.M.R. were supported by grants from the Medical Research Council. The interest and support of Professor R.H.S. Thompson in this work is gratefully acknowledged, and also the technical assistance of Miss J. Lumbers.

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