The kinetics of estrogen binding to rat α-fetoprotein[1,2]

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Summary. Fluid obtained from rat fetuses was utilized to characterize the affinity, number of binding sites, and the association and dissociation rate kinetics of the binding of estradiol and estrone to AFP. Statistical analysis demonstrated no differences when the values for the AFP-estradiol interaction were compared with those obtained for the AFP-estrone interaction. These data demonstrate that rat AFP specifically binds estradiol and estrone with a high capacity, high affinity, and similar binding kinetics.

In 1971 Soloff et al.[4] demonstrated that pregnant rat plasma contained a unique component which bound estrone and estradiol-17β and provided evidence that the estrogen-binding moiety in the plasma was distinct from that of rat uterine cytosol with respect to ligand specificity. Similar estrogen binding characteristics were later shown to be associated with rat amniotic fluid[5]. Additional evidence has shown that the estrogen binding moiety in these fluids is α-fetoprotein (AFP)[5-9]. In this report, we investigated the estrogen binding characteristics of rat AFP and examined the kinetics of this binding.

Materials and methods. Sprague-Dawley rat fetuses, with amnions intact, were obtained at 17–19 days gestation by Caesarean section. The fetuses were coarsely minced in their own amniotic fluid on ice. The preparation was centrifuged at 25,000 × g for 30 min at 4°C and the supernatant was then filtered through gauze. The resulting filtrate was termed fetal fluid (FF) and was used as the source of AFP as previously described[10]. Experiments were performed to determine the K_a and B_{max} values for the AFP-estrogen interaction as described elsewhere[11,12]. The data obtained from these experiments were then analyzed by the methods of Scatchard[13].

Reactions for the determination of k_{+1} were performed on ice in 0.1 M phosphate buffer, pH 8.2 containing 1.0 mM β-mercaptoethanol. At time zero, saturating amounts (1–2 nM) of either [^{3}H] estradiol-17β or [^{3}H] estrone were added to tubes containing 1 µg FF in a final volume of 1.0 ml. At intervals thereafter, 1.0 ml charcoal was added to each tube and the samples were allowed to incubate for 15 sec on ice. The bound radioactivity (supernatants) was separated immediately from the free radioactivity associated with charcoal by a Millipore 3025 sampling manifold (Millipore Corporation, Bedford, MA) and Millipore type HA filters (0.45 µm pore size), which were presoaked in phosphate buffer. The filtrates (bound steroid) were collected directly into scintillation vials while the charcoal precipitates (free steroid) were retarded on the filters. The entire sample was always filtered in less than 5 sec; this procedure enabled simultaneous filtering of up to 30 samples. The dissociation of the bound steroid-AFP complex due to the stripping action of charcoal therefore could be kept at a minimum using this rapid procedure.

A modification of the exchange method reported by Korach and Muldoon[14] was utilized to study the dissociation process (k_{-1}) of the steroid-AFP complex. FF was incubated with an excess of either [^{3}H] estradiol-17β or [^{3}H] estrone in phosphate buffer for 1–1.5 h on ice. After equilibriation, 2 1.0-ml aliquots (1 µg FF protein) of the reaction sample were removed, added to charcoal pellets, incubated for 15 sec, and filtered via the Millipore system. These duplicate aliquots were analyzed for zero-time concentration of the bound complex. The remainder of the reaction sample was added to a charcoal pellet, thoroughly mixed for 15 sec, drawn into an ice cold syringe and filtered through a Swinnex-25 filter Unit (Millipore Corporation, Bedford, MA.) containing a Millipore type HA filter. The filtrate was collected directly into a beaker containing a 500-fold molar excess of ice cold unlabeled steroid to prevent, by isotope dilution, reassociation of liberated [^{3}H] steroid with uncomplexed binding sites[14]. At appropriate time intervals, 1.0-ml aliquots (1 µg FF protein) were removed, added to charcoal pellets and quickly vortexed, incubated for 15 sec and then filtered via the Millipore system to separate bound from free. Analysis of the number and types of dissociating systems was performed according to the procedures recommended by Rodbard[15] as described by Korach and Muldoon[14].

Results. The in vitro binding data analyzed by Scatchard plots yielded linear plots indicating a single class of binding sites for estradiol and estrone. The association constants (K_a) at equilibrium were found to be $2.83 \pm 0.78 \times 10^8$ M^{-1} for estradiol and $5.5 \pm 1.01 \times 10^8$ M^{-1} for estrone. The numbers of binding sites (B_{max}/mg FF protein) were found to be 451.2 ± 37.1 10^{-12} moles for estradiol and $520.9 \pm 25.6 \times 10^{-12}$ moles for estrone. Statistical analysis showed no significant differences between either the K_a or the B_{max} values for estradiol and estrone.

Based on the results of the Scatchard analysis, which indicated a single class of high affinity binding sites, it was initially presumed that second-order kinetics (bi-molecular reaction) should adequately describe the reaction. The association reaction was therefore examined as a second-order rate function, as described by Korach and Muldoon[14],

$$2.3/[E]_o - [R]_o \log [E]_t/[R]_t = k_{+1}t + C \quad (1)$$

where $[E]_o$ and $[E]_t$ are the concentrations of free [^{3}H] estrogen at time zero and time t respectively; $[R]_o$ and $[R]_t$ are the concentrations of unbound AFP binding sites at time zero and time t, respectively. The contribution of nonspecific binding to the measured association reaction was studied by preincubating FF with a 500-fold molar excess of unlabeled estrogen prior to the addition of the [^{3}H] estrogen. The unlabeled estrogen successfully competed for [^{3}H] estrogen binding sites thereby decreasing the association rate to an unmeasurable level. As shown in figure 1, a plot of the data according to equation 1 yielded an initial linear increase for both estradiol and estrone; these increases began to plateau between 5–10 min. The slopes from these lines yielded k_{+1} values of $1.38 \pm 0.14 \times 10^6$ M^{-1} sec^{-1} and $1.13 \pm 0.05 \times 10^6$ M^{-1} sec^{-1} for estradiol and estrone, respectively (table). Statistical analysis demonstrated no significant difference in the k_{+1} values between estradiol and estrone.

In theory, the dissociation of a ligand from its binding site should follow first-order kinetics, as described by Korach and Muldoon[14]. However, deviation from a single exponential function could be attributed to the presence of unsaturable nonspecific binding sites. The contribution of this nonspecific binding to the measurement of the dissociation rate would be constant throughout the experiment. Therefore, equation 2 would be applicable[15],

$$[ER]_t = [ER]_o e^{-k-1^t} + N \quad (2)$$

where N is the nonspecific component and may be resolved as the horizontal asymptote of the arithmetic plot (fig. 2). When the data were analyzed by equation 2, a single negative exponential function was obtained (fig. 2, inset) indicating that the binding of estradiol and estrone to AFP could be resolved into a single specific and nonspecific system[14]. The results obtained from the corrected data yielded k_{-1} values of

$3.11 \pm 1.04 \times 10^{-3}$ sec^{-1} and $4.60 \pm 0.71 \times 10^{-3}$ sec^{-1} for estradiol and estrone, respectively (table). No statistical differences were detected in the k_{-1} values between estradiol and esterone. Independent measurements of the equilibrium association constants (K_a) for the estradiol and the estrone-AFP interaction, calculated as the ratio of the rate constants, are represented in the table. These data yield K_a values of $4.83 \pm 1.17 \times 10^8$ M^{-1} and $2.53 \pm 0.51 \times 10^8$ M^{-1} for estradiol and estrone, respectively. These values are statistically equivalent to the values of K_a obtained from the Scatchard analysis (table) of in vitro binding data. The calculated K_a values for estradiol and estrone are also statistically not different from each other.

Discussion. It has previously been shown that charcoal rapidly strips estradiol from AFP binding sites. Payne and Katzenellenbogen[16] reported that the rate of stripping was dependent upon the concentration of charcoal added. Toft and Tomasi[7] demonstrated that 90% of the AFP-estradiol complex became dissociated after 10 min of charcoal incubation and suggested

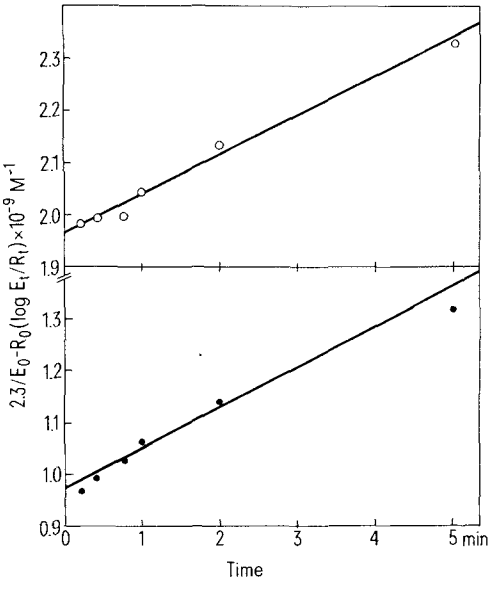

Figure 1. Second-order association rate plot of the interaction between estrogens and rat AFP. The methodology utilized is outlined in the 'Material and methods' section. The association rate constants (k_{+1}) were calculated from the slope of the plots. Upper panel, estradiol (○); lower panel, estrone (●). The above data are representative plots of 2 experiments; the correlation coefficients were > 0.98 in each assay.

Estrogen-AFP interactions: binding site concentrations, association and dissociation rate constants, and equilibrium association constants

Parameter[a]	Estradiol	Estrone
B_{max}	451.2 ± 37.1	520.9 ± 25.6
K_a	2.83 ± 0.78	5.51 ± 1.01
k_{+1}	1.38 ± 0.14	1.13 ± 0.05
k_{-1}	3.11 ± 1.04	4.60 ± 0.71
k_{+1}/k_{-1}	4.83 ± 1.17	2.53 ± 0.51

[a] B_{max} values determined by Scatchard analysis ($\times 10^{-12}$ moles/mg FF protein). K_a values determined by Scatchard analysis ($\times 10^8$ M^{-1}). k_{+1} values determined from association rate studies ($\times 10^6$ M^{-1} sec^{-1}). k_{-1} values determined from dissociation rate studies ($\times 10^{-3}$ sec^{-1}). k_{+1}/k_{-1} expressed as $\times 10^8$ M^{-1}.

that the half-time for hormone displacement was between 2 and 3 min. These findings suggest that the rate of dissociation (k_{-1}) of the AFP-estrogen complex must be very rapid, and that, when using the charcoal absorption technique for measuring estrogen-AFP binding, the incubation time must be brief and precisely timed, and separation of free from bound must be rapid and consistent. We have presented a technique which met all of the above conditions. Utilizing a Millipore filter sampling manifold, charcoal incubation times can be accurately timed and kept constant from sample to sample and assay to assay. Also, due to rapid filtration of the sample by this system (less than 5 sec), free and bound radioactivity can be separated quickly. This technique circumvented any erroneous loss of binding due to inconsistent and prolonged exposure of the AFP-estrogen complex to charcoal.

Since Scatchard analysis of the data showed only a single class of binding sites, the binding appeared to follow second-order kinetics. Significant contribution to the measurement of k_{+1} by low affinity, nonspecific binding was not observed, indicating that the only measurable association rate was accounted for by specific AFP-estrogen interaction[14]. The k_{+1} for AFP-estrogen reported here is 4–5 times greater than values reported for estradiol-receptor interactions[14]. Values obtained for estrone and estradiol were identical and indicate that estrone binds to AFP as rapidly as estradiol.

Measurement of the dissociation rate constant (k_{-1}) allowed the analysis of the high affinity component in the presence of an unsaturable level of nonspecific low affinity binding. The k_{-1} for the AFP-estrogen is 35–240 times greater than the values reported for the dissociation of the estradiol-receptor complex[14]; the values for estrone and estradiol were identical. This k_{-1} value for AFP-estradiol is somewhat different from that reported previously[18]. However, in that report k_{-1} was measured as a function of the dissociation of the bound complex in the presence of charcoal. Because of the detrimental effects of

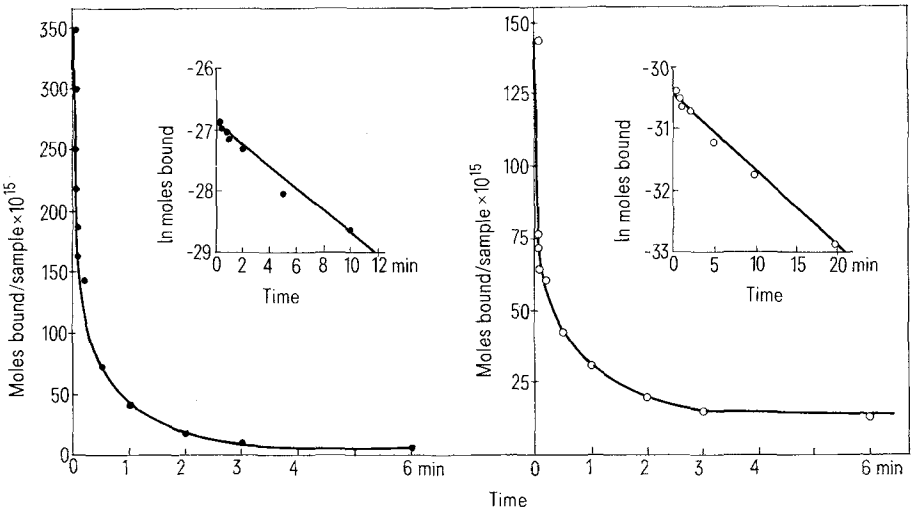

Figure 2. Analysis of dissociation rates for the rat AFP-estrogen complex. The methodology is outlined in 'Materials and methods'. The level of nonspecific binding for the curves was resolved as the horizontal asymptote of the arithmetic plot of the raw data. These data were then corrected for nonspecific binding and replotted as ln moles bound (inset). Left, estrone (●); right, estradiol (○). These data are representative plots for 2 experiments; the correlation coefficients were > 0.98 in each assay.

prolonged exposure of AFP-estrogen complexes to charcoal, we believe that the techniques reported here (Millipore system) allowed a more accurate determination of k_{-1} for AFP.

Calculations of the k_{+1} and k_{-1} values for the AFP-estrogen complex allowed independent determinations of the equilibrium association constants. Ratios of the rate constants yielded K_a values for estradiol and estrone that were statistically identical to the K_a values obtained for each estrogen from Scatchard analysis. Therefore, based on the results of Scatchard analysis, k_{+1} and k_{-1} determinations, and independent K_a calculations, it can be concluded that AFP has binding affinities and capacities that are identical for estradiol and estrone.

1 The abbreviations used are: AFP, α-fetoprotein; FF, fetal fluid; K_a, equilibrium association constant; B_{max}, number of binding sites: k_{+1}, association rate constant; k_{-1}, dissociation rate constant.

2 This work was supported by NSF grant PCM-8109847 and by a Grant-in-Aid of Research from Sigma Xi, The Scientific Research Society.

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0014-4754/84/050503-03$1.50 + 0.20/0

Decidual cell reaction in ovariectomized-adrenalectomized rats

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Summary. Enhancement of decidual cell reaction (DCR) following adrenalectomy was reversed by corticosterone as well as indomethacin. The results suggest the adrenal involvement in DCR through uterine prostaglandin production.

Decidual cell reaction (DCR) can be induced by endometrial traumatization or intrauterine oil instillation in pseudopregnant as well as suitably hormone-primed rodents[3]. Previous studies have suggested the involvement of prostaglandins (PGs) in the induction of DCR[4-7]. Glucocorticoids, known to inhibit synthesis and/or release of PGs in a cell culture system[8,9], prevent estradiol-induced implantation in hypophysectomized pregnant rats[10]. However, possible in vivo effects of corticosteroids on decidualization have not yet been adequately studied. The present experiments reveal the involvement of the adrenals in DCR following instillation of oil into the uterine lumen in adequately sensitized ovariectomized adult rats.

Five groups of female rats of the T strain used in the present study were maintained in a temperature- and light-controlled room (lights on from 05.00 to 19.00 h). The rats were ovariectomized on the day of the first vaginal estrus occurring after day 60 of age. 4 of the 5 groups were adrenalectomized at the time of ovariectomy (AX-OX rats), while the adrenals were left intact in the remaining one (OX rats). All the rats were given s.c. injections of 3 mg progesterone (P) in 0.1 ml sesame oil for 7 consecutive days commencing on the day after the operation. A single s.c. injection of 0.1 μg estradiol-17β (E_2) in 0.05 ml oil was given between 18.00 and 19.00 h on the 3rd day of the P injection period. 16 h after the E_2 injection, 0.1 ml sesame oil was instilled into the right uterine horn from its tubal end as a deciduogenic stimulus. 2 of the 4 AX-OX groups were given s.c. injections of 500 μg corticosterone or 50 μg aldosterone, each dissolved in 0.1 ml oil, twice daily (08.00 and 16.00 h) during the P injection period. The remaining 2 groups receiving no corticoid were provided with 0.9% NaCl as drinking water, instead of tap water. 1 of these 2 groups were given s.c. injections of 1 mg indomethacin in 0.2 ml oil, one 2 h before and the other 6 h after oil instillation. On the day following the last P injection, the animals were sacrificed. The weight of the stimulated horn bearing deciduomata was used to estimate the size of DCR. Data were analyzed by Student's t-test and Fisher's exact probability test.

As shown in the table, both OX and AX-OX rats invariably formed deciduomata. In the AX-OX rats, however, the treated horn bore massive deciduomata along its entire length, 2–3 times heavier than in the OX rats with their adrenals intact ($p < 0.001$). 9 of 10 AX-OX rats formed deciduomata not only in the treated horns but also in the contralateral untreated horns. Thus, the absence of adrenals resulted in a marked increase in uterine responsiveness to the oil instillation stimulus. Under the conditions of the present study, administration of 500 μg corticosterone twice daily during the P injection period significantly reduced the size of DCR in AX-OX rats ($p < 0.001$). There was no difference in mean weight of the treated horns between OX rats with their adrenals and AX-OX rats given corticosterone ($0.2 > p > 0.1$). However, the reduction in number of rats with positive DCR in the untreated horns in latter group was not significant ($p = 0.151$). By contrast, all AX-OX rats given a similar treatment with 50 μg aldosterone produced deciduomata in both treated and untreated horns, incidence and size of DCR being approximately the same as in AX-OX rats receiving no corticoid (size of response: treated horn, $0.6 > p > 0.4$, untreated horn, $0.2 > p > 0.1$; incidence in untreated horns: $p = 0.588$). These results suggest that the increase in DCR following adrenalec-