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CONDITIONS FOR THE MEASUREMENT OF NUCLEAR ESTROGEN RECEPTOR AT LOW TEMPERATURE

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This study was undertaken to determine optimum conditions for the extraction and measurement of uterine nuclear estrogen receptor at low temperature. We measured the influence of glycerol, 0.5 M KCl, 10 mM pyridoxal 5′-phosphate, and 0.5 M NaSCN on the dissociation of estradiol from the receptor at 0°C. The half-time ($t_{1/2}$) of estradiol dissociation from the receptor in 0.5 M KCl nuclear extracts containing 30% glycerol was very slow (greater than 250 h). Exclusion of glycerol from the extract (Tris buffer) increased the dissociation rate ($t_{1/2} = 35$ h). The inhibitory effect of glycerol on estradiol dissociation kinetics predominated over the mild stimulatory effect of KCl; and both effects were independent of the electrical conductivity of the buffer. When pyridoxal phosphate was added to a nuclear KCl extract (barbital buffer) lacking glycerol, dissociation of the estrogen-receptor complex increased such that the $t_{1/2}$ decreased from 20 to 7.6 h; the receptor extracted from nuclei with 10 mM pyridoxal phosphate exhibited these same rapid dissociation kinetics. The $t_{1/2}$ of estradiol dissociation from the receptor at 0°C in the presence of 0.5 M NaSCN was 5.6 h. Following extraction of uterine receptor by KCl, pyridoxal phosphate, or NaSCN, we measured the number of estradiol binding sites at each of two incubation temperatures: 30°C for 1 h and 0°C for 24 h. We verified that unoccupied receptor was measured reliably in KCl extract during incubation at 0°C in the presence of glycerol. Total receptor can be determined using either pyridoxal phosphate extract or NaSCN extract at low temperature. However, the number of sites recovered in either pyridoxal phosphate or NaSCN extract was twice the number obtained with the KCl procedure at elevated temperature. It is noteworthy that pyridoxal phosphate and NaSCN increased the number of sites when added directly to nuclear KCl extract, and the effect of pyridoxal phosphate and NaSCN was reversed by treatment with L-lysine and dialysis against KCl, respectively. Thus, the lower receptor recovery with the KCl procedure is not due to the inability of KCl to extract these sites from the nucleus but rather is ascribable to the assay procedure itself. Although total receptor can be measured at low temperature with either NaSCN or pyridoxal phosphate, the pyridoxal phosphate method can be used to assay nuclear progesterone receptor in the same extract.

Introduction

Recent work in our laboratory has focused on the mechanism of progesterone antagonism of estrogen action in the uterus [1]. We have shown that progesterone treatment rapidly inhibits retention of nuclear estrogen receptor in the hamster [2,3] and rat [4], and we have postulated that this effect depends on a progesterone-induced estrogen receptor-regulatory factor [1,5] which acts preferentially on occupied nuclear estrogen receptor [3,6]. Receptor-regulatory factor is extractable from hamster uterine nuclei with 0.5 M KCl, and its

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activity is detectable in vitro as enhanced inactivation at 37°C of endogenous receptor present in the nuclear extract. The receptor remaining after this incubation has been measured by a subsequent ligand exchange assay at 30°C [2,6,7].

The assay for receptor-regulatory factor described above might be improved if conditions were such that progesterone-independent losses of estradiol-17β binding by receptor were reduced, e.g., by minimizing dissociation of the estrogen-receptor complex during the 37°C incubation or by preventing nonspecific receptor inactivation due to elevated temperature, particularly during the exchange assay. Regarding the dissociation problem, two buffer components which may be important are KCl and glycerol [8]. In addition, in developing an in vitro assay for receptor-regulatory factor, (Okulicz, W.C. and Leavitt, W.W., unpublished data) it is important that we have the ability to achieve ligand exchange under conditions of low temperature and low salt. Studies by others have demonstrated the feasibility of ligand exchange for steroid receptors at low temperature using sodium thiocyanate (NaSCN) [9,10] and mercurial reagents [11–13] to promote dissociation of steroid-receptor complexes. Pyridoxal 5′-phosphate extracts progesterone receptor from hamster uterine nuclei [14], estrogen receptor from rat uterine nuclei [15], and nuclear androgen receptor from mouse kidney [16]. Thus pyridoxal phosphate may serve as a useful reagent for measuring estrogen receptor by ligand exchange in uterine nuclear extracts at low temperature and low ionic strength. The purpose of the present study was first, to investigate the effects of KCl, glycerol, pyridoxal phosphate and NaSCN on the dissociation kinetics of nuclear estrogen receptor, and second, to compare methods for the extraction and measurement of nuclear receptor using KCl, pyridoxal phosphate, and NaSCN.

Materials and Methods

Chemicals and buffers. [2,4,6,7-$^3H_4$]Estradiol-17β was obtained from New England Nuclear Corp., Boston, MA, and stored in ethanol (100 μCi/ml) at −10°C. Radioinert steroids and pyridoxal 5′-phosphate were from Sigma Chemical Co. (St. Louis, MO). Sodium barbital was from Mallinckrodt (St. Louis, MO). All other chemicals were obtained from standard commercial sources and were reagent grade or better.

Saline was buffered with 10 mM Tris-HCl (pH 7.4). Buffer A contained 50 mM Tris-HCl (pH 7.5)/1 mM EDTA/12 mM monothioglycerol; barbital buffer contained 20 mM sodium barbital (pH 8.0)/5 mM dithiothreitol. Buffer B contained 10 mM Tris-HCl (pH 7.5)/1 mM EDTA/1 mM dithiothreitol. The subscript following the letter designating a buffer (e.g., A_{30}) denotes the percentage glycerol (v:v) in the buffer. Dextran-coated charcoal contained 0.5 g Norit A (Sigma) and 50 mg Dextran T70 (Pharmacia, Piscataway, NJ) in 100 ml of 10 mM Tris-HCl (pH 7.5)/1 mM EDTA. Scintillation cocktail was toluene-Triton X-100 (2 : 1, v : v) with 5 g diphenyloxazole and 50 mg 1,4-bis[2-(5-phenyloxazolyl)]benzene per liter.

Tissue preparation. Adult female golden hamsters (Engle Labs, Farmersburg, IN) were housed under controlled conditions with a 14 : 10 photoperiod (lights on from 0500 to 1900 h). The regularity of estrous cycles was established according to the appearance of the postestrous vaginal discharge (morning, cycle day 1). Proestrous animals were killed by cervical dislocation. Uteri were removed rapidly, stripped of fat and mesentery, slit longitudinally, blotted, weighed and placed in ice-cold buffered saline. All subsequent procedures were carried out at 0–3°C unless otherwise indicated. Uteri were minced and then homogenized in buffer using a Polytron Pt-10 homogenizer (Brinkmann Instruments, Westbury, NY) as previously described [17]. The homogenate was centrifuged at $800 \times g$ for 15 min. The supernatant was discarded and the nuclear fraction was washed twice by resuspension with buffer with intervening centrifugations at $800 \times g$ for 15 min. Nuclear receptor was extracted from the washed pellet by resuspension in buffer containing either 0.5 M KCl, 0.5 M NaSCN, or various pyridoxal phosphate concentrations. In experiments involving pyridoxal phosphate, barbital buffer was used to prevent pyridoxal phosphate from interacting with free amino groups on Tris [18]. The nuclear suspension was incubated for 1 h, with mixing at 15 min intervals, and centrifuged at $170\,000 \times g$ for 1 h to remove nuclear debris. The resultant supernatant was the nuclear extract used for the study of nuclear receptor.

Analysis of nuclear estrogen receptor dissociation kinetics. Nuclear extracts were incubated for 1 h at 30°C with 3 nM [^{3}H]estradiol with (non-specific binding) or without (total binding) 750 nM unlabeled estradiol. After cooling to 0°C, dissociation was initiated (zero time) by the addition of 750 nM unlabeled estradiol in ethanol (final ethanol concentration: 1.0%, v : v). To a portion of the extract containing only [^{3}H]estradiol, ethanol which did not contain unlabeled estradiol was added as a control for stability of the estrogen-receptor complex. The extract was apportioned (300 μl) into test tubes and incubated at 0°C. Incubation was terminated by the addition of 700 μl dilute dextran-coated charcoal (2 : 5, buffer : dextran-coated charcoal) followed 10 min later by centrifugation at 1500 × *g* for 4 min. Radioactivity was measured in the supernatant. Specific binding was calculated by subtracting non-specific binding from total binding. Specific binding was then expressed as a percent of zero time. A semilogarithmic plot of percent specific [^{3}H]estradiol binding vs. time resulted in a linear relationship whose slope, calculated by least-squares logarithmic regression, is the dissociation rate constant (K_d). When necessary, percent bound was corrected for receptor loss by calculating percent bound as a function of the stability measurement at that time. The half-time ($t_{1/2}$) of estradiol dissociation from the receptor was then calculated mathematically using the equation:

$$t_{1/2} = \frac{0.693}{2.303\, K_d} \quad (1)$$

Assay of estrogen receptor in nuclear extracts. Receptor concentrations were determined by Scatchard plot analysis of specific binding data [1,19]. Aliquots (300 μl) of nuclear extract were incubated in a total volume of 500 μl with increasing concentrations of [^{3}H]estradiol (0.19–3 nM) for determination of total binding. A parallel set of samples was incubated with unlabeled diethylstilbestrol (0.088–1.4 μM) for determination of non-specific binding. Samples were incubated for 1 h at 30°C or 24 h at 0°C, or both as indicated in the text. Free steroid was then removed from each sample by incubation with 500 μl dextran-coated charcoal for 10 min, followed by centrifugation at 1500 × *g* for 4 min. Radioactivity was counted in the supernatant. Specific binding was calculated (total binding minus non-specific binding) and plotted according to the method of Scatchard [19]. The results were analyzed by least-squares linear regression.

General methods. Supernatants from the dextran-coated charcoal incubations were decanted into scintillation vials. In experiments with pyridoxal phosphate, 50 μl and 120 mM sodium borohydride in H_2O was added to blanch the characteristic yellow color of pyridoxal phosphate in order to prevent color quenching during scintillation counting. Scintillation cocktain (4 ml) was added to each vial and radioactivity was counted at 30% efficiency (determined by the channels ratio method) using a Packard Tri-Carb liquid scintillation spectrometer. Statistical analysis was done by Student's *t*-test.

Results

Both glycerol and KCl alter the dissociation kinetics of nuclear estrogen receptor. The effect of 10 and 30% glycerol on dissociation of labeled estradiol from the receptor is presented in Fig. 1A. At 0°C, the dissociation rate was 3-times faster in the absence of glycerol ($t_{1/2}$ = 35 h) than in the presence of 10% glycerol ($t_{1/2}$ = 140 h). Dissociation was extremely slow ($t_{1/2}$ > 250 h) when 30% glycerol was present in the buffer. The estrogen-receptor complex was extremely stable in the absence of unlabeled estradiol, and loss of specific binding sites was less than 8% over the course of the 50 h incubation at 0°C.

Increasing the KCl concentration from 0 to 0.5 M in buffer A resulted in a 70% increase in dissociation of the estrogen receptor complex at 0°C: the $t_{1/2}$ decreased from 53 to 32 h (Fig. 1B). We also tested the effect of KCl on receptor dissociation kinetics at 22°C, since the faster dissociation of the estrogen receptor complex at this temperature permitted a better estimate of K_d. In buffer A containing no glycerol or 10% glycerol, 0.5 M KCl had a similar stimulatory effect on the dissociation rate when added to extracts from which the KCl had been removed by dialysis (data not shown).

Glycerol and KCl affect the electrical conductivity of the buffer since this property is propor-

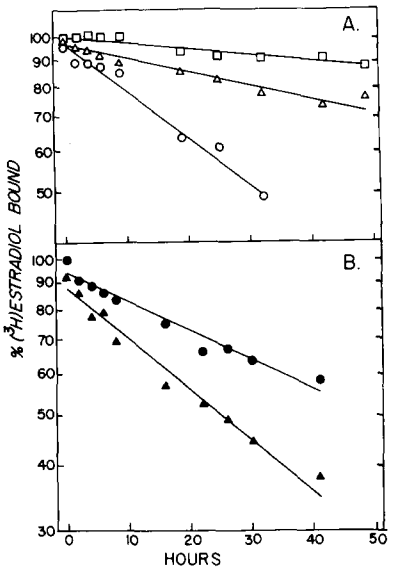

Fig. 1. Effect of glycerol and KCl on dissociation of [^{3}H]estradiol from nuclear estrogen receptor. A. Nuclear receptor was extracted with 4 vols. buffer A plus 0.5 M KCl. The nuclear extracts were then labeled with [^{3}H]estradiol. After labeling, the extract was divided into three equal fractions and then diluted with 2 vols. buffer A plus 0.5 M KCl containing various glycerol concentrations. Dissociation was initiated by the addition of 750 nM unlabeled estradiol and studied over a 50 h time period at 0°C. ○———○, (A_0) $K_d = 0.020$ h^{-1}; △———△, (A_{10}) $K_d = 0.0051$ h^{-1}; □———□, (A_{30}) $K_d = 0.0028$ h^{-1}. B. Nuclear receptor was extracted with 4 vols. buffer A plus 0.5 M KCl and the extract was then dialyzed for 1.5 h at 3°C against 100 vols. buffer A to remove the KCl. After dialysis, the receptor was labeled with [^{3}H]estradiol. After labeling, the extract was divided into two equal fractions and then diluted with 1 vol. buffer A, with or without 1.0 M KCl. Dissociation was initiated with 750 nM unlabeled estradiol and studied over a 40 h time period at 0°C. ●———●, (no KCl) $K_d = 0.013$ h^{-1}; ▲———▲, (0.5 M KCl) $K_d = 0.022$ h^{-1}.

tional to the ionic strength and inversely proportional to the viscosity of the medium [20]. This effect is demonstrated for our buffer system in Fig. 2: glycerol decreases and KCl increases the conductivity of buffer A. Interestingly, the effects of glycerol and KCl on nuclear receptor dissociation parallel their effects on conductivity, suggesting that changes in the dissociation kinetics of receptor due to glycerol and KCl might depend solely on the conductivity of the buffer. To test this hypothesis, the dissociation of estradiol from estrogen receptor was measured at 22°C under

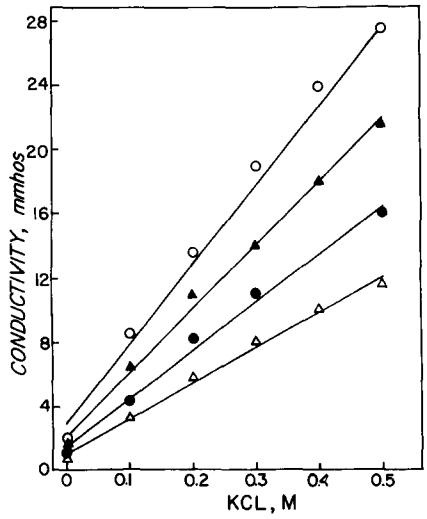

Fig. 2. Effect of KCl on conductivity of buffer A containing various glycerol concentrations. Conductance was measured with a Yellow Springs conductivity meter at room temperature. Lines were fitted by least-squares linear regression (r = 0.99). ○, A_0; ▲, A_{10}; ●, A_{20}; △, A_{30}.

conditions where the glycerol content of the buffer was raised but the conductivity of the medium was maintained by concomitantly increasing the KCl concentration. As shown in Table I, the dissociation rate decreased as the glycerol content of the buffer was raised, despite the fact that buffer conductivity was maintained at a constant level. Also, the suppressive effect of glycerol on the rate

TABLE I

DISSOCIATION KINETICS OF NUCLEAR ESTROGEN RECEPTOR AT 22°C

Estrogen receptor was extracted from nuclei with buffer A plus 0.5 M KCl. After labeling of estrogen receptor with [^{3}H]estradiol, the extracts were diluted 2:1 with buffer A containing various glycerol and KCl concentrations such that the conductivity of the buffer was maintained at 12000 μmhos. Dissociation was then initiated with 750 nM unlabeled estradiol and monitored at 22°C for 120 min.

Buffer	K_d (h^{-1})	$t_{1/2}$ (h)
A + 0.18 M KCl	0.66	1.0
A_{10} + 0.25 M KCl	0.47	1.5
A_{20} + 0.35 M KCl	0.37	1.8
A_{30} + 0.5 M KCl	0.28	2.5

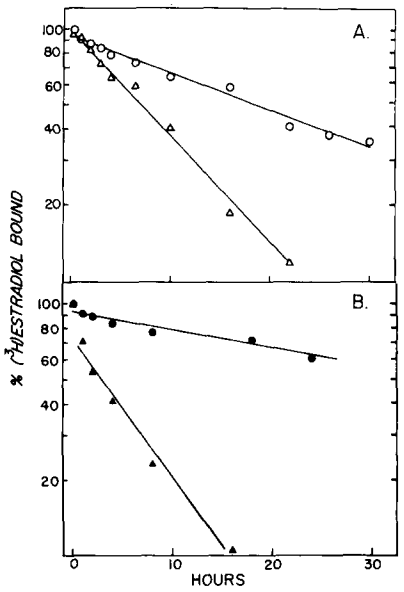

Fig. 3. Effect of pyridoxal 5'-phosphate and sodium thiocyanate on the dissociation of [^{3}H]estradiol from nuclear estrogen receptor. A. Nuclear receptor was extracted with 6 vols. barbital buffer plus 0.5 M KCl and then labeled with [^{3}H]estradiol. The extracts were then diluted with 1 vol. barbital plus 0.5 M KCl with or without 20 mM pyridoxal phosphate. Dissociation was initiated with 750 nM unlabeled estradiol and studied over a 30 h time period at 0°C. O——O, (no pyridoxal phosphate) $K_d = 0.034$ h^{-1}; △——△, (10 mM pyridoxal phosphate) $K_d = 0.091$ h^{-1}. B. Nuclear receptor was extracted with 6 vols. buffer B plus 0.5 M KCl and then labeled with [^{3}H]estradiol. The extracts were then diluted with 1 volume buffer B plus 0.5 M KCl, with or without 0.5 M NaSCN. Dissociation was initiated with 750 nM unlabeled estradiol and studied over a 24 h time period at 0°C. ●——●, (no NaSCN) $K_d = 0.016$ h^{-1}; ▲——▲, (0.5 M NaSCN) $K_d = 0.12$ h^{-1}.

of estrogen receptor dissociation predominated over the stimulatory effect of KCl.

The data in Fig. 3A illustrate the effect of 10 mM pyridoxal phosphate on the dissociation kinetics of the receptor which had been extracted from nuclei with 0.5 M KCl in barbital buffer. The addition of pyridoxal phosphate to a nuclear KCl extract dramatically enhanced the dissociation of labeled estradiol from the receptor: the $t_{1/2}$ decreased from 20 to 7.6 h. Fig. 3B demonstrates the dissociation kinetics of the receptor in the presence of 0.5 M NaSCN: the $t_{1/2}$ decreased from 42 to 5.6 h. The rapid dissociation rate at low temperature in the presence of the chaotropic salt NaSCN permits the assay of total receptor [9,10]. Since the half-life of estrogen-receptor complex at 0°C in 10 mM pyridoxal phosphate (approx. 7 h, Fig. 3A) is comparable to that in 0.5 M NaSCN, it appeared possible to measure total nuclear receptor by exchange assay at both low temperature and low salt using pyridoxal phosphate. We found that pyridoxal phosphate extracts high-affinity estrogen binding sites from hamster uterine nuclei in a concentration-dependent manner where maximal extraction was achieved with 5–10 mM pyridoxal phosphate (data not shown), and the dissociation rate of the estrogen receptor complex in 10 mM pyridoxal phosphate extract (data not shown) was similar to that observed when 10 mM pyridoxal phosphate was added to KCl nuclear extract (Fig. 3A).

From the half-life of dissociation, we estimated that approx. 90% exchange of ligand bound to the receptor should be possible during a 24 h incubation at 0°C in the presence of pyridoxal phosphate. This possibility was tested by quantifying the receptor using Scatchard analysis of equilibrium binding data following incubation of nuclear extract in the presence or absence of pyridoxal phosphate for 1 h at 30°C and for 24 h at 0°C (Table II). In buffer A_{30} plus 0.5 M KCl, the number of high-affinity estrogen binding sites measured at 0°C was only 53% of that detected after incubation at 30°C. Since dissociation of estrogen receptor complex is negligible during the 24 h incubation at 0°C (Fig. 1A), the binding sites detected at 0°C must represent unoccupied receptor and the binding sites calculated by difference (30–0°C) represent occupied receptor. In contrast, a similar number of estrogen binding sites was detected in nuclear pyridoxal phosphate extract at both 0 and 30°C (Table II), indicating that virtually complete exchange of [^{3}H]estradiol with bound estradiol is promoted by pyridoxal phosphate at low temperature. Table II also demonstrates that extraction of the receptor by either pyridoxal phosphate or NaSCN yields the same number of binding sites at 0°C. However, specific estrogen binding sites are lost during a brief (1 h) incubation at elevated temperature in the presence of NaSCN whereas no loss was observed with pyridoxal phosphate (Table II).

The data in Table II show that the number of

TABLE II

MEASUREMENT OF NUCLEAR ESTROGEN RECEPTOR BY EQUILIBRIUM BINDING ANALYSIS

Uteri were homogenized in buffer A. Nuclei were prepared, washed, and divided into three equal fractions. The nuclear pellets were extracted with either buffer A_{30} plus 0.5 M KCl, barbital buffer plus 10 mM pyridoxal phosphate (PLP), or buffer B plus 0.5 M NaSCN. The extracts were diluted with 1 vol. of the same buffer. Measurement of the receptor was performed by Scatchard plot analysis of specific binding after incubating extracts for either 1 h at 30°C or 24 h at 0°C. Steroid was added to the incubation tubes dissolved in the same buffer used for extraction except for the case of extract made with A_{30} plus 0.5 M KCl, where buffer B was used. Data are presented as the mean ± S.E. of three observations. *N* represents the number of binding sites expressed as pmol/g tissue.

Nuclear extract	1 h at 30°C		24 h at 0°C	
	N	Slope (nM^{-1})	*N*	Slope (nM^{-1})
A_{30} + 0.5 M KCl	1.56 ± 0.06	− 1.84 ± 0.04	0.83 ± 0.02	− 1.49 ± 0.02
Barbital + 10 mM PLP	3.16 ± 0.10	− 1.21 ± 0.03	2.89 ± 0.08	− 1.64 ± 0.19
B + 0.5 M NaSCN	0	0	2.96 ± 0.18	− 1.69 ± 0.13

high-affinity estrogen binding sites measured by extraction with either pyridoxal phosphate or NaSCN is higher than that obtained under exchange conditions (1 h, 30°C) after extraction with KCl. Therefore, it was important to determine if the effect of these agents was on the extraction or the measurement of binding sites.

Addition of pyridoxal phosphate to nuclear KCl extract significantly ($P < 0.05$) enhanced the number of binding sites detected (Table IV). The addition of KCl (final concentration: 0.5 M) to nuclear pyridoxal phosphate extract did not significantly alter the number of binding sites measured (data not shown). Since Tris [18] and L-lysine [15] have been found to reverse the pyridoxal phosphate inhibition of progesterone receptor binding to ATP-Sepharose and nuclear binding of estrogen receptor, respectively, we employed these reagents to determine whether the apparent pyridoxal phosphate-induced increase in number of estrogen binding sites was reversible. Addition of Tris (100 mM) slightly reduced the number of binding sites in pyridoxal phosphate nuclear extracts (data not shown). However, addition of L-lysine (100 mM) reduced significantly the number of sites, whereas there was no effect of this agent on the number of sites measured in KCl extract (Table IVB).

The effect of NaSCN on measurement of the receptor in nuclear KCl extract was assessed by dialyzing a KCl extract against NaSCN. Table V shows that replacing KCl in the extract by dialysis against NaSCN resulted in a significant enhancement of total binding sites to the level detected in a nuclear NaSCN extract. In addition, the effect of

TABLE III

COMPARISON OF METHODS FOR MEASUREMENT OF NUCLEAR ESTROGEN AND PROGESTERONE RECEPTOR

	KCl (0.5 M)	NaSCN (0.5 M)	PLP (10 mM)
Total receptor	30°C, 1 h	0°C, 24 h [a]	0°C, 24 h [a] (or 30°C, 1 h)
Unoccupied receptor	0°C, 24 h [b]	No	No
Progesterone receptor	0°C, 24 h [c]	No [d]	0°C, 24 h [e]

[a] More total binding sites compared to KCl (Table II).
[b] Occupied receptor can be estimated as the difference between total receptor and unoccupied receptor (Table II).
[c] Refs. 2 and 17.
[d] No detectable binding sites (unpublished results).
[e] Ref. 14.

TABLE IV

EFFECT OF PYRIDOXAL PHOSPHATE ON DETECTION OF ESTROGEN BINDING SITES IN NUCLEAR EXTRACTS AND ITS REVERSAL BY L-LYSINE

A. Nuclear estrogen receptor was extracted with 6 vols. of either 0.5 M KCl or 10 mM pyridoxal phosphate in barbital buffer. The extracts were then diluted with 1 vol. same buffer. The diluent buffer which was added to half of the KCl extract contained 20 mM pyridoxal phosphate. Measurement of total estrogen receptor was performed by Scatchard plot analysis. The results represent the mean ± S.E. ($n = 3$). B. Uteri were homogenized in barbital buffer and the nuclear fraction was divided into two equal parts after the last wash. The nuclear pellets were extracted with either 0.5 M KCl or 10 mM pyridoxal phosphate. Each extract was further divided into two equal portions and diluted with 1 vol. buffer. After dilution, 4 ml extract was treated with 444 μl of either barbital buffer or 1.0 M L-lysine-HCl in barbital. The samples were incubated for 1 h at 3°C. Total receptor was then assayed by Scatchard plot analysis after incubation for 1 h at 30°C. The addition of L-lysine had no effect on the slope of the Scatchard plot. Data are presented as the mean ± S.E. ($n = 3$ or 4).

Extract	Addition	Estrogen receptor (pmol/g tissue)	
		A	B
0.5 M KCl		1.09 ± 0.10	1.16 ± 0.10
0.5 M KCl	10 mM pyridoxal phosphate	1.71 ± 0.10 [a]	
10 mM pyridoxal phosphate		2.41 ± 0.08 [a]	2.30 ± 0.21 [a]
0.5 M KCl	0.1 M lysine		1.20 ± 0.20
10 mM pyridoxal phosphate	0.1 M lysine		1.39 ± 0.21

[a] $P < 0.05$.

TABLE V

EFFECT OF NaSCN ON NUCLEAR KCl EXTRACTS AND ITS REVERSAL BY DIALYSIS

Uteri were homogenized in buffer A, and the nuclei were pooled and divided into two portions after the last wash. The nuclear pellets were extracted with either buffer B plus 0.5 M KCl or buffer B plus 0.5 M NaSCN. The KCl extract was dialyzed for 3 h at 3°C against 200 vols. buffer B plus 0.5 M NaSCN. The NaSCN extract was dialyzed against either buffer B plus 0.5 M NaSCN or buffer B plus 0.5 M KCl. The estrogen receptor assay was performed by incubating the dialyzed extracts for 24 h at 0°C. Total receptor was measured in undialyzed KCl extract by incubation for 1 h at 30°C. Data are presented as the mean ± S.E. ($n = 3$).

Extract	Dialysis	Estrogen receptor (pmol/g tissue)
0.5 M KCl	–	1.78 ± 0.04
0.5 M KCl	0.5 M NaSCN	2.66 ± 0.08 [a]
0.5 M NaSCN	0.5 M NaSCN	2.84 ± 0.05
0.5 M NaSCN	0.5 M KCl	1.52 ± 0.10 [b]

[a] $P < 0.01$ vs. undialyzed KCl extract.
[b] $P < 0.01$ vs. NaSCN extract.

NaSCN on the measurement of nuclear estrogen binding sites could be reversed by dialyzing an NaSCN extract against KCl (Table V).

Discussion

This study demonstrates that dissociation of the uterine estrogen-receptor complex is impeded by glycerol and enhanced by KCl. The relatively strong effect of glycerol predominated over the weaker influence of KCl. Moreover, the effects of both these agents on estrogen receptor dissociation kinetics were independent of their opposing effects on solvent conductivity. Hansen et al. [8] observed similar effects of these reagents on the dissociation kinetics of the chick oviductal progesterone receptor, postulating that glycerol might retard steroid diffusion by increasing solvent viscosity. Another possibility is that glycerol may alter the stability of the estrogen-receptor complex by changing the properties of the receptor rather than the steroid. Glycerol-dependent stabilization of proteins is a general phenomenon previously studied in detail for many proteins, e.g., β-lactoglobulin [21], ribonuclease [22] and chymotrypsin [23]. Gekko and Timasheff [22,23] proposed that proteins tend to compact in glycerol-containing aqueous solutions in order to minimize the protein-solvent interface. This situation arises from a thermodynamically unfavorable ordering of water molecules around the protein surface in the presence of

glycerol, resulting in glycerol-induced stabilization of a highly folded, compressed form of the protein. For the case of the estrogen-receptor complex in buffer A_{30}, tightening of the tertiary structure of the receptor protein around the steroid binding site would be expected to suppress dissociation of the complex. The influence of KCl on estrogen receptor dissociation, although small, may also depend on changes in protein-solvent interactions, in this case, solvent disordering around the protein [24]. If true, then the receptor protein may unfold and form a more 'relaxed' steroid binding site in solutions containing 0.5 M KCl. However, these salt effects are usually expected only at ionic strengths greater than about 2 M [25]. It is also possible that KCl has a direct effect on the receptor conformation, e.g., by disrupting electrostatic interactions between charged groups on the protein. These weak ionic bonds, either proximal or distal to the steroid binding site, may contribute to receptor conformation involved in steroid binding.

De Boer and Notides [25] observed that dissociation of estradiol from calf uterine receptor is faster after the receptor is extracted from nuclei with KCl than when the receptor is bound to intact nuclei. They concluded from other work [26] that this difference was probably due to the faster dissociation of steroid from the solubilized form of the receptor relative to the matrix-bound form. Our results suggest that the presence of KCl in the nuclear extract may also contribute to the enhanced dissociation of the extracted nuclear receptor.

On a more practical note, our observations on the effects of KCl and glycerol on estrogen receptor dissociation have important implications for the measurement of occupied and unoccupied forms of the receptor. Ideally, unoccupied receptor should be determined under conditions where dissociation of endogenous ligand bound to the occupied receptor is minimized. Unoccupied receptor is customarily measured by overnight incubation of nuclear KCl extract in buffer A_{30} with [^{3}H]estradiol at 0°C [2]. Since dissociation is only slightly affected by KCl, ionic strength is not a significant factor in determining conditions for unoccupied receptor measurement. However, our results clearly show that in order to prevent overestimation of unoccupied receptor, buffers containing glycerol should be used. Since pyridoxal phosphate and NaSCN enhance the rate of dissociation of the occupied receptor, these reagents cannot be used to measure unoccupied receptor.

We have shown for the first time that pyridoxal phosphate substantially increases the dissociation rate of the estrogen-receptor complex to the same extent as that observed with NaSCN. Furthermore, this property of pyridoxal phosphate can be exploited to assay total receptor at low temperature and low ionic strength by incubation of nuclear pyridoxal phosphate extract with labeled estradiol at 0–4°C overnight. However, the number of estrogen binding sites detected in the presence of pyridoxal phosphate or NaSCN at low temperature is higher than the amount measured in nuclear KCl extract at elevated temperature. Thus, caution must be exercised when comparing estimates of the receptor using these different methods. Table III summarizes the three procedures that were evaluated in the present study. The KCl method can be used to distinguish between occupied and unoccupied forms of the receptor, but the total number of binding sites appears to be underestimated with this method. The NaSCN method can measure total receptor at low temperature, but nuclear progesterone receptor is unstable under the conditions of this assay. Thus, the pyridoxal phosphate method has the advantage over the NaSCN procedure of being able to measure both nuclear estrogen receptor and nuclear progesterone receptor at low temperature and low ionic strength.

It is believed that pyridoxal phosphate interacts directly with the steroid receptor molecule to form a Schiff's base with free amino residues [15,18,27]. Previous studies on the interaction on pyridoxal phosphate with steroid hormone receptors have demonstrated a dramatic inhibitory effect of the vitamin on the DNA binding domain of the receptor [15,18,27–30]. With this in mind, our results suggest that pyridoxal phosphate does not extract more receptor than KCl, but instead may interact directly with the extracted receptor to affect measurement of the steroid binding sites. This interpretation is supported by the observations that: (1) agents which reverse the interaction of pyridoxal phosphate with proteins, i.e., lysine [15], partially suppress the number of binding sites measured in

nuclear pyridoxal phosphate extract; and (2) addition of pyridoxal phosphate to nuclear KCl extract increases the number of binding sites detected. Similarly, NaSCN does not appear to extract more receptor than KCl since: (1) replacement of KCl by NaSCN results in an increase in binding sites, and (2) replacement of NaSCN by KCl decreases the level of measurable binding sites. Thus, it appears that KCl, pyridoxal phosphate and NaSCN all extract an equivalent amount of the receptor from uterine nuclei. However, some of these sites are not detected in the KCl procedure, and the reason for this is unknown. It is possible that binding sites are masked or inactivated in the KCl procedure during exchange at elevated temperature. Alternatively, these latent estrogen binding sites might represent nuclear receptor which is in an altered state as a result of processing during the normal course of estrogen action in the nucleus [31]. Another possibility is that pyridoxal phosphate may expose latent binding sites which had been inactivated as a consequence of progesterone action mediated by receptor-regulatory factor (1,2,4–7). The present results support the conclusion that pyridoxal phosphate may serve as a useful reagent for the extraction and measurement of nuclear estrogen receptor at low temperature. However, further study is required to determine the biological significance of the estrogen binding sites detected with the pyridoxal phosphate procedure.

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References

1 Leavitt, W.W., Evans, R.W., Okulicz, W.C., MacDonald, R.G., Hendry, W.J., III and Robidoux, W.F., Jr. (1982) in Hormone Antagonists (Agarwal, M.K., ed.), pp. 213–232, Walter de Gruyter, New York
2 Evans, R.W., Chen, T.J., Hendry, W.J., III and Leavitt, W.W. (1980) Endocrinology 107, 383–390
3 Okulicz, W.C., Evans, R.W. and Leavitt, W.W. (1981) Science 213, 1503–1505
4 Okulicz, W.C., Evans, R.W. and Leavitt, W.W. (1981) Steroids 37, 463–470
5 Evans, R.W. and Leavitt, W.W. (1980) Proc. Natl. Acad. Sci., U.S.A. 77, 5856–5860
6 Okulicz, W.C., MacDonald, R.G. and Leavitt, W.W. (1981) Endocrinology 109, 2273–2275
7 MacDnald, R.G., Okulicz, W.C. and Leavitt, W.W. (1982) Biochem. Biophys. Res. Commun. 104, 570–576
8 Hansen, P.E., Johnson, A., Schrader, W.T. and O'Malley, B.W. (1976) J. Steroid Biochem. 7, 723–732
9 Sica, V., Puca, G.A., Molinari, A.M., Buonaguro, F.M. and Bresciani, F. (1980) Biochemistry 19, 83–88
10 Sica, V., Weisz, A., Petrillo, A., Armetta, I. and Puca, G.A. (1981) Biochemistry 20, 686–693
11 Coty, W.A. (1980) J. Biol. Chem. 255, 8035–8037
12 Banerji, A. and Kalimi, M. (1981) Steroids 37, 409–421
13 Traish, A.M., Müller, R.E. and Wotiz, H.H. (1981) J. Biol. Chem. 256, 12028–12033
14 Chen, T.J., MacDonald, R.G., Robidoux, W.F., Jr. and Leavitt, W.W. (1981) J. Steroid Biochem. 14, 1023–1028
15 Müller, R.E., Traish, A. and Wotiz, H.H. (1980) J. Biol. Chem. 255, 4062–4067
16 Isomaa, V., Pajunen, A.E.I., Bardin, C.W. and Jänne, O.A. (1982) Endocrinology 111, 833–843
17 Chen, T.J. and Leavitt, W.W. (1979) Endocrinology 104, 1588–1597
18 Nishigori, H. and Toft, D. (1979) J. Biol. Chem. 254, 9155–9161
19 Scatchard, G. (1949) Ann. N.Y. Acad. Sci. 51, 660–672
20 Edsall, J.T. and Wyman, J. (1958) Biophysical Chemistry, pp. 386–404, Academic Press, New York
21 DiPaola, G. and Belleau, B. (1978) Can. J. Chem. 56, 848–850
22 Gekko, K. and Timasheff, S.N. (1981) Biochemistry 20, 4677–4686
23 Gekko, K. and Timasheff, S.N. (1981) Biochemistry 20, 4667–4676
24 Tanford, C. (1961) Physical Chemistry of Macromolecules, pp. 240–245, John Wiley and Sons, New York
25 De Boer, W. and Notides, A.C. (1981) Biochemistry 20, 1290–1294
26 De Boer, W. and Notides, A.C. (1981) Biochemistry 20, 1285–1289
27 Cake, M.H., Di Sorb, D.M. and Litwack, G. (1978) J. Biol. Chem. 253, 4886–4891
28 Hiipakka, R.A. and Liao, S. (1980) J. Steroid Biochem. 13, 841–846
29 Cidlowski, J.A. and Thanassi, J.W. (1978) Biochem. Biophys. Res. Commun. 82, 1140–1146
30 Henrikson, K.P., Gross, S.C. and Dickerman, H.W. (1981) Endocrinology 109, 1196–1202
31 Horwitz, K.B. and McGuire, W.L. (1978) J. Biol. Chem. 253, 8185–8191