Molybdate Interaction with the Estrogen Receptor: Effects on Estradiol Binding and Receptor Activation†

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ABSTRACT: The effects of sodium molybdate on the equilibrium and kinetics of [^{3}H]estradiol binding, and on the differential ammonium sulfate precipitation and activation of the calf uterine estrogen receptor, were investigated. The magnitude of the fast component of the biphasic [^{3}H]estradiol–receptor dissociation, which is proportional to the fraction of the receptor in the nonactivated state, was markedly increased by the presence of 10–20 mM molybdate, indicating an inhibition of receptor activation. A small fraction of the receptor (10–20%) was consistently found in the slower [^{3}H]estradiol-dissociating (activated) form because a very slow rate of receptor activation still occurred in the presence of molybdate, or a small fraction of the receptor was initially present in the activated state, even in the absence of estradiol. The degree of inhibition of receptor activation by molybdate was proportionally reduced by the ionic strength of the buffer: in 0.4 M KCl, there was no inhibition of receptor activation by molybdate. Dialysis of the molybdate-treated cytosol reversed the inhibitory action of molybdate on receptor activation; however, once activation had occurred, the addition of molybdate did not shift the equilibrium between the two forms of the receptor toward the nonactivated state. The [^{3}H]estradiol equilibrium binding analysis of the receptor in the presence of molybdate showed a decrease in the positive cooperative binding of estradiol. The Hill coefficient (n_H) was reduced from 1.62 ± 0.08 to 1.40 ± 0.03. Pretreatment of the cytosols with DNA–cellulose prior to the [^{3}H]estradiol equilibrium binding assay further reduced positive cooperativity: n_H = 1.13 ± 0.06 in the presence and n_H = 1.58 ± 0.05 in the absence of molybdate. DNA–cellulose pretreatment of the cytosol removed the small fraction of the receptor that was initially present in the activated state. Sedimentation analyses with sucrose gradients containing 0.4 M KCl showed that molybdate inhibited the transformation of the receptor from the 4S to the activated 5S form. A small fraction (10–20%) of the receptor in the presence of molybdate was seen consistently in the activated 5S form in agreement with the results of the [^{3}H]estradiol–receptor dissociation assay. The sedimentaton coefficients of the estrogen receptor in low-salt buffers were not significantly affected by the molybdate. In the absence of molybdate, the receptor was precipitated at 30% ammonium sulfate and was shown to be transformed to the activated receptor, while in the presence of molybdate the receptor was precipitated at 30–50% ammonium sulfate saturation and shown to be in the nonactivated form. This differential ammonium sulfate precipitation can be used to provide approximately a 30-fold purification of the activated receptor. These data indicate that molybdate maintains and interacts exclusively with the nonactivated state of the receptor.

The activation (transformation) of the cytoplasmic, nonactive form of the estrogen receptor by an estrogen- and temperature-promoted reaction results in the translocation of the receptor to the nucleus where the receptor, by a mechanism that remains to be resolved, regulates chromatin function (Jensen, 1979; Gorski & Gannon, 1976). The biochemical studies of the estrogen receptor, particularly in cell-free systems, have contributed to an understanding of the molecular basis of the receptor activation that is initiated by estrogen binding. The transformation from the cytoplasmic 4S monomer with a molecular weight of (7–8) × 10^4 into the activated, nuclear 5S receptor with a molecular weight of (13–14) × 10^4 is accomplished by an estrogen- and temperature-promoted dimerization reaction (Notides & Nielsen, 1974; Notides et al., 1975). For each form of the estrogen receptor, a finite affinity state exists, readily measurable by the biphasic [^{3}H]estradiol dissociation kinetics of the receptor; estrogen binding shifts the equilibrium between these two states toward the higher affinity state, the activated 5S form (Weichman & Notides, 1977, 1979; de Boer & Notides, 1981). The relative biological potency and nuclear retention of the receptor correlate well with the dissociation rate constants and with the proportion of the slower [^{3}H]estrogen-dissociating component (activated state) for several different estrogens (Weichman & Notides, 1980). The equilibrium for estradiol binding to the receptor is a positive cooperative reaction reflecting the two affinity states of the receptor (Notides et al., 1981).

Molybdate has been shown to stabilize the unoccupied steroid hormone receptors against thermal denaturation (Nielsen et al., 1977a,b; Toft & Nishigori, 1979; Marver, 1980; Chen et al., 1981; Wright et al., 1981). It also inhibits the activation of the progesterone receptor (Toft & Nishigori, 1979; Nishigori & Toft, 1980), the glucocorticoid receptor (Leach et al., 1979), and the estrogen receptor (Shyamala & Leonard, 1980; Mauck & Notides, 1980). Although several mechanisms of molybdate action have been suggested, none has been established. This report investigates the action of molybdate upon the interaction of estradiol with its receptor and the relationship of estrogen binding to receptor activation.

Materials and Methods

Materials

The 17β-[6,7-^{3}H]estradiol (52 or 58 Ci/nmol) and [*methyl*-^{14}C]ovalbumin (9.94 μCi/mg) were obtained from New England Nuclear. Alkaline phosphatase (*Escherichia coli*) and sodium molybdate were obtained from Sigma Chemical Co. Sucrose and ammonium sulfate were ultrapure grades

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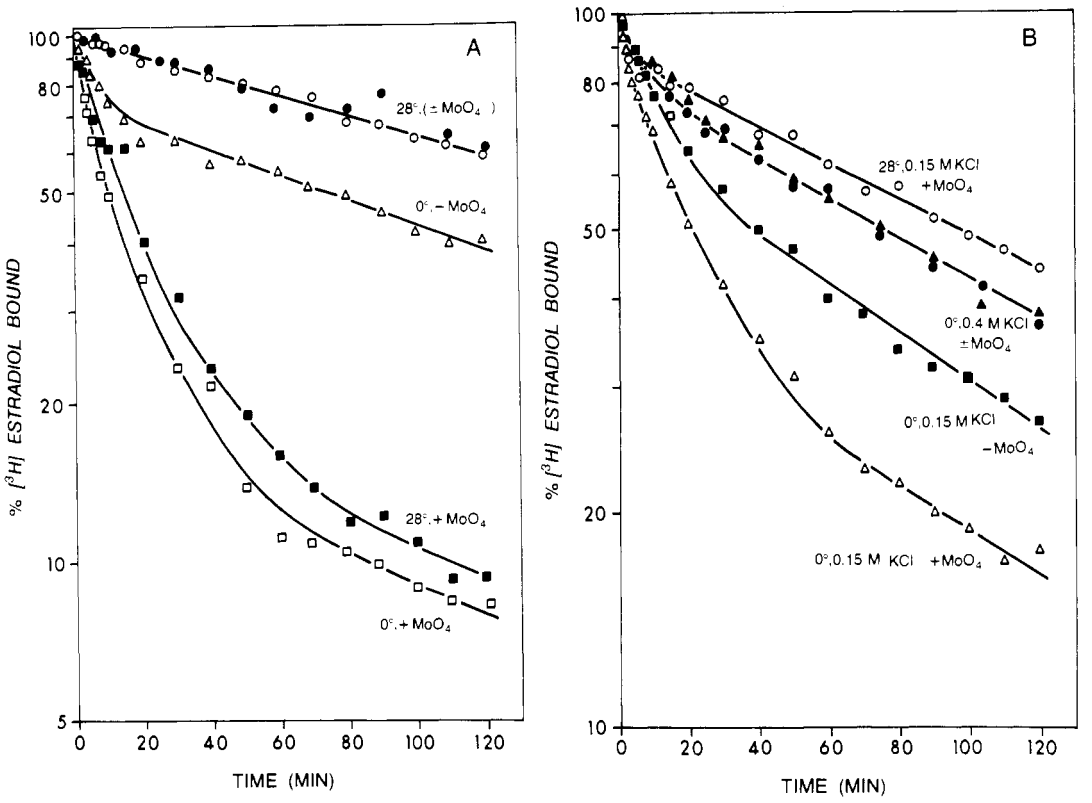

FIGURE 1: Effect of molybdate on the [^{3}H]estradiol–receptor dissociation kinetics. Calf uterine cytosol prepared in buffer TD without (○, ●, △) or with 10 mM molybdate (□, ■) was equilibrated with 10 nM [^{3}H]estradiol, with or without 1 μM unlabeled estradiol, for 1 h at 0 °C. Aliquots were incubated for 30 min at 0 °C (△, □) or 30 min at 28 °C (○, ●, ■), and then molybdate was added to one aliquot (●) for a final concentration of 10 mM molybdate. The dissociation of [^{3}H]estradiol from the receptor in cytosol was measured at 28 °C after the addition of 1 μM unlabeled estradiol. Receptor inactivation was measured by incubating the receptor at 28 °C without the addition of unlabeled estradiol. The dissociation rate constants are given in Table I (A). Calf uterine cytosol prepared with buffer TD–0.15 M KCl (■), buffer TD–0.15 M KCl with 10 mM molybdate (○, △), buffer TD–0.4 M KCl (●), or buffer TD–0.4 M KCl with 10 mM molybdate (▲) was equilibrated with 10 nM [^{3}H]estradiol with and without 1 μM unlabeled estradiol for 1 h at 0 °C. The cytosol prepared with TD–0.15 M KCl and 10 mM molybdate (○) was preincubated for 30 min at 28 °C. The remaining cytosol samples were preincubated for 30 min at 0 °C. The dissociation of [^{3}H]estradiol from the receptor was assayed as described above (B).

obtained from Schwarz/Mann. All other reagents were analytical grade.

Methods

Preparation of Calf Uterine Cytosol. Calf uteri stored at −80 °C were reduced to a powder by using a liquid nitrogen cooled, stainless-steel mortar and pestle. All subsequent procedures were carried out at 0–4 °C. The tissue homogenized, using a Polytron PT-10, in 5 volumes of 40 mM Tris-HCl–1 mM dithiothreitol, pH 7.5 (buffer TD). The homogenate was centrifuged at 20000*g* for 10 min, and then the supernatant was removed and centrifuged at 220000*g* for 45 min. The supernatant from the second centrifugation is referred to as "the cytosol". The protein concentration of the cytosol, 6–7 mg/mL, was determined by the method of Bradford (1976) with bovine serum albumin as the standard.

Assay of [^{3}H]Estradiol Dissociation from the Estrogen Receptor. The dissociation of [^{3}H]estradiol from the receptor was measured at 28 °C by exchange of [^{3}H]estradiol with an excess of unlabeled estradiol. Details of the method of assay and analyses of the data have been published (Weichman & Notides, 1977, 1979).

Sucrose Density Centrifugation. Free [^{3}H]estradiol was removed from the samples to be analyzed, by adsorption to 0.25% activated charcoal Norit A–0.0025% dextran 500 for 5 min at 0 °C. Aliquots (0.2 mL) were centrifuged on 3.8-mL gradients of 5–20% sucrose in buffer TD or buffer TD–0.4 M KCl or 10–30% sucrose in buffer TD without KCl (low-salt sucrose gradients). The samples were then centrifuged at 50 000 rpm for 19 h at 1 °C with an SW 56 Beckman rotor. *E. coli* alkaline phosphatase and [*methyl*-^{14}C]ovalbumin were used as internal standards. The gradients were fractionated, and the sedimentation coefficients of the [^{3}H]estradiol–receptor complexes were determined (Notides & Nielsen, 1974).

Results

Effect of Molybdate on Estradiol Interaction with the Receptor. The effect of molybdate on receptor activation, measured by the kinetics of [^{3}H]estradiol dissociation from the receptor at 28 °C, is shown in Figure 1 and Table I. In buffer TD, in the presence or absence of 10 mM molybdate, the dissociation kinetics in cytosol showed two first-order components. The magnitude of the faster component, which reflects the fraction of the receptor in the nonactivated state (Weichman & Notides, 1977), was increased 58% in the cytosol treated with molybdate (Figure 1A). The rate constants for the two components were not significantly affected by molybdate. In 0.15 M KCl, 10 mM molybdate produced a 29% increase in the magnitude of the faster component; 0.15 M KCl reduced the effectiveness of molybdate in suppressing receptor activation (Figure 1B).

Conditions that succeed in inducing *complete* receptor activation (preincubation of the [^{3}H]estradiol–receptor complex for 30 min at 28 °C) fail in the presence of molybdate. The [^{3}H]estradiol–receptor complex when preincubated for 30 min at 28 °C in buffer TD with 10 mM molybdate produced a fast-dissociating component with a magnitude only 10% less than that for the [^{3}H]estradiol–receptor complex not preincubated but treated with molybdate (Figure 1A). Thus, only

Table I: Effect of Molybdate on the [^{3}H]Estradiol Receptor Dissociation Kinetics at 28 °C

cytosol prepared in TD buffer	$-Na_2MoO_4$				+10 mM Na_2MoO_4			
	k-1[a]	%[b]	k-2[a]	%	k-1	%	k-2	%
−KCl	0.17	25	4.8×10^{-3}	75	6.9×10^{-2}	83	8.0×10^{-3}	17
+0.15 M KCl	6.9×10^{-2}	33	5.8×10^{-3}	67	6.0×10^{-2}	72	6.6×10^{-3}	38
+0.4 M KCl	5.4×10^{-2}	20	6.2×10^{-3}	80	5.4×10^{-2}	20	6.2×10^{-3}	80
−KCl (preincubated 30 min at 28 °C)			4.5×10^{-3}	100	6.9×10^{-2}	78	7.6×10^{-3}	22
+0.15 M KCl (preincubated 30 min at 28 °C)			5.9×10^{-3}	100	6.9×10^{-2}	12	5.8×10^{-3}	88

[a] The rate constants k-1 and k-2 are expressed as min^{-1}. [b] The percent indicates the fraction of the receptor dissociating with dissociation rate constant k-1 or k-2.

a very small fraction of the receptor was activated during preincubation in buffer TD containing 10 mM molybdate. The presence of 0.15 M KCl mitigated the suppression of receptor activation by molybdate during preincubation. In buffer TD–0.15 M KCl with 10 mM molybdate preincubated for 30 min at 28 °C, the fast component was reduced by 50% when compared with the unheated control (Figure 1B). In buffer TD–0.4 M KCl, molybdate had no effect on the magnitude of the fast component (Figure 1B). These data indicate that in buffer TD or buffer TD–0.15 M KCl, in the presence of molybdate, the activation of the receptor occurred at a greatly reduced rate. Furthermore, the inhibition of the rate of receptor transformation was greater in buffer TD than in buffer TD–0.15 M KCl.

When the [^{3}H]estradiol–receptor was activated by preincubation for 30 min at 28 °C or by precipitation with 30% saturated ammonium sulfate, the dissociation kinetics showed only a single first-order component. The rate constant, $k = 4.5 \times 10^{-3}$ min^{-1}, with or without the addition of 10 mM molybdate, was the same as that found for the slower component in the cytosol (Figure 1A). Receptor transformation whether brought about by incubation at 28 °C or by ammonium sulfate precipitation was not reversed by molybdate.

The [^{3}H]estradiol equilibrium binding of the estrogen receptor is a positive cooperative reaction indicative of the two states, activated and nonactivated, of the receptor (Notides et al., 1981). Equilibrium binding analysis of the estrogen receptor in the presence of 10 mM molybdate showed a decrease in the degree of cooperativity. The convexity of the Scatchard plot in the presence of molybdate was reduced. The Hill coefficient (n_H) was reduced from 1.62 ± 0.08 to 1.40 ± 0.03 ($n = 4$). Incubation of the cytosol with DNA–cellulose for 1 h at 0 °C prior to the [^{3}H]estradiol equilibrium binding assay resulted in $n_H = 1.13 \pm 0.06$ ($n = 3$) in the presence of molybdate and $n_H = 1.58 \pm 0.05$ ($n = 5$) in the absence of molybdate (Figure 2). Approximately 10–30% of the estrogen receptor (with or without molybdate) was retained by the DNA–cellulose following incubation with uterine cytosol.

Effect of Molybdate on Receptor Transformation. We analyzed the effect of molybdate on receptor tranformation by sedimenting the receptor, which was previously incubated under activating conditions with and without molybdate, through high-salt sucrose gradients. In the presence of molybdate, the receptor remained predominately in the nonactivated 4S form after incubation for 30 min at 28 °C. Nevertheless, a small fraction (10–20%) of the receptor was seen consistently in the activated 5S form (Figure 3). Molybdate inhibition of transformation was not seen when the receptor incubation was carried out in buffer TD–0.4 M KCl: high ionic strength retarded the action of molybdate (data not shown).

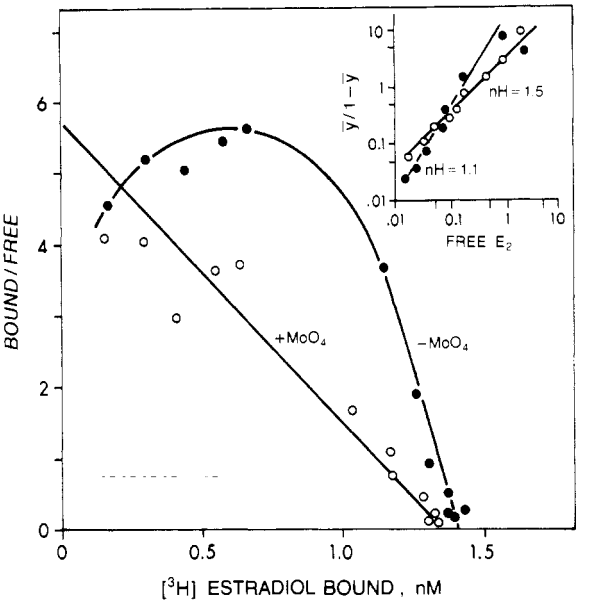

FIGURE 2: Scatchard plot of [^{3}H]estradiol binding by the receptor in the presence or absence of molybdate. Calf uterine cytosol was prepared in buffer TD containing 0.5 mM leupeptin without (●) or with (○) 10 mM molybdate. Each cytosol was pretreated with DNA–cellulose (1 mL per 10 mL of cytosol) for 1 h at 0 °C. The supernatant was then equilibrated with 0.2–15 nM [^{3}H]estradiol for 22 h at 0–4 °C. Inset: Hill plot of the [^{3}H]estradiol receptor binding curve; $\bar{y}$ is the fractional saturation of the receptor.

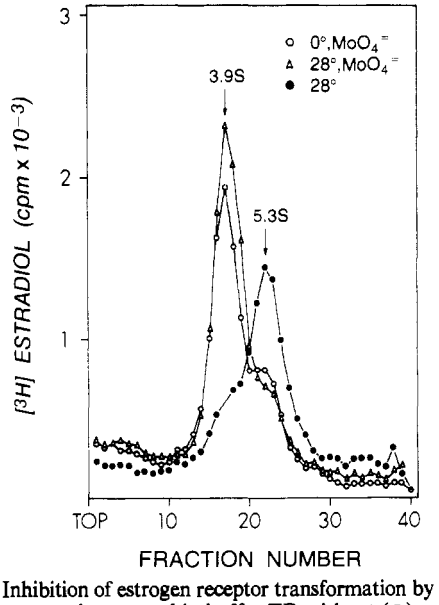

FIGURE 3: Inhibition of estrogen receptor transformation by molybdate. Calf uterine cytosol, prepared in buffer TD without (●) and with (○, △) 10 mM molybdate, was equilibrated with 10 nM [^{3}H]estradiol for 1 h at 0 °C. Aliquots were then incubated for 30 min at 30 °C (●, △) or for 30 min at 0 °C (○). Gradient analysis was carried out in a 5–20% sucrose gradient by using buffer TD–0.4 M KCl.

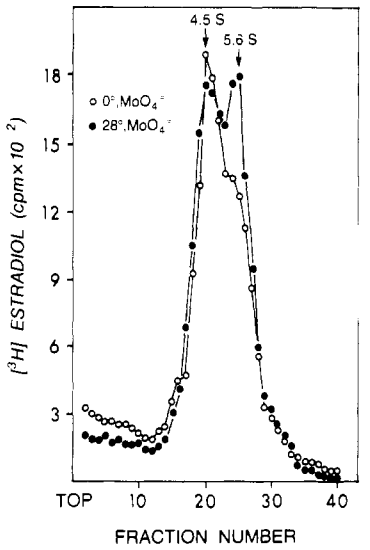

FIGURE 4: Inhibition of estrogen receptor transformation by molybdate in 0.15 M KCl. Calf uterine cytosol, prepared in buffer TD–0.15 M KCl with 10 mM molybdate, was equilibrated with 10 nM [^{3}H]estradiol for 1 h at 0 °C. Aliquots were incubated for 45 min at 0 °C (O) or for 45 min at 28 °C (●). Gradient analysis was carried out in a 5–20% sucrose gradient with buffer TD–0.4 M KCl.

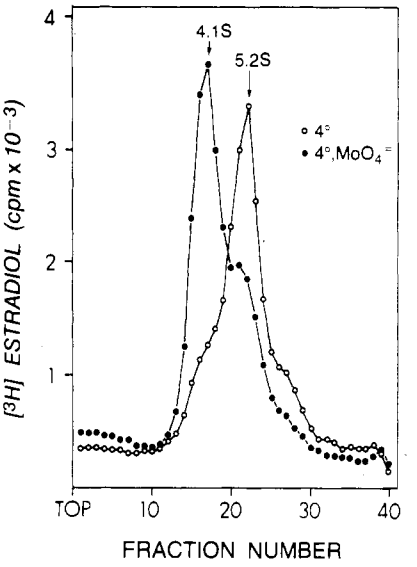

FIGURE 5: Reversibility of the molybdate inhibition of receptor transformation. Calf uterine cytosol, prepared in buffer TD with 10 mM molybdate, was equilibrated with 10 nM [^{3}H]estradiol for 1 h at 0 °C. Aliquots were dialyzed overnight at 4 °C against buffer TD (O) or buffer TD with 10 mM molybdate (●). Gradient analyses were carried out in a 5–20% sucrose gradient with buffer TD–0.4 M KCl.

Receptor tranformation was retarded less by molybdate when the calf uterine cytosol was prepared in buffer TD–0.15 M KCl than in buffer TD. In sucrose gradients with buffer TD–0.15 M KCl and molybdate, the receptor sedimented predominately as the 4S form, while in cytosol prepared in the absence of molybdate the receptor sedimented predominately as the 5S form. Incubating the receptor in the presence of molybdate for 45 min at 28 °C transformed only a small fraction of the receptor to the 5S form, while in the absence of molybdate transformation was complete (Figure 4).

Uterine cytosol prepared in buffer TD or buffer TD–0.1 M KCl and then subjected to sucrose gradient analysis in buffer TD–0.1 M KCl showed the receptor sedimenting as a single peak, predominately at 6.2 to 6.6 S. Occasionally, the receptor sedimented as an aggregation in a broad band from 7.6 to 10.6 S. Nevertheless, no difference in receptor sedimentation in buffer TD–0.1 M KCl was noted whether 10 mM molybdate was present or not in the homogenization or gradient buffer (data not shown). The sedimentation of the receptor in sucrose gradients containing buffer TD without KCl was also not significantly affected by molybdate. The receptor sedimented at 7.8 ± 0.28 S (n = 5) without molybdate and at 8.0 ± 0.28 S (n = 5) with molybdate (10 mM) (data not shown).

Figure 5 shows that molybdate inhibition of receptor activation is reversible. The estrogen–receptor complex, prepared in buffer TD with 10 mM molybdate, converted to the 5S form during overnight dialysis against buffer TD at 4 °C. An aliquot dialyzed as above against buffer TD containing 10 mM molybdate remained predominately in the 4S form of the receptor, although a small increase in the 5S form was seen. Receptor prepared in buffer TD with 10 mM molybdate and then heated for 30 min at 28 °C also tranformed into the 5S form after overnight dialysis against buffer TD at 4 °C (data not shown).

Effect of Molybdate on Ammonium Sulfate Fractionation of the Receptor. In the absence of molybdate, the receptor was found predominately in the 30% saturated ammonium sulfate precipitate; in the presence of 10–20 mM molybdate, a larger fraction of the receptor precipitated in the 30–50% saturated ammonium sulfate fraction (Table II). The effect of molybdate was reversed by dissolving the 30–50% ammonium sulfate precipitate in buffer TD and then reprecipitating the receptor by adding ammonium sulfate until 30% saturation was reached.

Table II: Effect of Molybdate on the Ammonium Sulfate Fractionation of the Estrogen Receptor[a]

fraction	specific binding (pmol/mg of protein)	total receptor (pmol)	x-fold purification	recovery (%)
cytosol ($+MoO_4^{2-}$)	0.20	45.0	1.0	100
0–30% $(NH_4)_2SO_4$	0.36	6.4	1.8	14.3
30–50% $(NH_4)_2SO_4$	0.16	19.1	0.8	42.4
(30–50%) + 30% $(NH_4)_2SO_4$	5.20	4.9	26.0	10.9
cytosol ($-MoO_4^{2-}$)	0.11	112.4	1.0	100
0–30% $(NH_4)_2SO_4$	0.56	63.0	5.1	56.0
30–50% $(NH_4)_2SO_4$	0.30	7.7	2.7	6.9

[a] Calf uterine cytosol in the presence and absence of 10 mM sodium molybdate was equilibrated with 10 mM [^{3}H]estradiol in the presence or absence of 1 μM unlabeled estradiol for 1 h at 0 °C. The experimental group, "(30–50%) + 30% $(NH_4)_2SO_4$", designates the 30–50% ammonium sulfate fraction of the cytosol (the cytosol contained 10 mM sodium molybdate). The ammonium sulfate precipitate was then dissolved in buffer TD (one-tenth the original volume of the cytosol) and reprecipitated with 30% ammonium sulfate. Specifically bound [^{3}H]estradiol was determined after dissolving the ammonium sulfate precipitated fractions in buffer TD and treating the aliquots with charcoal-dextran to remove the unbound [^{3}H]estradiol.

The overall purification of the receptor in the absence of protein carrier was limited and variable: 2–30-fold with only a 10–20% yield. The purification and yield of the receptor were not improved by dialysis of the 30–50% ammonium sulfate fraction before reprecipitation. In the presence of a carrier protein (1 mg of γ-globulin per mL), the overall yield of receptor increased to 20–52% after the second precipitation; however, its purity could not be measured because of the

presence of the coprecipitant, γ-globulin. The precipitation pattern of the unoccupied receptor in the presence of molybdate was the same as that for the [^{3}H]estradiol–receptor complex (data not shown).

Ammonium sulfate precipitation in the absence of molybdate activated the estrogen receptor as demonstrated by its transformation into the 5S sedimenting form (De Sombre et al., 1972) and by the appearance of monophasic, slow, estradiol dissociation kinetics (Weichman & Notides, 1979). The 30–50% saturated ammonium sulfate fraction, dissolved in and dialyzed overnight at 0–4 °C against buffer TD containing 20 mM molybdate, showed predominantly the 4S receptor in high-salt sucrose density gradients and biphasic dissociation kinetics, characteristics of the nonactivated receptor. An aliquot of the same 30–50% ammonium sulfate fraction dialyzed against buffer TD overnight at 4 °C showed the 5S receptor and monophasic [^{3}H]estradiol receptor dissociation kinetics (data not shown).

Discussion

Molybdate affects several molecular properties of the receptor that are related to the receptor's activation process: the receptor's biphasic [^{3}H]estradiol dissociation kinetics; the loss of the receptor's cooperative [^{3}H]estradiol binding; the inhibition of transformation of the nonactivated 4S receptor into the activated 5S form; and the differential precipitation of the receptor by ammonium sulfate. Previous reports of receptor binding to isolated nuclei, DNA–cellulose, or ATP–Sepharose have demonstrated the inhibition of activation of the glucocorticoid receptor (Leach et al., 1979), progesterone receptor (Nishigori & Toft, 1980), and estrogen receptor (Noma et al., 1980) by molybdate.

The biphasic [^{3}H]estradiol dissociation kinetics are a sensitive indicator of the receptor's activation process, in which the magnitude of the fast component is proportional to the fraction of the receptor in the nonactivated 4S form (Weichman & Notides, 1977, 1979). Molybdate markedly increased the magnitude of the fast [^{3}H]estradiol-dissociating component of the receptor, thereby indicating an inhibition of receptor activation, although a small fraction of the receptor (10–20%) consistently showed the slower, activated form. Preincubation of the estrogen–receptor complex at 28 °C, before assaying [^{3}H]estradiol dissociation, produced a small decrease (~10%) in the magnitude of the fast component, indicating that receptor activation had occurred in buffer TD with molybdate but at a very low rate. These data suggest that a slow rate of receptor activation had occurred during the receptor isolation procedure or during the [^{3}H]estradiol dissociation assay, or a small fraction of the receptor was initially present in the activated form in vivo even in the absence of estradiol.

When molybdate was added after receptor activation had occurred, the molybdate was incapable of shifting the activated receptor to the nonactivated state. The failure of molybdate to reverse receptor tranformation has also been found for the glucocorticoid, progesterone, and estrogen receptors (Leach et al., 1979; Nishigori & Toft, 1980; Shyamala & Leonard, 1980). We observed that removal of the molybdate by dialysis was sufficient to reverse the inhibition of the activation of the calf uterine estrogen receptor (Figure 5). Reversal of the molybdate inhibition of the progesterone receptor has been reported (Nishigori & Toft, 1980), but not for the mouse uterine estrogen receptor (Shyamala & Leonard, 1980).

Previously, this laboratory had demonstrated that the nonactivated and activated forms of the estrogen receptor from calf uteri are each a different affinity state (Weichman & Notides, 1977, 1979) and, recently, that estradiol is bound with positive equilibrium cooperativity (Notides et al., 1981). In the absence of molybdate, the receptor binds estradiol with positive cooperativity [the Hill coefficient (n_H) was 1.64], while in the presence of molybdate, n_H decreased to 1.40. The minimal effect of molybdate on the positive equilibrium cooperativity of the estradiol–receptor interactions suggests that the small fraction of the receptor initially present in the activated state may be sufficient to generate a near-maximal effect on the slope of the Hill plot. The presence of a small fraction of the receptor in the activated state in uterine cytosol prepared with buffer TD containing molybdate is also indicated by the fraction of the receptor in the slower [^{3}H]estradiol-dissociating state (Figure 1) and by the sedimentation analysis showing the presence of the 5S form of the receptor (Figure 3).

Heterotropic effectors, such as molybdate, may show a marked shift in ligand binding or enzymatic activity while only a negligible effect on the slope of the corresponding Hill plot (Rubin & Changeux, 1966; Blangy et al., 1968). The change in the Hill coefficent (n_H) is chiefly dependent upon the following: the intrinsic allosteric constant (i.e., the equilibrium constant of the two states of the receptor in the absence of ligand); the ratio of the affinities of the ligand for the two states of the receptor; the apparent allosteric constant (i.e., the equilibrium constant of the two states of the receptor in the presence of all effectors except the homotropic effector, estradiol); and the extent of the conformational transition induced by the homotropic ligand, estradiol. The values of these constants may be such that the cooperativity of the ligand binding is near-maximal, even with a slight change in one or more of these constants. This is particularly true for allosteric proteins that are nonexclusive ligand binding; e.g., estradiol is bound by both the nonactivated and activated states of the receptor, but with different affinities, and this imposes limits on the extent to which equilibrium can be shifted. Pretreatment of the uterine cytosol (prepared with buffers containing molybdate) with DNA–cellulose resulted in [^{3}H]estradiol binding that showed a linear Scatchard plot and a Hill coefficient of 1.13, which indicated a marked reduction in the degree of positive cooperativity and the predominance of one state of the receptor (Figure 2). Presumably, the DNA–cellulose removed the small fraction of the receptor initially resent in the activated form while molybdate maintained the receptor in the nonactivated form during the [^{3}H]estradiol equilibrium binding assay.

Sedimentation analysis of molybdate action upon estrogen receptor transformation agrees with the conclusions of the [^{3}H]estradiol dissociation kinetics assay of receptor activation: molybdate inhibited the transformation of the receptor from the nonactivated 4S into the activated 5S form during incubation for 30–45 min at 28 °C. Sedimentation analysis showed that molybdate inhibition was maximal in buffer TD, less pronounced in buffer TD with 0.15 M KCl, and absent in buffer TD with 0.4 M KCl. The inhibition of the receptor transformation by molybdate was reversed following removal of the molybdate by dialysis. In contrast to several reports (Nishigori & Toft, 1980; Shyamala & Leonard, 1980; Chen et al., 1981) suggesting that molybdate increased receptor aggregation, we found that molybdate had little or no effect on the sedimentation of the estrogen receptor in low-salt sucrose gradients or in sucrose gradients containing 0.1 M KCl. This may reflect a difference between species, a difference between the interactions of the individual steroid hormone

receptors with molybdate, or a difference in the experimental conditions; e.g., we have used Tris whereas the higher sedimenting forms are generally seen in dilute phosphate buffers (Nishigori & Toft, 1980; Shyamala & Leonard, 1980).

The differential precipitation of the receptor by ammonium sulfate in the presence and absence of molybdate was unexpected, considering the absence of molybdate inhibition of receptor transformation in 0.4 M KCl (Figure 1) and the ability of ammonium sulfate precipitation itself to promote receptor activation (De Sombre et al., 1972; Weichman & Notides, 1979). It is possible that molybdate inhibition of receptor transformation is minimal at intermediate salt concentrations but increases at higher concentrations when the receptor is aggregated and salted out of solution. Pratt and co-workers (Dahmer et al., 1981) have noted a differential ammonium sulfate precipitation of the glucocorticoid receptor.

The mechanism of molybdate inhibition of receptor transformation is unknown. Molybdate is known as an inhibitor of alkaline phosphatase activity (Paigen, 1958) and possibly phosphoprotein phosphatases (Swarup et al., 1981); its inhibition of receptor dephosphorylation or a factor associated with the receptor has been suggested (Sando et al., 1979a,b). Molybdate interactions with sulfhydryl groups or phosphate moieties of the receptor have also been proposed (Leach et al., 1979; Nishigori & Toft, 1980). Our results demonstrate that molybdate interacts directly and exclusively with the nonactivated form of the receptor to influence its conformational state. This is indicated by the effects of molybdate on the equilibrium and kinetics of estradiol binding by the receptor and the differential salting out of the receptor from the cytosol.

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