Inhibition of Progesterone Receptor Activation by Sodium Molybdate†

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ABSTRACT: The effects of sodium molybdate on the cytosol progesterone receptor from the avian oviduct were examined. Sodium molybdate will retard denaturation of the receptor by elevated temperature. However, this stabilizing effect is evident only before the receptor has been activated or transformed to the state which can bind to nuclei, DNA–cellulose, phosphocellulose, or ATP–Sepharose. When molybdate is added to freshly prepared receptor, it blocks the receptor activation process that occurs with incubation at 23 °C. However, only a minor inhibitory effect is evident when the receptor is activated by exposure to high salt concentrations. The inhibitory effect of molybdate on receptor activation is easily reversed when the compound is removed by dialysis. Activation of the progesterone receptor at 23 °C is accompanied by a change in its size. The nonactivated receptor sediments on sucrose gradients as 6–8S aggregates, but a 4S species is observed after activation. This apparent disaggregation of the receptor is also blocked by molybdate. Although molybdate is a potent phosphatase inhibitor, several other such compounds, including levamisole, fluoride, phosphate, and arsenate, do not inhibit receptor activation. The activation process is inhibited by tungstate and vanadate, indicating that this effect on the receptor is specific for those compounds that are chemically similar to molybdate. While the mechanism of action of these inhibitors remains unknown, they present very interesting probes for studying the process of steroid receptor activation.

It is now well-known that when steroid receptors are initially extracted in the tissue cytosol, they do not have the capacity to bind to nuclear sites. However, the nuclear binding activity can be generated in vitro by incubating the receptor (plus hormone) at elevated temperatures or under high ionic conditions. After this activation[1] process, the avian progesterone receptor is able to bind to isolated nuclei or chromatin (Spelsberg et al., 1971; Lohmar & Toft, 1975; Buller et al., 1975) and also acquires the ability to bind to DNA–cellulose (Schrader et al., 1972), phosphocellulose (Schrader et al., 1975), and ATP–Sepharose (Miller & Toft, 1978). While it is generally assumed that the binding of steroid promotes conformational changes in the receptor that lead to the activated state, little is known concerning chemical or physical changes that might occur during the receptor activation process.

Over the past few years, we have used various chemical probes to characterize the avian progesterone receptor. Several agents such as *o*-phenanthroline, rifamycin AF/013, and pyridoxal 5′-phosphate have been identified as inhibitors of progesterone receptor binding to isolated nuclei or to ATP–Sepharose (Lohmar & Toft, 1975; Toft et al., 1976; Nishigori et al., 1978). A similar attempt is being made to identify compounds which alter the receptor activation process. Such compounds could provide very useful clues about the biochemical events in receptor activation.

Recently, Nielson et al. (1977a,b) described the stabilization of glucocorticoid receptor by sodium molybdate. The present study illustrates the effects of this compound on the avian progesterone receptor. We have found that sodium molybdate not only stabilizes the progesterone receptors but also blocks the receptor activation process that is induced by elevated temperature. A preliminary report of these findings has been presented (Toft & Nishigori, 1979; Moudgil et al., 1979).

Experimental Procedures

[1,2-$^3H_2$]Progesterone (50 Ci/mmol) and [^{14}C]formaldehyde (10 Ci/mmol) were obtained from New England Nuclear; progesterone, cortisol, diethylstilbestrol, aminophylline, l-tryptophan, and l-phenylalanine were from Sigma; sodium arsenate was from Baker Chemical Co.; dithiothreitol was from Biochemical Laboratories, Inc.; glycerol was from Eastman Kodak; Tris and sucrose (density gradient grade) were from Schwarz/Mann. l-*p*-Bromotetramisole oxalate was from Aldrich Chemical Co., Inc.; *p*-nitrophenyl phosphate disodium salt was from Boehringer Mannheim; l-homoarginine was from Calbiochem. Sodium salts of metavanadate, tungstate, and chromate were from Alfa Div., Ventron Corp. Levamisole (Tramisol, l-tetramisole) was from American Cyanamid Co. Sodium molybdate and other reagents were purchased from Fisher Scientific Co. Distilled/deionized water was used to prepare all reagents.

Buffers. The following buffers were used: Tris buffer I, 50 mM Tris, 10% (v/v) glycerol, 5 mM dithiothreitol, and 10 mM KCl; Tris buffer II, 10 mM Tris and 10% glycerol; Tris buffer III, 10 mM Tris, 10% glycerol, 25 mM KCl, and 5 mM dithiothreitol; Tris buffer IV, 10 mM Tris, 10% glycerol, 5 mM dithiothreitol, and 1 mM EDTA. All buffers were pH 8.0 at 23 °C.

Preparation of [^3H]Progesterone-Labeled Receptor. Oviducts were removed from White Leghorn chicks treated with diethylstilbestrol for 2–4 weeks as described previously (Toft & O'Malley, 1972). The tissue was rinsed in normal saline and then homogenized in 4 volumes of Tris buffer I by using a "Polytron" homogenizer (Brinkmann). The temperature was kept at 0–4 °C. After centrifugation of the homogenate for 10 min at 23000*g*, the supernatant was centrifuged at 84000*g* for 30 min. For convenience, this fraction is termed "cytosol" even though the centrifugation time and speed are somewhat less than normally used to prepare the cytosol fraction.

The cytosol receptor was complexed with progesterone by adding 1/100 volume (in ethanol) of 10^{-6} M [^{3}H]progesterone and 2×10^{-4} M cortisol (to saturate binding sites of corticoid-binding globulin). This preparation was incubated for

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[1] The term activation refers to the in vitro alteration of receptor to the form which binds to nuclei, DNA–cellulose, phosphocellulose, or ATP–Sepharose. The same term has been used by Pratt and co-workers to refer to the change of glucocorticoid receptor from a form which does not bind steroid to a steroid-binding form (Nielson et al., 1977a,b). This could be a point of confusion.

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2 h in ice before further experimentation.

ATP-Sepharose Binding Assay. ATP–Sepharose was prepared as described previously (Moudgil & Toft, 1975). Our preparation contained 2–4 μmol of ATP per mL of packed Sepharose. A batch-type assay was used to measure ATP–Sepharose binding as previously described (Nishigori & Toft, 1979). The assay tubes were prepared and treated in duplicate as follows. Aliquots of reaction mixtures (0.2 mL) were combined with an equal volume of 50% (v/v) ATP–Sepharose in Tris buffer III and incubated at 0–4 °C for 5 min with gentle shaking. The reaction mixtures were then diluted with 2 mL of Tris buffer III and centrifuged at 600*g* for 5 min. The pellets were washed once with 2 mL of Tris buffer III. For extraction of bound receptor, the pellets were resuspended in 1.0 mL of Tris buffer III containing 1 M KCl and incubated for 10 min at 0–4 °C. After centrifugation, 0.5 mL of the supernatant was mixed with 5 mL of scintillation cocktail I to determine radioactivity. All experiments included incubation tubes for background determination which were prepared and treated exactly as described above, but using nonactivated receptor samples that were not exposed to elevated temperature or high ionic conditions. The background values were ~10% of the maximal binding values (see zero time points for Figures 2 and 5) and were normally subtracted to provide the illustrated data.

Charcoal Adsorption Binding Assay. Progesterone–receptor complex was quantitated in samples by using the charcoal adsorption method as described previously (Toft & O'Malley, 1972). Briefly, 0.5 mL of dextran–charcoal suspension (0.25% charcoal and 0.025% dextran T-70 in Tris buffer IV) was added to 0.2–0.5 mL of reaction mixture. Following incubation in ice for 5 min, the samples were centrifuged for 5 min. The supernatants were mixed with 5 mL of scintillation cocktail I to determine radioactivity. All assays were performed in duplicate and included background determinations (plus a 1000-fold excess of unlabeled progesterone) which were generally less than 5% of the total radioactivity.

Sucrose Gradient Analysis. Linear 5–20% sucrose gradients (4.5 mL) in Tris buffer IV (without glycerol) containing 10 mM KCl for low salt gradients or 300 mM KCl for high salt gradients were prepared by the layering diffusion method (Stone, 1974). Three 1.5-mL layers of sucrose solution were prepared containing 22, 12.1, and 2.2% sucrose (w/v). The layers were allowed to diffuse in the horizontal position for 2 h at 23 °C, and the tubes were then chilled for 5 h at 4 °C. Receptor preparation (0.25 mL) mixed with [^{14}C]ovalbumin (Rice & Means, 1971) was layered on the gradients which were then centrifuged at 149000*g* for 16 h, 4 °C. The fractions were collected by piercing the bottom of each tube. Radioactivity from [^{3}H]progesterone was first determined in 5 mL of scintillation cocktail I, where [^{14}C]ovalbumin remained in the aqueous phase. Ten milliliters of scintillation cocktail II was then added to each vial, and the dissolved [^{14}C]ovalbumin was determined. This procedure, with appropriate channel selection, assured a quantitative determination of ^{14}C and ^{3}H without cross-interference.

Phosphatase Assay. Phosphatase activity was determined under the same conditions used for receptor analysis (Tris buffer I, pH 8.0, plus 10^{-8} M progesterone and 2×10^{-6} M cortisol). Cytosol aliquots (0.1 mL) were mixed with 0.05 mL of Tris buffer II containing inhibitors as indicated (adjusted to pH 8.0) and incubated for 30 min at 0–4 °C. After addition of 0.05 mL of 20 mM *p*-nitrophenyl phosphate, incubation was carried out for 30 min at 23 °C. The samples were then chilled in ice and mixed with 1 mL of Tris buffer II, and the absorption at 410 nm was determined.

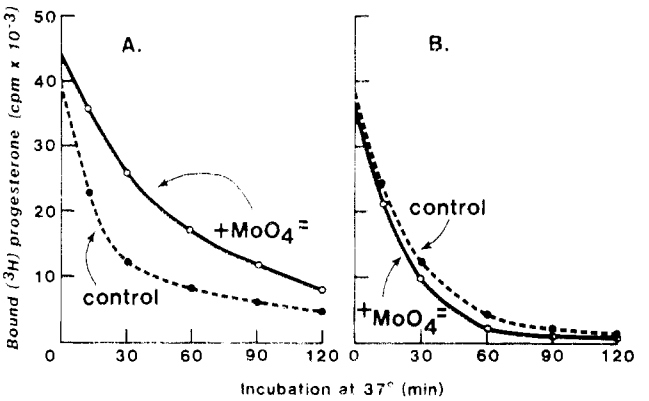

FIGURE 1: Stabilization of the progesterone receptor at 37 °C by sodium molybdate. Chick oviduct cytosol was incubated for 2 h at 4 °C with 10^{-8} M [^{3}H]progesterone and 10^{-6} M cortisol. (A) Treatment of nonactivated receptor. Assay tubes were prepared containing 0.1 mL of cytosol plus 0.4 mL of buffer with or without the addition of 10 mM sodium molybdate. The samples were incubated for 30 min in ice and then transferred to 37 °C for the times indicated. They were then chilled in ice, and the amount of hormone–receptor complex was measured by charcoal adsorption. (B) Treatment of activated receptor. After the 2-h labeling period, the cytosol was incubated at 23 °C for 30 min to activate the receptor. The cytosol was then chilled in ice, and assay groups were prepared and treated exactly as described in (A).

Other Methods. Radioactivity was determined by combining the sample with 5 mL of either scintillation cocktail I [toluene and Scintiprep I, 95:4 (v/v)] or scintillation cocktail II [toluene, Triton X-100, and Scintiprep I, 190:99:8 (v/v/v)]. The counting efficiency for tritium was 48% with cocktail I and 33% with cocktail II on a Beckman LS 250 liquid scintillation counter. All experiments were performed 2–4 times with complete reproducibility.

Results

Effect of Molybdate on Stability. Sodium molybdate has been used as a stabilizing agent in studies on the glucocorticoid receptor (Nielson et al., 1977a,b). Cytosol samples were incubated at 37 °C in the presence or absence of 10 mM sodium molybdate to see if a similar effect could occur with the avian progesterone receptor (Figure 1A). As noted previously (Buller et al., 1975), the cytosol receptor is very unstable at 37 °C. However, when sodium molybdate is added, the loss of progesterone binding activity is considerably more gradual.

Since temperature elevation activates the progesterone receptor, it was of interest to see if sodium molybdate could stabilize both the nonactivated and activated forms of the receptor. Figure 1B shows the effect of 37 °C incubation on cytosol receptor that has been first activated by incubation at 23 °C for 30 min (Lohmar & Toft, 1975; Buller et al., 1975). In this case, sodium molybdate has no stabilizing effect on the activated receptor. Similar results were observed when the receptor was first activated by incubation in 0.3 M KCl for 1 h at 4 °C (results not shown). Therefore, it appears that sodium molybdate is able to stabilize the receptor only when added before receptor activation.

Effects on Receptor Activation. The above results indicated that molybdate acts selectively on the nonactivated form of the receptor and may therefore alter the receptor activation process. For a test of this possibility, sodium molybdate was added to the cytosol at various times during the course of receptor activation at 23 °C (Figure 2). The extent of receptor activation was determined by quantitating the amount of receptor that could bind to ATP–Sepharose. Previous

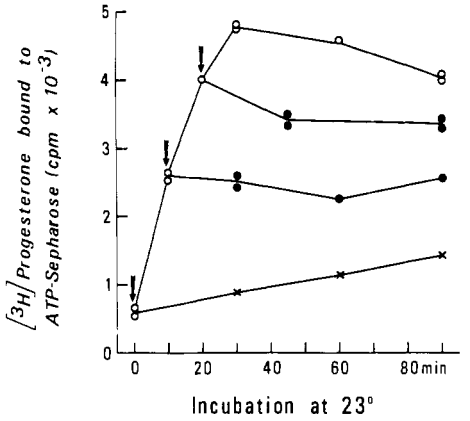

FIGURE 2: Inhibition of receptor activation by sodium molybdate. Chick oviduct cytosol was prepared and labeled as described under Experimental Procedures. Aliquots (0.1 mL) were incubated at 23 °C for the times indicated. 0.1 mL of 20 mM sodium molybdate in Tris buffer II was added to some samples after 0, 10, or 20 min of incubation (indicated by arrows). 0.1 mL of Tris buffer II was added to the control samples at zero time. Following incubation at 23 °C, the samples were chilled in ice and the amount of receptor that could bind ATP–Sepharose was determined as a measure of receptor activation (see Experimental Procedures). Control (○); molybdate at zero time (×); molybdate at 10 or 20 min (●).

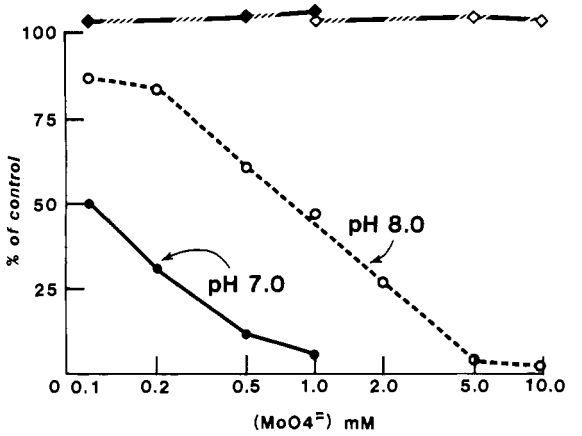

FIGURE 3: Concentration dependency for molybdate inhibition of receptor activation. Chick cytosol was prepared and labeled by the standard procedure (pH 8.0). One portion was maintained at pH 8.0, and the second was adjusted to pH 7.0 with HCl. Both portions were diluted 1.5-fold by the addition of Tris buffer II (pH 7.0 or 8.0). Cytosol aliquots (0.15 mL) were mixed with 0.05 mL of Tris buffer II plus molybdate (pH 7.0 or 8.0) as indicated (final concentration), and these were incubated for 30 min at 23 °C. The samples were then chilled in ice, and the amount of receptor that could bind ATP–Sepharose was measured. Parallel samples were treated in the same way, and [^{3}H]progesterone binding was measured by charcoal adsorption. ATP–Sepharose binding, pH 7.0 (●); ATP–Sepharose binding, pH 8.0 (○); progesterone binding, pH 7.0 (♦); progesterone binding, pH 8.0 (◇).

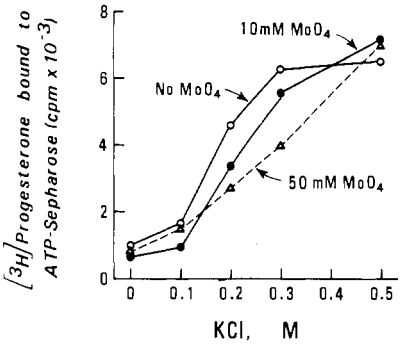

FIGURE 4: Effect of molybdate on receptor activation by salt. Cytosol labeled with [^{3}H]progesterone was divided into 0.5-mL aliquots. These were mixed with 0.5 mL of Tris buffer II plus molybdate to give final concentrations of 0, 10, or 50 mM molybdate. After incubation for 1 h at 4 °C, 0.5 mL of KCl (plus 0, 10, or 50 mM molybdate) was added to provide the final KCl concentrations indicated. Incubation was continued for 1 h at 4 °C to allow receptor activation. Each sample was then dialyzed in Tris buffer II (plus 0, 10, or 50 mM molybdate) for 2 h, 4 °C, to remove the KCl. Finally, the amount of activated receptor was measured in 0.3-mL aliquots of each sample by binding to ATP–Sepharose.

studies have shown that only the activated receptor can bind to this affinity resin (Miller & Toft, 1978). Sodium molybdate (10 mM) clearly inhibits receptor activation when it is added before or during the course of activation at 23 °C. This inhibition appears to occur immediately upon addition of the compound. Once the receptor is in the activated form, its binding to ATP–Sepharose is not altered by molybdate. This is shown by the delayed addition of molybdate after 10 or 20 min at 23 °C (Figure 2). Therefore, molybdate does not affect receptor binding to ATP–Sepharose directly nor does it reverse the activation process once it has occurred.

The concentration dependency for this molybdate effect on activation is illustrated in Figure 3. Under the conditions normally employed, a 50% inhibition occurs with 1 mM molybdate and it is maximum with a 5 mM concentration. However, if the incubations are conducted at pH 7 instead of pH 8, the sensitivity toward molybdate inhibition is increased 10-fold. Thus, the molybdate interaction is very dependent upon the pH of the reaction mixture. Figure 3 also shows that molybdate has no effect on steroid binding under these conditions. Additional experiments have shown that molybdate has no effect on the previously activated receptor at pH 7 (results not shown). Even though molybdate is more effective at pH 7, we have continued our studies at pH 8. The inhibition is readily apparent at this pH, and the receptor is more stable at pH 8.

In these studies, ATP–Sepharose binding was used as a measure of receptor activation because of the efficiency and convenience of this technique (Miller & Toft, 1978; Nishigori & Toft, 1979). However, identical results were observed when receptor activation was measured by binding to isolated oviduct nuclei, DNA–cellulose, or phosphocellulose (results not shown). Therefore, we believe that the results illustrated here accurately reflect the receptor activation process as it occurs in a cell-free system.

In addition to temperature elevation, the progesterone receptor can be activated by exposure to high salt concentrations (Buller et al., 1975; Miller & Toft, 1978). In fact, this is the more efficient method for activation. It was therefore of interest to test the effect of molybdate on this method of activation. Aliquots of cytosol were incubated for 1 h in ice in the presence of various concentrations of KCl, and the extent of receptor activation was determined by ATP–Sepharose binding (Figure 4). At the intermediate salt concentrations (0.1–0.3 M KCl) 10 or 50 mM sodium molybdate was somewhat inhibitory. However, this was not evident in the presence of 0.5 M KCl. Additional experiments have shown that the extent of inhibition under high salt conditions cannot be improved by increasing or decreasing the molybdate concentration within the range of 1–100 mM. Molybdate at 100 mM is actually less effective than at 50 mM, perhaps because of the additional ionic strength. Therefore, salt activation is only partially blocked by molybdate and the inhibitor can be overcome completely if the salt concentration is sufficiently high. It is possible that the action of molybdate involves ionic interactions that are diminished by high salt conditions. While this may be the case, we have tested the inhibitory effect of

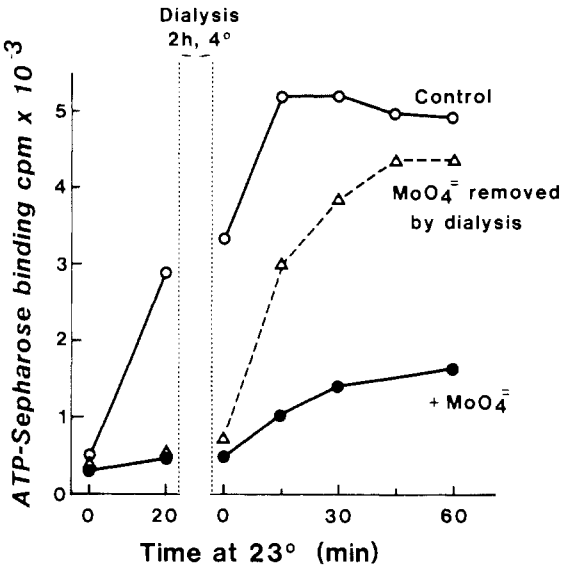

FIGURE 5: Reversal of molybdate inhibition by dialysis. Cytosol was prepared and labeled with [^{3}H]progesterone as usual. Aliquots (1.5 mL) were mixed with 1.5 mL of Tris buffer II with or without 5 mM sodium molybdate (final concentration). After 1 h of incubation at 4 °C, the samples were brought to 23 °C for 20 min. They were then chilled and dialyzed for 2 h at 4 °C in buffer (25 mM Tris, 10% glycerol, and 5 mM dithiothreitol, pH 8.0) with or without 5 mM molybdate. The samples were again incubated at 23 °C for 0–60 min and then chilled in ice. The amount of activated receptor was determined throughout the period of treatment in 0.2-mL aliquots by ATP-Sepharose binding. Control without molybdate (○); samples with molybdate throughout the experiment (●); samples with molybdate that was removed by dialysis in the absence of molybdate (△).

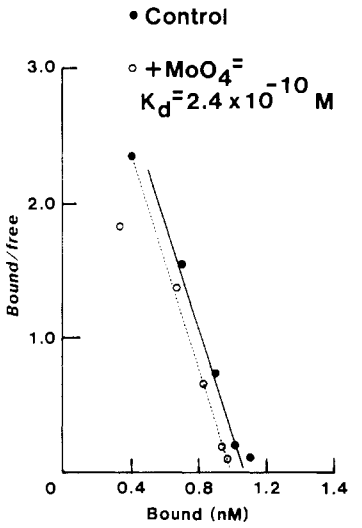

FIGURE 6: Comparison of control and molybdate-treated receptor by Scatchard analysis. Hormone binding was measured by preparing two series of incubation tubes (in duplicate) containing 0.05 mL of cytosol plus 6×10^{-10}–1.2×10^{-8} M [^{3}H]progesterone in a total volume of 0.5 mL. One series of tubes also contained 10 mM sodium molybdate, and all tubes contained 10^{-7} M cortisol. Parallel tubes containing 10^{-6} M unlabeled progesterone were used for background determination. After 16 h at 4 °C, binding was measured by the charcoal adsorption method.

molybdate on phosphatase activity in oviduct cytosol under various salt conditions (see Experimental Procedures). This inhibition was not diminished by the addition of 0.1–0.5 M KCl (results not shown).

The reversibility of molybdate inhibition of receptor activation at 23 °C was tested by dialysis (Figure 5). Receptor samples were incubated with or without molybdate for 20 min at 23 °C. The samples were then chilled in ice and dialyzed for 2 h at 4 °C. They were then elevated to 23 °C to continue the activation process. The period of dialysis did not alter the degree of receptor activation significantly. However, after dialysis the receptor that had been inhibited by molybdate could be activated to a degree which approached that of the control receptor. Receptor that was dialyzed in the presence of molybdate remained in the inhibited or nonactivated state.

Properties of the Inhibited Receptor. Efforts were made to identify any additional alterations in the receptor that might result from molybdate treatment. The progesterone binding properties of molybdate-treated and control (nonactivated) receptor were compared by Scatchard analysis (Scatchard, 1949) as shown in Figure 6. No difference was observed either in the binding affinity or capacity. Therefore, molybdate does not seem to alter the steroid binding region of the receptor at 0 °C even though it has a stabilizing effect which can be measured through steroid binding (Figure 1).

Figure 7 illustrates the sedimentation of progesterone receptor on 5–20% sucrose gradients. Under low ionic conditions, the nonactivated receptor migrates as aggregates in the 6–8S region (Figure 7A). However, after activation at 23 °C, the receptor changes to a 4S form. This apparent disaggregation of the receptor during activation is blocked by molybdate, and the inhibited receptor remains in the aggregated state (Figure 7B). In the presence of molybdate, the proportion of 8S to 6S receptor is generally somewhat higher than in control

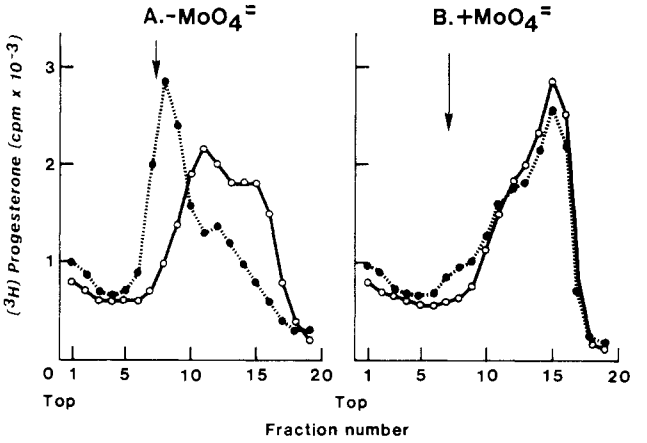

FIGURE 7: Sedimentation analysis of control and molybdate-treated receptor. Cytosol labeled with [^{3}H]progesterone was prepared as usual. Two portions were prepared with or without 10 mM sodium molybdate. Half of each portion was kept at 4 °C, and the other half was incubated for 30 min at 23 °C. Aliquots (0.25 mL plus 10 μL of [^{14}C]ovalbumin) were layered on 5–20% sucrose gradients and centrifuged for 16 h at 149000*g*. Sedimentation of the standard, [^{14}C]ovalbumin (3.7 S), is indicated by the arrow. (A) Without molybdate at 4 (○) and 23 (●) °C. (B) With molybdate at 4 (○) and 23 (●) °C.

nonactivated preparations. Under high ionic conditions (0.3 M KCl), the nonactivated, activated, and "inhibited" samples all migrate as 3.5S species (not shown). However, since all samples are exposed to high salt, they would be expected to actually be in the activated form.

Phosphatase Inhibitors. Since molybdate is a potent phosphatase inhibitor, it is possible that this action is involved in blocking receptor activation. This mode of action has been suggested from previous studies on the glucocorticoid receptor (Nielson et al., 1977a,b). For this reason, several other phosphatase inhibitors were tested (Table I). The effects of these compounds on receptor activation and on general phosphatase activity in the oviduct cytosol were tested under identical conditions. While it is known that all phosphatase activities are not blocked by any one inhibitor (Van Belle,

Table I: Effect of Phosphatase Inhibitors on Receptor Activation

inhibitor (mM)	ATP-Sepharose binding of PR[a] (% of control)	cytosol phosphatase act. (% of control)
levamisole (1)	107	114
1-*p*-bromotetramisole (1)	103	63
l-phenylalanine (10)	112	102
l-tryptophan (10)	109	97
homoarginine (10)	111	86
aminophylline (10)	126	48
NaN_3 (10)	112	80
NaF (10)	99	43
K_2HPO_4 (20)	107	16
Na_2HAsO_4 (10)	113	3
Na_2CrO_4 (10)	110	33
Na_2MoO_4 (10)	3	15
Na_2WO_4 (10)	2	7
$NaVO_3$ (1)	27	0

[a] PR = progesterone receptor.

1972), this is particularly true of the first five compounds in Table I. For example, levamisole and bromotetramisole inhibit alkaline phosphatase of most tissues of the rats, but they do not inhibit the enzyme in rat intestine (Borgers, 1973). In the human, homoarginine blocks alkaline phosphatase from bone or liver but not from intestine or placenta (Lin & Fishman, 1972). The first seven compounds in Table I were, at best, weak phosphatase inhibitors in the oviduct cytosol, even though the concentrations used were high and should have blocked enzymes that were sensitive to the inhibitors. These compounds also showed no inhibitory effect on receptor activation. However, cytosol phosphatase activity was clearly inhibited by fluoride, arsenate, and phosphate but these compounds did not reduce receptor activation.

Since arsenate is an inhibitor of several enzymes that involve phosphate metabolism, it was studied through a range of concentrations (Figure 8). This compound almost completely blocks cytosol phosphatase activity when a 10 mM concentration is used (Figure 8). However, concentrations in the range of 1–10 mM have no effect on receptor activation (Figure 8).

In addition to molybdate, two compounds, tungstate and vanadate, were found to be potent inhibitors of both phosphatase activity and receptor activation (Table I). However, chromate had no effect on receptor activation even though it inhibited phosphatase activity. The concentration dependencies for tungstate and vanadate are illustrated in Figure 9. Metavanadate is approximately fivefold more potent than molybdate, and an intermediate potency is displayed by tungstate. These results argue that the inhibition of receptor activation depends upon the common physicochemical properties of molybdate, tungstate, and vanadate and not upon the general property of these compounds as phosphatase inhibitors.

Discussion

The present study was based upon the earlier observations of Pratt and co-workers (Nielson et al., 1977a,b), who observed a stabilizing effect of molybdate on the glucocorticoid receptor. The glucocorticoid receptor is very unstable and is easily converted to a form which is unable to bind steroid. This loss of steroid binding is blocked effectively by molybdate. Our studies show that molybdate not only stabilizes the avian progesterone receptor but also blocks the transformation or activation process by which the receptor is converted to the "nuclear" form. In more recent studies, Pratt and co-workers

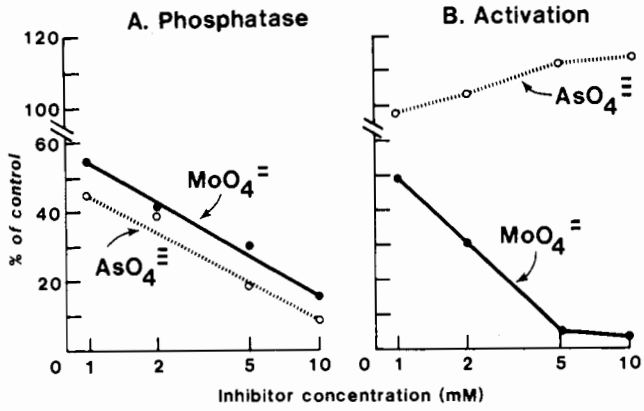

FIGURE 8: Effects of arsenate and molybdate on phosphatase activity and receptor activation. Aliquots (0.1 mL) of labeled cytosol were mixed with 0.1 mL of Tris buffer II plus arsenate or molybdate as indicated (final concentration). The samples for testing receptor activation were incubated for 1 h at 4 °C and then for 30 min at 23 °C. After being chilled in ice, the samples were analyzed for binding to ATP–Sepharose. Additional samples were analyzed for phosphatase activity (see Experimental Procedures). (A) Phosphatase activity: plus arsenate (○); plus molybdate (●). (B) ATP–Sepharose binding (receptor activation): plus arsenate (○); plus molybdate (●).

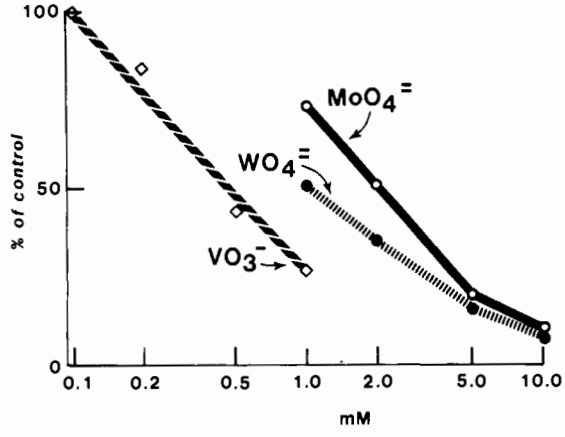

FIGURE 9: Inhibition of receptor activation by vanadate, tungstate, and molybdate. Aliquots (0.1 mL) of labeled cytosol were mixed with 0.1 mL of Tris buffer II plus inhibitor to make the final concentrations indicated. The samples were incubated for 1 h at 4 °C and for 30 min at 23 °C. The samples were then chilled in ice, and the amount of activated receptor was measured by binding to ATP–Sepharose. The results are expressed as a percentage of the control (without inhibitor). Molybdate (○); tungstate (●); metavanadate (□).

have also found that molybdate inhibits transformation of the glucocorticoid receptor to the form which binds to DNA–cellulose (Dahmer et al., 1979; Leach et al., 1980). Therefore, the effects of this compound on two classes of steroid receptors from avian and mammalian sources are quite similar.

It now appears that the stabilizing action of molybdate may be a universal effect on steroid receptors. Molybdate has recently been shown to stabilize aldosterone receptors in the rat kidney (Grekin & Sider, 1979), androgen receptors in the rat prostate (Tremblay et al., 1979), glucocorticoid receptors in mouse mammary tissue (McBlain and Shyamala, personal communication), and vitamin D receptors in rat intestinal mucosa (McCain et al., 1979). Whether molybdate also blocks receptor activation in these systems remains unknown.

At the present time, there is no information on whether molybdate acts on the receptor directly or indirectly through other cytosol components. This is a difficult problem to address since the inhibitor apparently affects only the nonactivated receptor form that must be studied in freshly prepared cytosol. Efforts to purify steroid receptors in the nonactivated

state have been quite unsuccessful. However, the use of molybdate or similar compounds to stabilize this receptor form may offer new advantages for its purification.

Molybdate has been known as a potent phosphatase inhibitor for many years. It inhibits acid phosphatase from a wide variety of sources (Courtois & Bossard, 1944; Bossard, 1947; Courtois & Anagnostopoulos, 1948; Spencer, 1954; Van Etten et al., 1974). Its action is reversible and, at least in some cases, its action has been shown to be competitive with regard to the substrate (Spencer, 1954; Van Etten et al., 1974). Molybdate has also been shown to inhibit cell surface phosphatases of yeast (Rothstein & Meier, 1949), glucose-6-phosphatase from rat liver (Nordlie & Arion, 1964), 6-phosphogluconate dehydrogenase from *Candida utilis* (Rippa et al., 1978), and phosphoprotein phosphatase from mouse liver (Paigen, 1958) and bovine tracheal smooth muscle (Poietta & Sands, 1978). The action of molybdate is somewhat selective since there are a wide variety of enzymes that are not affected by this inhibitor (Bossard, 1947). Tungstate and vanadate are also potent phosphatase inhibitors (Van Etten et al., 1974; Lopez et al., 1976). However, the common physicochemical features of these compounds may be more important factors with regard to receptor inhibition.

Since molybdate is a very effective inhibitor of phosphatase, its action on the steroid receptor may also involve phosphatase inhibition. For example, a dephosphorylation of the receptor or a component associated with the receptor may occur during the activation process. This possibility has been suggested previously by Pratt and co-workers (Nielson et al., 1977a,b; Sando et al., 1979a,b). However, activation of the progesterone receptor is not blocked by many other phosphatase inhibitors. Therefore, if a phosphatase is involved in progesterone receptor activation, it seems to be sensitive only to inhibitors that are chemically similar to molybdate. At this point, several other possibilities must also be considered. For example, an interaction of molybdate with heavy metals has been postulated in its inhibitory effect on particulate phosphoprotein phosphatase from mouse liver (Paigen, 1958). A heavy-metal interaction could be involved in a variety of protein activities, and there is some evidence that steroid receptors may be metalloproteins (Shyamala, 1975; Lohmar & Toft, 1975). Also, molybdate may modify protein activities through an interaction with amino acid residues. Molybdate has been shown to interact with the thiol group of cysteine and the imidazole group of histidine (Weathers et al., 1979).

Another possibility is that molybdate could complex phosphate groups which may reside on the receptor or on a component associated with receptor activity. This has been recently suggested by Pratt and co-workers as a possible mechanism for molybdate action on glucocorticoid receptor (Leach et al., 1980). Molybdate, tungstate, and vanadate are all capable of forming heteropoly structures with inorganic phosphate at acid pH (Cotton & Wilkinson, 1972). We are not aware of any well-defined interactions of these compounds with phosphate under alkaline conditions, but suggestive evidence for such interactions has been reported by Leach et al. (1980).

Whatever its mechanism of action, molybdate should be an important probe in analyzing receptor activation. Molybdate, tungstate, and vanadate are the only known inhibitors of this process. The fact that molybdate blocks activation of the avian progesterone receptor by elevated temperature but not by high salt suggests that these two means of activation are not identical even though the final results seem to be the same. The use of inhibitors might provide important clues regarding this and other aspects of steroid receptor activation.

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Effect of Chemical Perturbation with NaSCN on Receptor–Estradiol Interaction. A New Exchange Assay at Low Temperature†

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ABSTRACT: When 0.5 M sodium thiocyanate is added to uterine cytosol previously labeled with excess [^{3}H]-17β-estradiol, no change can be detected in the steady-state cytosol concentration of [^{3}H]estradiol–receptor complex for at least 20 h at 4 °C. However, the rate of exchange of bound estradiol in the presence of NaSCN was found to be substantially higher than that in the absence of the chaotropic salt. In the presence of NaSCN, the dissociation rate of the complex increases about 10-fold (K_{-1}^{SCN} = 1.10 × 10^{-2} min^{-1} vs. K_{-1} = 1.07 × 10^{-3} min^{-1}) while the rate of association increases about 2-fold (K_1^{SCN} = 1.2 × 10^7 min^{-1} M^{-1} vs. K_1 = 7.4 × 10^6 min^{-1} M^{-1}). The K_d changes 6.4-fold (K_d^{SCN} = 9 × 10^{-10} M vs. K_d = 1.4 × 10^{-10} M) with no decrease in the number of binding sites as shown by Scatchard plots of saturation experiments. This effect of NaSCN can be exploited to assay preformed estrogen–receptor complex by exchange with [^{3}H]estradiol at low temperature. When the sample containing preformed complex is incubated overnight (16 h) at 4 °C with excess [^{3}H]estradiol in the presence of 0.5 M NaSCN, there is a quantitative exchange of nonlabeled for labeled estradiol without loss of binding sites. Hormonal steroids other than estrogens do not interfere, and the exchanged estradiol is bound with high affinity. Precision, accuracy, and linearity of the method are highly satisfactory.

Specific ion effects on macromolecules have been recognized since Hofmeister (1888) noted that salts differ greatly in their ability to salt out proteins. A wide variety of salt effects on biological systems have since been found, and specifically those ions that are the most effective precipitants lead to folding, coiling, and aggregation while those least effective promote unfolding, extension, and dissociation. The latter ions have been termed "chaotropic" (tending to disorder) by Hamaguchi & Geiduschenk (1962). The effects of chaotropic ions have been interpreted as weakening or disrupting the hydrophobic bonding and at the same time as affecting hydrogen bonding and electrostatic attractions (Dandliker et al., 1967; Dandliker & de Saussure, 1971). Such salts are known to increase the solubility of nonpolar molecules in water, and thermodynamic evidence suggests that they make water more "disordered" or lipophilic (Hatefi & Haustein, 1969; Haustein et al., 1971).

Contrary to agents like urea, guanidine hydrochloride, or detergents, chaotropic agents produce dissociation of proteins at concentrations which do not cause major shifts in protein conformation (Swayer & Puckridge, 1973).

Recently, we have used chaotropic salts to prevent the age-dependent aggregation of estradiol receptor in cytosol. We have found that sodium thiocyanate up to 0.5 M is compatible with a stable estradiol–receptor complex during sucrose gradient centrifugation; however, the maximum permissible concentration is 0.1 M during Sephadex G-100 and G-200 chromatography. In fact, at higher thiocyanate concentrations the estradiol–receptor complex dissociates (Sica et al., 1976). This strongly suggests that chaotropic salt concentrations higher than 0.1 M weaken the binding of estradiol to the receptor. In this paper, we have investigated the kinetics of association and dissociation of the estradiol–receptor complex in the presence of sodium thiocyanate and have found that both association and dissociation rates are increased. Furthermore, we have investigated the possibility of using this increased turnover to facilitate the exchange of radioactive estradiol into binding sites previously filled with "cold" estradiol.

Materials and Methods

Materials. All reagents were of analytical grade. NaSCN (ACS) was purchased from C. Erba. [6,7-$^3H_2$]-17β-Estradiol

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