Uterine Progesterone Receptor: Stabilization and Physicochemical Alterations Produced by Sodium Molybdate†

Tong J. Chen,‡ Richard G. MacDonald, and Wendell W. Leavitt*

ABSTRACT: Incubation of hamster uterine cytosol with millimolar concentrations of sodium molybdate prior to addition of labeled steroid increased recovery of progesterone receptor 2-fold. This stabilizing effect of molybdate was also manifest on gel electrophoresis of the receptor. In the absence of molybdate, no specific [^{3}H]progesterone binding was detectable on polyacrylamide gel electrophoresis. But, in the presence of 5 mM sodium molybdate, a [^{3}H]progesterone-binding species was clearly evident on the gels. The radioactivity associated with this binder was displaceable by unlabeled progesterone but not by cortisol and depended on the concentration of [^{3}H]progesterone employed, suggesting that this binding species is a progesterone receptor. Molybdate treatment produced a small increase in receptor size on low-salt sucrose gradients (from 6–7 S to 7.5 S). There was no effect of molybdate on receptor sedimentation in the presence of high salt (0.3 M KCl). Further analysis of this phenomenon by gel filtration suggested that this molybdate-mediated increase in receptor size was due to receptor aggregation. In low-salt buffers, molybdate treatment markedly increased the proportion of receptors contained in large aggregates (Stokes radius >8.0 nm). Again, this effect was abolished in the presence of high salt. In conjunction with receptor stabilization, molybdate prevented binding of uterine progesterone receptor to DNA–cellulose. These findings suggest that sodium molybdate stabilizes the unliganded, unactivated form of the receptor. Moreover, these effects seem to be mediated through a direct interaction of molybdate with the receptor, one which results in receptor aggregation.

It is generally accepted that steroid hormone effects are mediated in target cells by hormone-specific intracellular receptors (Buller & O'Malley, 1976). Critical to our understanding of the mechanism of steroid hormone action are purification and physicochemical characterization of these receptors. These goals have often been hindered by the lability of receptor molecules under cell-free conditions. Recent evidence from several laboratories suggests that certain agents, including pyridoxal phosphate (Nishigori & Toft, 1979; Toft & Nishigori, 1979), sodium fluoride (Nielsen et al., 1977a; Grody et al., 1980), and sodium molybdate (Nielsen et al., 1977a,b; Leach et al., 1979; Nishigori & Toft, 1980; McBlain & Shyamala, 1980), can stabilize receptors from inactivation at elevated temperatures and during prolonged incubation in vitro. Previous work from our laboratory has demonstrated the presence of progesterone receptors (Rp)[1] in the uterus of the golden hamster (Leavitt et al., 1974). The steroid binding and physicochemical properties of these receptors have been analyzed by saturation with [^{3}H]progesterone, sucrose–glycerol gradient centrifugation, ATP–Sepharose binding, and ion-exchange chromatography on DEAE-cellulose (Leavitt et al., 1974, 1979). Due to the extreme lability of the receptors under cell-free conditions, our previous attempts to characterize the properties of these molecules by using other, more stringent, analytical methods were predominantly unsuccessful. Through the use of sodium molybdate to stabilize the receptors during analysis, appropriate conditions were found for electrophoresis of progesterone receptor in polyacrylamide gels. Additionally, we examined the effects of sodium molybdate on other physicochemical properties of the receptors, namely, sucrose gradient centrifugation, binding to DNA–cellulose, and gel filtration, in an effort to understand the receptor-stabilizing action of molybdate.

Experimental Procedures

Animals. Adult female golden hamsters were kept under controlled conditions with a 14-h light, 10-h dark 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 of cycle day 1). All animals for these studies were killed between 0800 and 1100 h on cycle day 4, since the tissue concentration of uterine cytosol progesterone receptor is maximal during this period (Leavitt et al., 1974).

Preparation of Uterine Cytosol Containing Progesterone Receptors. Hamster uterine cytosol was prepared as described previously (Leavitt et al., 1974). Uterine tissue was homogenized in 2 or 4 volumes (v/w) of buffer with a Polytron Pt-10 homogenizer (Brinkmann). The tissue homogenate was centrifuged at 170000*g* for 1 h to yield the cytosol fraction. These and all subsequent procedures were conducted at a temperature of 0–4 °C. The following buffers were used for homogenizing the tissue: *barbital buffer*, 20 mM barbital (pH 8.0 at 22 °C), 5 mM dithiothreitol, and 15% (v/v) glycerol; *Tris buffer* B_{10}, 10 mM Tris (pH 7.5), 1 mM EDTA, 12 mM monothioglycerol, and 10% (v/v) glycerol; *phosphate buffer*, 50 mM sodium phosphate (pH 7.5), 10 mM monothioglycerol, and 10% (v/v) glycerol. The barbital buffer, which is similar to that used by Nishigori & Toft (1979), excluding 10 mM KCl, was found to be very satisfactory for gel electrophoretic analysis of [^{3}H]progesterone receptor complexes. Phosphate buffer was superior to Tris buffer B_{10} in promoting the binding of [^{3}H]progesterone receptors to DNA–cellulose. Cytosol was preincubated with or without sodium molybdate for the times specified in the figure legends prior to labeling with [^{3}H]-

† From the Worcester Foundation for Experimental Biology, Shrewsbury, Massachusetts 01545. *Received December 1, 1980.* This work was supported by Grant PCM 77-25630 from the National Science Foundation and Grants CA-23362 and HD-15132 from the U.S. Public Health Service.

‡ Present address: Department of Obstetrics and Gynecology, University of Massachusetts Medical Center, Worcester, MA 01605.

[1] Abbreviations used: Rp, progesterone receptor; cRp, cytosol progesterone receptor; DEAE, diethylaminoethyl; EDTA, ethylenediaminetetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate.

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progesterone. After this incubation, [1,2,6,7-$^3H_4$]progesterone (97 Ci/mmol, New England Nuclear) was added in ethanol to a final concentration of 10 nM (final ethanol content 2% v/v). Parallel samples of cytosol were incubated with 10 nM [^{3}H]progesterone plus 4 μM unlabeled progesterone for calculation of the nonspecific steroid binding component. All cytosol samples contained 4 μM unlabeled cortisol to inhibit [^{3}H]progesterone binding to corticosteroid-binding globulin-like molecules present in uterine cytosol (Milgrom & Baulieu, 1970; Faber et al., 1972; Davies & Ryan, 1972; Do & Leavitt, 1978).

Progesterone Receptor Quantification by Scatchard Analysis. Receptor concentrations and steroid binding characteristics were measured by Scatchard analysis of specific [^{3}H]progesterone binding (Scatchard, 1949; Chen & Leavitt, 1979). Aliquots (500 μL) of cytosol prepared at 1:20 (w/v) dilution in barbital buffer were incubated with five concentrations of [^{3}H]progesterone (0.75–12 nM) for determination of total binding. Parallel samples were incubated with these same concentrations of radioactive steroid plus 4 μM unlabeled progesterone. Following an 18–21-h incubation at 0 °C, free and loosely bound steroids were removed by addition of 500 μL of dextran-coated charcoal (0.5 g of Norit A charcoal and 50 mg of Dextran T70 (Pharmacia) per 100 mL of Tris buffer B). After a 40-s incubation with charcoal, the samples were centrifuged for 4 min at 1500g. The supernatant fractions were decanted into scintillation vials for radioactivity measurement. Specific binding, computed by subtracting the amount of bound radioactivity measured in the presence of excess unlabeled steroid from that in its absence, was plotted according to the method of Scatchard (1949). The data were subjected to linear regression analysis for determination of the equilibrium association constant (K_A) for progesterone binding and the binding site concentration. Statistical treatment of the data was by Student's t test.

Sucrose Gradient Centrifugation. [^{3}H]Progesterone receptor complexes were analyzed by sedimentation on 4.5-mL linear 5–20% sucrose gradients prepared in Tris buffer B_{10} with or without 0.3 M KCl, as described before (Chen & Leavitt, 1979). Bovine serum albumin (4.6 S) was employed as a reference for estimation of the receptor sedimentation coefficient by the method of Martin & Ames (1961).

Polyacrylamide Gel Electrophoresis. Electrophoretic analysis of [^{3}H]progesterone receptor complexes under nondenaturing conditions on highly cross-linked polyacrylamide gels was conducted according to a modification of the procedure of Miller et al. (1975). Cylindrical gels (5 × 83 mm) consisting of 5% (w/v) total acrylamide with 15% bis-(acrylamide) were prepared in 0.375 M Tris (pH 8.9) and 10% glycerol. The electrode buffer was 20 mM Tris (pH 8.3), 154 mM glycine, and 10% glycerol. Samples of cytosol (100 μL) prepared in barbital buffer were applied to the top of the gels, with an underlayer (50 μL) of 65 mM Tris–thioglycolate (pH 8.0) and 40% glycerol. Current (2 mA/gel) was applied for 4 h at 2 °C. Following electrophoresis, the gels were sliced into 1.8-mm sections. The slices were placed into scintillation vials, and the radioactivity in each slice was extracted into 0.8 mL of 1.0% sodium dodecyl sulfate with continuous shaking for 16 h.

Gel Filtration. Sephacryl S-300 (Pharmacia) was equilibrated with *Tris buffer A* (50 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 12 mM monothioglycerol) and poured into a 2.5 × 45 cm column. Samples of 3-mL volume in Tris buffer B_{10} (1:4 dilution) were applied and eluted with Tris buffer A with or without 0.5 M KCl at a flow rate of about 30 mL/h. Fractions of 2-mL volume were collected and assayed for radioactivity (1 mL) and protein (0.1 mL). The column void volume (V_0, 62–63.5 mL) was measured with Blue Dextran 2000 (Pharmacia), and the total column volume (V_t, 175 mL) was determined by elution of [^{14}C]leucine. Stokes radii (R_s) of [^{3}H]progesterone receptor complexes were estimated by the method of Laurent & Killander (1964). The following proteins, obtained from Sigma as highly purified preparations, were employed as standards: myoglobin (R_s = 2.0 nm), bovine serum albumin (3.5 nm), yeast alcohol dehydrogenase (4.6 nm), liver catalase (5.2 nm), and ferritin (7.9 nm). Values for Stokes radii of these proteins were taken from Riveros-Moreno & Wittenberg (1972) and Siegel & Monty (1966). Sodium molybdate, when included in the elution buffers at 5 mM concentration, did not change the elution of standard proteins or the column parameters V_t and V_0.

DNA–Cellulose Binding Assay. Binding of [^{3}H]progesterone receptor complexes to DNA was assessed by a modification of the DNA–cellulose batch procedure of Kalimi et al. (1975). DNA–cellulose was prepared from denatured calf thymus DNA (Millipore) and Munktell 410 cellulose (Bio-Rad) by the method of Alberts & Herrick (1971) as modified by Fox & Pardee (1971). Duplicate aliquots (0.1–0.5 mL) of cytosol prepared in phosphate buffer were mixed with pellets from 1 mL of a 50% (w/v) slurry of DNA–cellulose in phosphate buffer. The DNA content of each assay tube was approximately 150 μg, as measured by the method of Burton (1956). The mixtures were incubated at 3 °C for 1 h with occasional agitation. One set of the duplicate samples was then incubated for 15 min at 37 °C to destroy receptor binding, whereas the second set was kept at 3 °C. The DNA–cellulose was then washed twice with 10 mL of phosphate buffer followed by centrifugation at 1500g for 6 min. DNA-bound [^{3}H]progesterone was extracted for 1 h with 1 mL of phosphate buffer containing 0.5 M KCl. The mixtures were centrifuged at 1500g for 6 min, and the supernatant solutions were decanted into counting vials for radioactivity measurement. Specific [^{3}H]progesterone binding to DNA–cellulose was calculated by subtracting the radioactivity extracted from the heat-treated samples from that extracted from the samples kept in the cold. Parallel incubations of cytosol with cellulose alone were employed to correct for a small amount of receptor interaction with cellulose.

General Methods. Protein was measured in cytosol and column fractions by the method of Sedmak & Grossberg (1977) with crystalline bovine serum albumin as standard. Radioactivity was measured in aqueous samples (1.0-mL volume) after addition of 7 mL of scintillation cocktail: toluene–Triton X-100 (2:1 v/v) containing 5 g of diphenyloxazole and 50 mg of 1,4-bis[2-(5-phenyloxazolyl)]benzene per L. Radioactive samples were counted in a Beckman LS-350 liquid scintillation spectrometer in the efficiency range of 25–40% for tritium. Correction for sample quenching was made by use of the external standard of the instrument.

Results

Sodium molybdate increased the recovery of cytosolic uterine progesterone receptor (cRp) measured by saturation with [^{3}H]progesterone after a 4-h incubation at 0 °C in the absence of exogenous steroid (Table I). The stabilizing effect was dependent on the concentration of molybdate employed. Maximal enhancement of receptor recovery (89% increase over cytosol incubated with no molybdate) occurred at a molybdate concentration of 5 mM. Raising the molybdate concentration beyond this level gave no further enhancement of recovery, and actually decreased [^{3}H]progesterone binding relative to

Table I: Effect of Molybdate Concentration on Cytosol Progesterone Receptor Binding[a]

Na_2MoO_4 concn (mM)	cRp (pmol/g of tissue)	K_A ($\times 10^9$ M^{-1})
0	47.5 ± 2.4	0.65 ± 0.04
1.0	52.3 ± 4.6[b]	0.50 ± 0.05[b]
5.0	80.4 ± 4.7[c]	0.53 ± 0.03[b]
10.0	72.3 ± 2.1[d]	0.62 ± 0.03[b]
20.0	56.9	0.55

[a] Aliquots (1 mL) of uterine cytosol were incubated at 0 °C for 4 h with increasing concentrations of sodium molybdate. The concentration of specific [^{3}H]progesterone binding sites (cRp) and equilibrium association constants (K_A) were then measured in each of these solutions by saturation analysis with increasing concentrations of [^{3}H]progesterone (see Experimental Procedures). Values are mean ± SEM of measurements made in three separate experiments. Differences between molybdate-treated and control (0 mM Na_2MoO_4) [^{3}H]progesterone binding parameters were analyzed by Student's t test: [b] $P > 0.10$; [c] $P < 0.01$; [d] $P < 0.05$. Differences have been considered significant at $P \leq 0.05$.

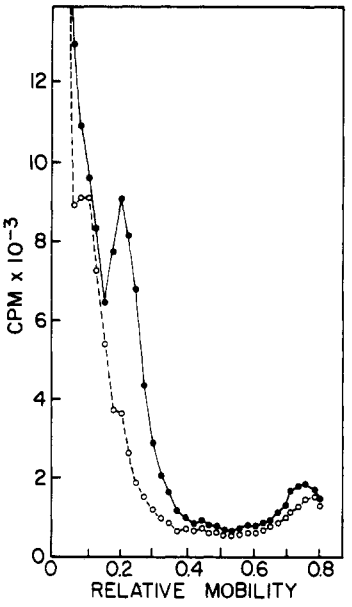

FIGURE 1: Stabilization of progesterone receptor during polyacrylamide gel electrophoresis by sodium molybdate. Uterine cytosol prepared in barbital buffer (4:1 v/w) was incubated at 0 °C for 4 h with (●) or without (○) 5 mM sodium molybdate. [^{3}H]Progesterone (10 nM) was added, followed by further incubation at 0 °C for 18 h. Aliquots (0.1 mL) of each sample were electrophoresed on highly cross-linked polyacrylamide gels. Following electrophoresis, the gels were sliced into 44 1.8-mm sections, and radioactivity was measured in each slice after extraction with 1.0% $NaDodSO_4$. Mobility was determined relative to migration of bromphenol blue (dye marker).

the optimal molybdate concentration. There was no significant effect of sodium molybdate at any concentration on the equilibrium association constant for the progesterone-receptor interaction.

The observation that molybdate improves recovery of uterine cRp led us to suspect that this agent would be effective in maintaining receptor integrity during electrophoresis. Our previous efforts to demonstrate specific [^{3}H]progesterone binding following electrophoresis of uterine cytosol on highly cross-linked polyacrylamide gels had given unsatisfactory results. These experiments had included the use of glycerol (10% v/v) and pyridoxal phosphate (10 mM) as receptor-stabilizing agents. The data in Figure 1 show that pretreatment of cytosol with 5 mM molybdate does indeed permit demonstration of a [^{3}H]progesterone-binding component from uterine cytosol by electrophoresis. There is a single peak of

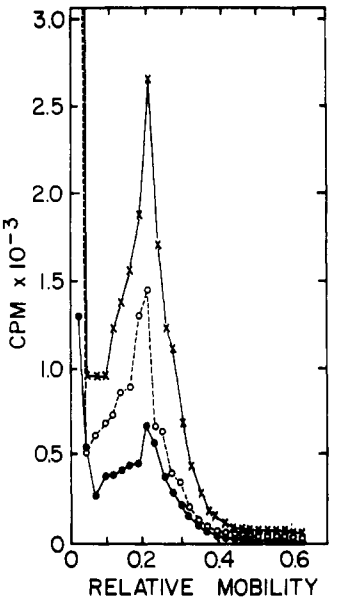

FIGURE 2: Effect of increasing [^{3}H]progesterone concentration on progesterone receptor electrophoresis in the presence of sodium molybdate. Uterine cytosol prepared in barbital buffer (4:1 v/w) was incubated at 0 °C for 4 h in the presence of 5 mM sodium molybdate. Increasing amounts of [^{3}H]progesterone were added to 0.5-mL aliquots of cytosol, giving final [^{3}H]progesterone concentrations of 2.5 (●), 5.0 (○), or 10.0 nM (×). Following incubation at 0 °C for 18 h, 0.1-mL aliquots were subjected to polyacrylamide gel electrophoresis.

[^{3}H]progesterone binding detectable in this gel system, having a mobility relative to the tracking dye of 0.2 (R_f = 0.19 ± 0.004, mean ± SEM, n = 23). In the absence of sodium molybdate, the majority of the radioactivity found in the gel represents that of the free [^{3}H]progesterone, which either diffuses into the gel or dissociates from the receptor during electrophoresis (relative mobility between 0 and 0.3). A small shoulder is observed in this radioactivity profile at a mobility of 0.2, but it is not known whether this represents steroid binding. The quantity of radioactivity measured under the gel peak in the presence of sodium molybdate varies between 30 and 45% of the initial bound [^{3}H]progesterone present in the cytosol. This finding suggests either that some receptor degradation occurs during electrophoresis even in the presence of molybdate or that dissociation of steroid from the receptor occurs during the run.

Inclusion of [^{3}H]progesterone in the gels without molybdate did not aid in electrophoresis of progesterone receptor but did permit identification of two other [^{3}H]progesterone binders in the cytosol. These were easily distinguished from the receptor by their high electrophoretic mobilities and lack of hormonal binding specificity. One of these binders exhibited the steroid binding specificity of corticosteroid binding globulin (i.e., [^{3}H]progesterone displaceable by cortisol) and had an R_f of 0.61 ± 0.008 (n = 11). The second binder, which co-migrated with serum albumin, had an R_f of 0.53 ± 0.006 (n = 14) and bound [^{3}H]progesterone in a nonsaturable manner.

The studies shown in Figures 2 and 3 establish more firmly that the [^{3}H]progesterone peak observed at a mobility of 0.2 in the gel electrophoresis profile represents progesterone receptor binding. Figure 2 shows the effect of increasing [^{3}H]progesterone concentration on the amount of radioactivity subsequently recovered in the gel peak after electrophoresis of molybdate-treated cytosol. As the concentration of labeled steroid was raised from 2.5 to 10 nM, the amount of radioactivity in this peak increased proportionally: 3100 cpm at 2.5 nM, 7800 cpm at 5.0 nM, and 14 800 cpm at 10.0 nM. The data in Figure 3 show that this progesterone-binding

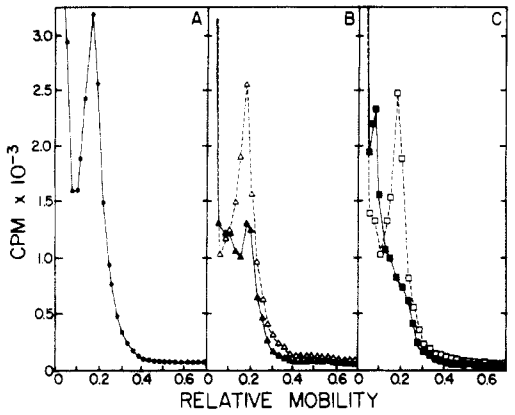

FIGURE 3: Specificity of [^{3}H]progesterone receptor binding analyzed by polyacrylamide gel electrophoresis. Uterine cytosol prepared in barbital buffer (4:1 v/w) was incubated for 4 h at 0 °C in the presence of 5 mM sodium molybdate. Aliquots (0.3 mL) of cytosol were then incubated for 18 h with 10 nM [^{3}H]progesterone with or without unlabeled steroids. Following this incubation, portions of each sample (0.1 mL) were subjected to polyacrylamide gel electrophoresis. The unlabeled steroids used were (A) ethanol vehicle control (●), (B) 1 nM cortisol (△) or 1 nM progesterone (▲), and (C) 100 nM cortisol (□) or 100 nM progesterone (■).

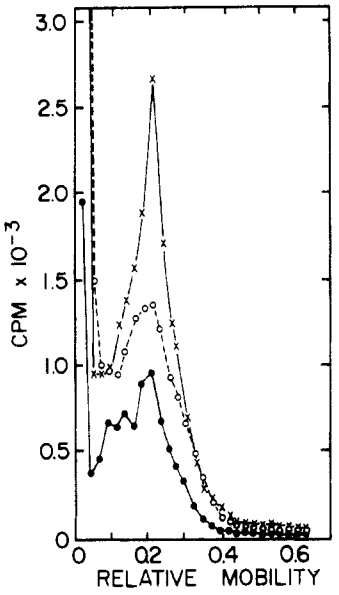

FIGURE 4: Time course of sodium molybdate stabilization of progesterone receptor analyzed by polyacrylamide gel electrophoresis. Aliquots (0.3 mL) of uterine cytosol prepared in barbital buffer (4:1 v/w) containing 5 mM sodium molybdate were incubated at 0 °C for 0 (●), 1 (○), or 4 h (×). After these times, [^{3}H]progesterone (10 nM) was added to each sample. The samples were incubated with the steroid at 0 °C for 16–20 h, and 0.1-mL aliquots were subjected to electrophoresis.

component represents specific binding of [^{3}H]progesterone. The radioactivity present in this peak was reduced by incubation of cytosol with 10 nM [^{3}H]progesterone in the presence of either 1.0 or 100 nM unlabeled progesterone, suggesting displaceable binding. Incubation of cytosol with 1.0 or 100 nM unlabeled cortisol had no effect on the amount of [^{3}H]-progesterone associated with this peak. Thus, the progesterone-binding component represented by this peak of radioactivity represents cytosolic progesterone receptor (cRp).

The data in Figure 4 show that the stabilizing effect of molybdate on cRp, as measured by polyacrylamide gel electrophoresis, is a time-dependent process. Samples of uterine cytosol were incubated with sodium molybdate for different times (0, 1, or 4 h) prior to the addition of [^{3}H]progesterone. The time of molybdate exposure required for maximal [^{3}H]-

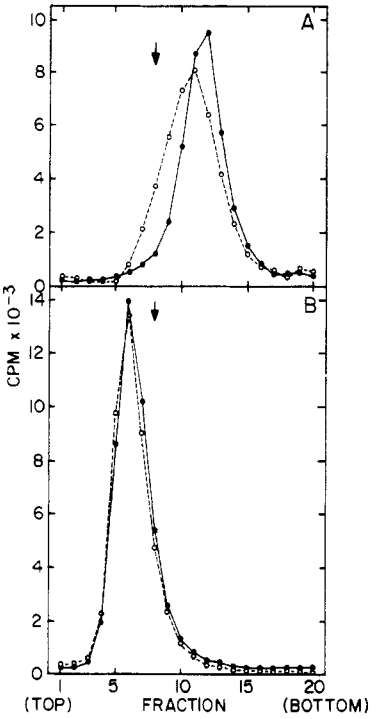

FIGURE 5: Effect of sodium molybdate on sedimentation of [^{3}H]-progesterone receptor complexes on sucrose gradients. Aliquots of uterine cytosol (0.5 mL) prepared in barbital buffer (4:1 v/w) were incubated for 4 h at 0 °C with (●) or without (○) 5 mM sodium molybdate. [^{3}H]Progesterone (5 nM) was added to each sample followed by incubation at 0 °C for 20 h. The samples were layered on top of linear 5–20% sucrose gradients prepared in Tris buffer B_{10} containing no KCl (A) or 0.3 M KCl (B). Following centrifugation at 220000*g* for 19 h at 2 °C, the gradients were fractionated into 20 0.3-mL portions. Loosely bound and free steroids were removed from each fraction by exposure to dextran-coated charcoal. Radioactivity was then measured in the postcharcoal supernatant solutions from each fraction. Arrows indicate the position of bovine serum albumin (4.6 S) which was sedimented in companion gradients in each run.

progesterone binding on the gels was 4 h. Longer periods of incubation in the absence of steroid were not tested. These findings suggest that molybdate stabilization of uterine cRp and the molybdate-mediated increase in steroid binding capacity are best effected in the *absence* of the ligand. These data favor the conclusion that sodium molybdate stabilizes the unbound or ligand-free form of the receptor.

In order to gain some insight into and determine the nature of the molybdate effect on receptor stability, the physicochemical properties of the molybdate-treated uterine cRp were compared to those of receptor prepared in the absence of molybdate.

Figure 5 illustrates the effect of sodium molybdate on the sedimentation of uterine cRp on sucrose gradients. Treatment of uterine cytosol with 5 mM sodium molybdate produced some small changes in cRp sedimentation on low-salt (no KCl) sucrose gradients (Figure 5A). In the absence of sodium molybdate, the receptor sedimented as a single species having a sedimentation coefficient of 6–7 S (peak fraction 11). Molybdate-treated cRp sedimented as a sharper, slightly heavier species with a sedimentation coefficient of about 7.5 S (peak fraction 12). These small changes were consistently observed (four trials) under low-salt conditions, regardless of the buffer employed for cytosol preparation (barbital buffer and Tris buffer B_{10} give identical results). In contrast, there was no apparent effect of molybdate treatment on Rp sedimentation in high-salt (0.3 M KCl) sucrose gradients (Figure 5B). Both control and molybdate-treated Rp forms sedimented under these conditions as a single, sharp peak (fraction 6) at

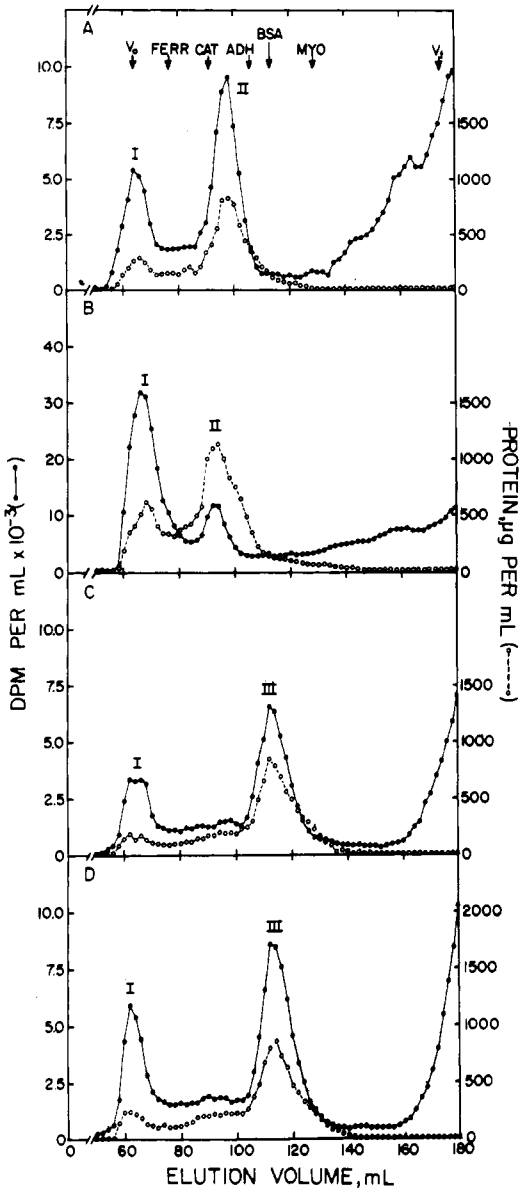

FIGURE 6: Gel filtration of [^{3}H]progesterone receptor complexes. Aliquots (3.0 mL) of uterine cytosol (4:1 v/w) prepared in Tris buffer B_{10} (A and B) or in Tris buffer B_{10} containing 0.5 M KCl (C and D) were incubated at 0 °C for 4 h with (B and D) or without (A and C) sodium molybdate. [^{3}H]Progesterone (10 nM) was added, followed by further incubation at 0 °C for 17–18 h. The samples were then chromatographed on a 2.5 × 45 cm column of Sephacryl S-300, which was equilibrated and eluted with Tris buffer A containing the corresponding concentration of KCl. Fractions of 2-mL volume were collected at a flow rate of 30 mL/h. Portions of each fraction were assayed for radioactivity (●) and protein (○). The arrows designate the positions of marker proteins used for column calibration: FERR = ferritin, BSA = bovine serum albumin, CAT = catalase, ADH = alcohol dehydrogenase, MYO = myoglobin. The void volume (V_0) was 62–63.5 mL. The total column volume (V_t) was 175 mL. Sodium molybdate had no effect on the elution of the standard proteins or on the column parameters V_0 and V_t. Roman numerals designate forms of the receptor differing in molecular size.

about 3.5 S. Thus, the molybdate effect on cRp size on sucrose gradients is overcome by incubation with high salt.

The effect of sodium molybdate on the molecular size of cRp was also analyzed by gel filtration on Sephacryl S-300 in the presence and absence of high salt (Figure 6). At least two distinct molecular forms of cRp were observed after gel filtration of cytosol in the absence of sodium molybdate in low-salt buffer (Figure 6A). Form I, eluting just after the void volume of the column, probably represents receptor present in large aggregates (Stokes radius (R_s) >7.9 nm). Form II had a R_s of 5.42 ± 0.30 (mean ± SEM, n = 5). A number of smaller molecules (R_s <2.0 nm) eluted between 140 and 180 mL in low-salt profiles. These may represent proteolytic fragments of the receptor containing the steroid binding site. The relative amounts of nonspecific [^{3}H]progesterone binding (determined in cytosol incubated with excess unlabeled progesterone) on the Sephacryl S-300 column paralleled the elution of cytosol protein. Thus, the radioactivity peak associated with form II contained more nonspecific binding (about 60% of the disintegrations per minute in Figure 6A) than that of form I (about 20%). The ratio of disintegrations per minute under the peak for form I to that of form II is 0.55 under these conditions in the absence of sodium molybdate. Treatment with 5 mM sodium molybdate under low-salt conditions (Figure 6B) did not produce a significant change in the molecular radius of any of the [^{3}H]progesterone binding components detected on Sephacryl S-300 (R_s for form II equals 5.03 ± 0.39, n = 5, $P > 0.10$; compared to control with Student's t test), and there was no effect of molybdate on the elution of cytosol protein (Figure 6A,B). However, a substantial difference in recovery and distribution of radioactivity associated with forms I and II was observed with molybdate treatment. The ratio of radioactivity represented by form I to that of form II in this case was 1.94 ± 0.32 (n = 4), suggesting that molybdate increases aggregation of the receptor in low-salt buffers. In conjunction with this enhancement of aggregation, molybdate produced a marked increase in receptor recovery on gel filtration (approximately 500% increase in form II). Thus, molybdate may stabilize uterine cRp by increasing the extent of receptor aggregation. In agreement with the observation that molybdate has no effect on cRp sedimentation in high-salt buffer, this agent caused no detectable changes in the molecular size distribution or recovery of cRp forms upon gel filtration in the presence of high salt (Figure 6C,D). In contrast to the selective effect of molybdate on receptor, 0.5 M KCl caused a general shift in the protein elution toward smaller sized molecules, which resulted from disruption of electrostatic protein–protein interactions. Gel filtration in high salt reduced the amount of aggregated receptors (form I) but did not eliminate these species. Form II was absent in high-salt gel filtration profiles, replaced by a smaller form III (R_s = 3.44 ± 0.03, n = 4). Although it is not certain whether this molecule is derived from form II, it is possible that form III represents the monomeric species resulting from dissociation of a form II dimer. The form I to form III disintegrations per minute ratios for these experiments were 0.45 for control cytosol and 0.49 for molybdate-treated cytosol. Thus, molybdate does not promote receptor aggregation in high-salt buffer.

Much work from other laboratories has shown that molybdate blocks "activation" or "transformation" of steroid receptors to forms capable of binding to DNA, DNA–cellulose, or ATP–Sepharose (Leach et al., 1979; Mierendorf & Mueller, 1979; Toft & Nishigori, 1979; Nishigori & Toft, 1980; Shyamala & Leonard, 1980). Thus, it was of considerable interest to determine if sodium molybdate inhibits activation of hamster uterine cRp. Activation was gauged by binding to DNA–cellulose. The data in Figure 7 clearly show that, under the conditions of our experiments, molybdate prevents activation of cRp. After a 25-h incubation in phosphate buffer at 0 °C, up to 30% of cRp present in control cytosol was capable of binding to DNA–cellulose (Figure 7A). In contrast, little or no DNA binding of [^{3}H]progesterone receptor complexes was observed after this same period of incubation with 5 mM sodium molybdate (Figure 7B). Similar results were

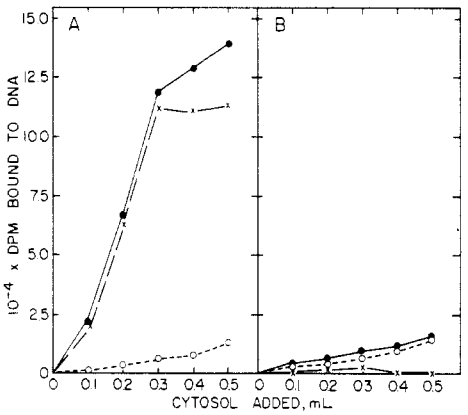

FIGURE 7: Inhibition of progesterone receptor binding to DNA–cellulose by sodium molybdate. Aliquots (3.4 mL) of uterine cytosol prepared in phosphate buffer (4:1 v/w) were incubated for 4 h at 0 °C with (B) or without (A) 5 mM sodium molybdate. [^{3}H]Progesterone (20 nM) was added to the samples, followed by a further incubation at 0 °C for 21 h. Duplicate aliquots of cytosol, ranging in volume from 0.05 to 0.50 mL, were mixed with pellets from 1 mL of a 50% (w/v) slurry of DNA–cellulose. The volume of each assay was then made up to 1.0 mL with phosphate buffer. The mixtures were incubated at 3 °C for 1 h with occasional agitation. One of the duplicate samples of each cytosol dilution was heated to 37 °C for 15 min. All the DNA–cellulose pellets were washed twice with 10 mL of phosphate buffer. [^{3}H]Progesterone receptor complexes were extracted from the pellets by incubation for 1 h with 1 mL of phosphate buffer containing 0.5 M KCl. Radioactivity was measured in these extracts: total bound radioactivity (non-heat-treated samples) (●); nonspecifically bound radioactivity (heat-treated samples) (○); and specific radioactivity (total minus nonspecific) (×).

obtained with Tris buffer B_{10}, except that this buffer did not promote receptor activation as well as phosphate buffer. Inclusion of 5 mM sodium molybdate in the wash buffer after incubation of receptor with DNA–cellulose did not reduce the amount of receptor bound to DNA (data not shown). These findings indicate that molybdate prevents activation of uterine cRp in conjunction with its enhancement of receptor stability.

Discussion

In this report, we have shown that sodium molybdate stabilizes uterine progesterone receptors under cell-free conditions, improving receptor yield and permitting analysis and measurement of [^{3}H]progesterone receptor complexes by electrophoresis. The stabilizing effect of sodium molybdate has been observed for many steroid receptor systems, including avian oviduct progesterone receptors (Toft & Nishigori, 1979; Grody et al., 1980), rat thymocyte glucocorticoid receptors (Nielsen et al., 1977b; Leach et al., 1979), mouse mammary glucocorticoid receptors (McBlain & Shyamala, 1980), and rat prostatic androgen receptors (Gaubert et al., 1980). The obvious and exciting practical implications for the use of molybdate to stabilize receptors toward in vitro inactivation during receptor purification and analysis are only beginning to be explored. Our results provide a clear demonstration of the utility of molybdate in this regard. In the absence of this agent, analysis of uterine cRp by a modification of the polyacrylamide gel electrophoresis procedure of Miller et al. (1975) was impossible. Because of receptor degradation or rapid steroid dissociation during electrophoresis, no specific [^{3}H]-progesterone binding was demonstrable by this technique. Treatment of uterine cytosol with sodium molybdate permitted detection of a [^{3}H]progesterone-binding species possessing many of the characteristics of the progesterone receptor (i.e., specificity for binding of progesterone but not cortisol, limited binding capacity, and concentration-dependent binding of [^{3}H]progesterone). Recovery of the receptor (about 30% of that applied to the gels) was reasonably good, considering that steroid dissociation from the receptor certainly occurs during the 4-h time of electrophoresis.

Molybdate stabilization of uterine cRp was found to be a time-dependent process. Addition of molybdate to cytosol simultaneously with [^{3}H]progesterone did not cause as great an increase in receptor recovery upon gel electrophoresis as that observed after a 4-h preincubation with molybdate prior to steroid addition. These findings suggest that sodium molybdate stabilizes the unliganded form of the receptor (aporeceptor), having little protective effect on preformed steroid–receptor complexes. Similar conclusions have been reached by other investigators (Leach et al., 1979; Grody et al., 1980; Gaubert et al., 1980).

Pratt and co-workers (Nielsen et al., 1977a,b) originally hypothesized that the effect of molybdate on receptor stability was indirect, resulting from inhibition of phosphatases, which were proposed to inactivate receptors by dephosphorylation. The observations that highly purified alkaline phosphatase can accelerate glucocorticoid receptor inactivation (Nielsen et al., 1977b) and that the phosphatase inhibitors sodium fluoride and glucose 1-phosphate also stabilize receptors favored this theory. However, more recent evidence from these and other workers suggests that molybdate inhibition of steroid receptor inactivation results from a direct interaction of molybdate with the receptor itself. Molybdate blocks receptor inactivation which occurs during precipitation with ammonium sulfate or upon exposure to high salt concentrations (Leach et al., 1979; Grody et al., 1980). Other phosphatase inhibitors have no protective effect in these cases. Moreover, Grody et al. (1980) have shown that the protective effect of molybdate is lost if the receptors are first dissociated into subunits by ammonium sulfate precipitation or high salt prior to molybdate addition. Thus, these finding are more consistent with the recent hypothesis that molybdate complexes with phosphate groups on a putative phosphorylated aporeceptor to produce stabilization (Leach et al., 1979; Nishigori & Toft, 1980).

Although we have no evidence to support the contention that steroid receptors are phosphoproteins, our data on the effects of sodium molybdate upon the physicochemical properties of hamster uterine cRp are in accord with this hypothesis. Molybdate treatment of cRp causes a shift in the sedimentation coefficient measured on low-salt sucrose gradients from 6–7 S to 7.5 S. A small increase in the receptor sedimentation coefficient upon molybdate treatment was also observed by Grody et al. (1980) for the chick oviduct progesterone receptor and by McBlain & Shyamala (1980) for mouse mammary glucocorticoid receptor. These observations have been interpreted to mean that molybdate stabilizes aggregated or large forms of receptors. In the presence of 0.3 M KCl, a single 3.5 S receptor was observed on sucrose gradient analysis, irrespective of the presence of sodium molybdate. A similar salt-dependent reduction in the sedimentation coefficient was observed for rabbit uterine progesterone receptor by Fleischmann & Beato (1978). We have further analyzed molybdate-dependent increases in cRp size by gel filtration chromatography. In contrast to the results of sucrose gradient analysis, where the receptors sediment as a single species, at least two distinct molecular forms of cRp are demonstrable following gel filtration of uterine cytosol under all conditions tested. The major species observed were an aggregated form eluting just after the column void volume and an included peak with a Stokes radius of 5.4 (low salt) or 3.4 nm (high salt). Incubation of cytosol with sodium molybdate in low-salt buffers produced a dramatic increase in the aggregated species

in conjunction with increased receptor recovery. There was no apparent effect of molybdate on receptor size or recovery after analysis in the presence of high salt. In this instance, a nonaggregated 3.4-nm form, perhaps a product of dissociation of the low-salt 5.4-nm species, predominated. As an explanation for the differences in the number of progesterone-binding species observed on sucrose gradients vs. gel filtration, we propose that the receptors exist in cytosol as a heterogeneous mixture of different-sized molecules, monomers, dimers, and aggregates, which are in reversible equilibrium. The basic difference between the two methods of size analysis is one of time. Whereas sucrose gradient centrifugation required 19 h, gel filtration takes 5 h. Thus, cRp sediments as a mixture of individual and aggregated molecules in equilibrium with each other, and the measured sedimentation coefficient really represents the weighted average for these different-sized interacting species. Since molybdate enhances receptor aggregation, it would shift the equilibrium toward these forms, yielding a higher average sedimentation coefficient than that for untreated receptor. Gel filtration possesses greater resolving power than sucrose gradient analysis and is rapid enough to permit separation of the different forms. These studies provide further evidence for a direct interaction of molybdate with steroid receptors and suggest that the existence of the receptors in aggregated form is a prerequisite for molybdate stabilization of these molecules.

In agreement with the findings of other investigators (Leach et al., 1979; Mierendorf & Mueller, 1979; Nishigori & Toft, 1980; Shyamala & Leonard, 1980), sodium molybdate at a 5 mM concentration completely blocks uterine cRp activation to the DNA-binding form. The mechanism of this inhibition is also incompletely understood. Since the receptors exist as large aggregates under these conditions, it is tempting to suggest that molybdate inhibition of receptor activation is simply a function of the inability of these large molecules to complex with DNA. Within the aggregates, the putative receptor transconformation necessary for activation may be prevented. Alternatively, the DNA binding site(s) on the receptor may be shielded by interaction with neighboring proteins in the aggregate. Such a hypothesis is favored by the findings of Nishigori & Toft (1980), who observed that molybdate blocks the 6–8S to 4S disaggregation of chick oviduct progesterone receptor which occurs upon receptor activation. The molecular mechanism(s) by which molybdate induces aggregation or maintains the receptors in this state is (are) not yet known.

In summary, these studies show that sodium molybdate stabilizes hamster uterine progesterone receptor. Molybdate enhances the recovery of receptors in cytosol and permits receptor measurement by a method which is too stringent for analysis of untreated receptors, polyacrylamide gel electrophoresis. Studies on the physicochemical properties of the molybdate-stabilized receptor suggest that molybdate protects the unliganded, unactivated (untransformed) aporeceptor and that molybdate favors receptor aggregation.

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