Inactivation of rat liver glucocorticoid receptor by molybdate

Effects on both non-activated and activated receptor complexes

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When freshly prepared glucocorticoid–receptor complex from rat liver cytosol was incubated at 23°C in the presence of sodium molybdate, its subsequent binding to isolated nuclei, DNA–cellulose and ATP–Sephadex was blocked. In addition, binding to these acceptors by cytosol receptor complex fractionated with $(\text{NH}_4)_2\text{SO}_4$ was also blocked by incubation of the complexes with 50 mM-sodium molybdate. However, molybdate had no effect on the binding of activated receptor complexes to ATP–Sephadex. Molybdate was also effective in extracting the nuclear- and DNA–cellulose-bound glucocorticoid–receptor complexes in a dose-dependent manner. Molybdate appears to exert its effects directly on the receptor by interacting with both non-activated and activated receptor forms.

Receptors in freshly prepared cytosol can be activated† by incubation at elevated temperatures in presence of hormones (Milgrom *et al.*, 1973), by exposure to high ionic strength (Baxter, 1972; Higgins *et al.*, 1973), or by an incubation with ATP at 4°C (John & Moudgil, 1979; Moudgil & John, 1980a; Moudgil & Eessalu, 1980). Once activated, receptors acquire an increased affinity for isolated nuclei (Lohmar & Toft, 1975; Buller *et al.*, 1975), DNA (Yamamoto & Alberts, 1972; Toft, 1972) and ATP–Sephadex (Miller & Toft, 1978; Moudgil & John, 1980b).

More recent studies have revealed that the addition of molybdate blocks the activation process of steroid receptors (Toft & Nishigori, 1979; Leach *et al.*, 1979; Moudgil *et al.*, 1980; Nishigori & Toft, 1980). However, no inhibitory effect was evident in these studies when molybdate was added to preparations that contained an already activated receptor. Present studies indicate that molybdate blocks the activation *in vitro* and the acceptor binding of activated glucocorticoid receptor. Therefore, both non-activated and ‘activated’ receptor forms are sensitive to molybdate action. Furthermore, the activated receptor bound to acceptors can be extracted by molybdate, thus suggesting an interaction between glucocorticoid receptor and molybdate.

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† The term activation refers to an *in vitro* alteration of cytosol receptor to a form which binds to isolated nuclei, DNA-cellulose or ATP–Sephadex.

Materials and methods

All reagents used were of analytical grade. [6,7(N)- ^3H]Triamcinolone acetonide (30–35 Ci/mmol) was obtained from New England Nuclear; NaF, Na_2MoO_4 and Na_2HAsO_4 were from Merck; levamisole hydrochloride was from Aldrich; K_2HPO_4 was from Baker Chemical Co., dextran T-70 and Sephadex 4B were from Pharmacia; cellulose powder (Cellex 410) was from BioRad; ATP was from Boehringer-Mannheim; Na_2WO_4 was from Fisher Chemical Co., and the other reagents were purchased from Sigma.

Buffers

The following buffers were prepared and stored at 4°C: buffer A: 50 mM-Tris/HCl/12 mM-monothioglycerol/10% (v/v) glycerol, pH 8.0; buffer B: 500 mM-sucrose/50 mM-Tris/HCl/25 mM-KCl/2 mM- MgCl_2 , pH 7.5; buffer C: 10 mM-Tris/HCl/2 mM- MgCl_2 /20% (v/v) glycerol, pH 7.5; buffer D: 50 mM-Tris/HCl/12 mM-monothioglycerol/10 mM-KCl/20% (v/v) glycerol, pH 8.0; buffer E: 10 mM-Tris/HCl/1 mM- MgCl_2 /10% (v/v) glycerol, pH 7.5; buffer F: 50 mM-Tris/HCl/12 mM-monothioglycerol/20% (v/v) glycerol/1 M-KCl, pH 8.0; buffer G: 50 mM-Tris/HCl/12 mM-monothioglycerol, pH 8.0.

Preparation of DNA–cellulose and ATP–Sephadex

DNA–cellulose was prepared according to the method of Alberts & Herrick (1971). Calf thymus DNA (type II) was linked to Cellex-410 and these preparations contained 1.5–2 mg of DNA/g of

packed DNA-cellulose as quantified by the method of Burton (1956).

ATP was covalently linked to Sepharose 4B as described previously (Moudgil & Toft, 1975, 1977) and preparations used here contained 7–10 μ mol of nucleotide per ml of packed Sepharose as determined by phosphate analysis (King, 1932).

Preparation of glucocorticoid receptor

Male albino rats of Yale strain (150–200 g) were obtained from King Animal Lab. Inc., Oregon, WI, U.S.A. The animals were bilaterally adrenalectomized, kept on 0.9% NaCl for 3 days and were sacrificed by cervical dislocation. The whole liver tissue was removed, rinsed with cold 0.9% NaCl, minced and homogenized in 4 vol. (w/v) of buffer A with a Tissumizer (Tekmar, model SDT). The homogenate was centrifuged at 12000 g for 10 min and then at 150000 g for 1 h. The resultant supernatant was incubated with 10 nM-[³H]triamcinolone acetonide at 4°C for 4 h to form a complex.

Activation of cytosol glucocorticoid-receptor complex

The freshly prepared cytosol glucocorticoid-receptor complexes were heat-activated by incubation at 23°C for 40 min and then were chilled for 15 min on ice. The mixtures were then mixed with pelleted dextran-coated charcoal (0.5% Norit A/0.05% dextran T-70 made in 10 mM-Tris/HCl/12 mM-monothioglycerol, pH 8.0) for 5 min to avoid dilution. The activated receptor complexes were recovered by centrifugation twice at 1000 g for 10 min. The [³H]triamcinolone acetonide-receptor complexes were also fractionated with (NH₄)₂SO₄ at 35% saturation. The resultant precipitate, after centrifugation at 12000 g for 20 min, was dissolved in buffer A and dialysed against the same buffer for 4 h at 4°C with buffer changes every hour. After centrifugation at 12000 g for 20 min the supernatant was used as the (NH₄)₂SO₄-activated complex. The heat-activated and (NH₄)₂SO₄-activated complexes were used for the nuclei, DNA-cellulose and ATP-Sepharose binding assay under various conditions.

Preparations of nuclei

Rat liver nuclei were prepared by method III of Spelsberg *et al.* (1974) with slight modifications. The nuclear fractions that sedimented through a 1.7–1.8 M-sucrose cushion were rinsed twice with buffer B, resuspended in buffer C using a Teflon/glass homogenizer and filtered through organza to remove cell debris and polysaccharides. The nuclear suspension was divided into 1 ml fractions and stored at –20°C.

To isolate nuclei labelled by administration *in vivo* of steroid, [³H]triamcinolone acetonide (15 μ Ci/

150 g body wt.) dissolved in 0.9% NaCl was injected intravenously and the whole liver was removed 1 h after injection. The nuclear fraction was prepared by the same procedure as described above. The DNA content of nuclear preparations was determined by the method of Burton (1956).

Determination of [³H]triamcinolone acetonide-receptor complex binding to nuclei, DNA-cellulose, and ATP-Sepharose

DNA-cellulose suspension (10 mg/ml) in buffer D was divided equally into a series of tubes (13 mm × 100 mm) and centrifuged at 1000 g for 10 min to obtain the DNA-cellulose pellet (15–30 μ g of DNA). ATP-Sepharose suspension (1 ml/tube) was centrifuged at 1000 g for 10 min and rinsed twice with buffer D and the pellet was used for assays. The nuclear preparation from rat liver (200–1500 μ g of DNA) was directly used for the assays. The [³H]triamcinolone acetonide-receptor complexes, activated either by incubation at 23°C for 40 min or by treatment with (NH₄)₂SO₄, were incubated with three different acceptors under various conditions. Following gentle shaking at 4°C for 40 min, the incubation mixtures were sedimented by centrifugation at 1000 g for 10 min, and rinsed three times with 3 ml of either buffer E (for nuclei) or buffer D (for DNA-cellulose and ATP-Sepharose).

Nuclear preparations after washing with buffer E were directly transferred into counting vials, whereas both DNA-cellulose and ATP-Sepharose were incubated with buffer F at 4°C for 30 min to extract the resin-bound [³H]triamcinolone acetonide-receptor complexes.

Levamisole, NaF, K₂HPO₄ and Na₂NaSO₄, Na₂MoO₄, Na₂WO₄, Na₂CrO₄, NaVO₃, Na₂Cr₂O₇, NaHCO₃ and Na₂B₄O₇ were dissolved in buffer G and used to determine their effect on the nuclear uptake of activated receptor complexes.

Effect of molybdate on the dissociation of [³H]triamcinolone acetonide-receptor complex

To determine the effect of molybdate on the dissociation of [³H]triamcinolone acetonide from the activated complex during the incubation period, heat-activated [³H]triamcinolone acetonide-receptor complexes were treated with charcoal and then incubated with different concentrations of molybdate at 4°C for 40 min in a final volume of 0.5 ml. After this the preparations were again mixed with dextran-coated charcoal and the radioactivity of macromolecule-bound [³H]triamcinolone acetonide was measured.

To determine whether the effect of molybdate is reversible, (NH₄)₂SO₄-activated [³H]triamcinolone acetonide-receptor complexes were incubated with buffer G (control) or 50 mM-sodium molybdate at 4°C for 30 min. Both control and molybdate-treated

samples were then dialysed against buffer A for 3 h at 4°C and centrifuged at 10000g for 20 min to remove the aggregated proteins. Following the above treatment, the aliquots containing [³H]triamcinolone acetonide–receptor complexes were incubated at 0°C or 23°C for 40 min. The extent of nuclear uptake of the complexes was determined as described earlier.

Extraction of [³H]triamcinolone acetonide–receptor complexes bound to nuclei, DNA–cellulose and ATP–Sephadex

The procedures of extraction of acceptor-bound [³H]triamcinolone acetonide–receptor complexes consisted of two steps. The complexes were first incubated with rat liver nuclear preparation, DNA–cellulose, and ATP–Sephadex as described previously. The pellets of each acceptor were incubated with different concentrations of molybdate or KCl dissolved in buffer G, at 4°C for 30 min with gentle shaking. After centrifugation at 1000g for 10 min, the radioactivity in the supernatant was measured directly. A small portion of the extracts was used for sucrose gradient analysis.

To investigate whether the molybdate-extracted receptor has the ability to rebind to nuclei or to DNA–cellulose, (NH₄)₂SO₄-activated [³H]triamcinolone acetonide–receptor complexes were incubated with nuclei or DNA–cellulose. Following the extraction of acceptor-bound complexes with 75 mM-molybdate, the resultant extracts from nuclei and DNA–cellulose were dialysed against buffer A. Equal portions of (NH₄)₂SO₄-fractionated complexes were also dialysed and served as control. The nuclear and DNA–cellulose binding assays were performed with the molybdate-extracted and the activated [³H]triamcinolone–receptor complex, according to the methods described previously.

Sucrose density gradient analysis

Linear 5–20% sucrose gradients (4.4 ml) were prepared in buffer containing 10 mM-Tris/HCl/12 mM-monothioglycerol/0.3 M-KCl (pH 8.0) by using a Beckman gradient former. Samples (0.25 ml) containing the molybdate-extracted [³H]triamcinolone acetonide–receptor complexes from nuclei and DNA–cellulose were layered on separate gradients. The heat-activated [³H]triamcinolone acetonide–receptor complexes from liver cytosol were diluted with buffer G to lower the glycerol concentration before layering on the gradient. The gradients were centrifuged at 150000g for 16 h on a Beckman LS-75 ultracentrifuge.

[¹⁴C]Ovalbumin (Rice & Means, 1971) was layered onto a separate gradient as a standard for the determination of sedimentation coefficients (Martin & Ames, 1961). The fractions were collected by piercing the bottom of each tube.

For measurement of radioactivity, aqueous samples were combined with 5 ml of scintillation fluid consisting of toluene (RPI)/Spectrafluor (Amersham) (1000:42, v/v), mixed thoroughly and cooled before counting.

Results

Effect of molybdate on the heat-activation of [³H]triamcinolone acetonide–receptor complex from rat liver cytosol

Based on several reports (Toft & Nishigori, 1979; Leach *et al.*, 1979; Moudgil *et al.*, 1980; Nishigori & Toft, 1980; Shyamala & Leonard, 1980; Noma *et al.*, 1980), it is now generally believed that heat activation of steroid–receptor complexes is blocked in the presence of sodium molybdate. The extent of activation in these studies was determined by measuring the binding of receptor complexes to ATP–Sephadex (Nishigori & Toft, 1980), DNA–cellulose (Leach *et al.*, 1979) and nuclear suspensions (Noma *et al.*, 1980). Fig. 1 illustrates a typical profile of the heat activation of glucocorticoid–receptor complexes in the presence of different concentrations of molybdate. The three acceptors used to measure receptor activation appeared to differ somewhat in their affinity for molybdate-treated preparations. A 50% inhibition in the binding of receptor to nuclei and DNA–cellulose was observed at 10 mM-molybdate, whereas much higher concentrations (>50 mM) of the inhibitor were required to inhibit receptor activation completely. The binding of receptor complexes to ATP–Sephadex was much more sensitive to the presence of molybdate during activation and was completely blocked by 5–10 mM-molybdate.

Effect of molybdate on acceptor binding by activated receptor complexes

The freshly formed cytosol [³H]triamcinolone acetonide–receptor complexes were activated by incubation at 23°C for 40 min. The effect of molybdate on the already activated preparations was determined by measuring the binding of receptor to nuclei, DNA–cellulose and ATP–Sephadex in the presence of different concentrations of molybdate (Fig. 2a). A 50% inhibition in the binding of receptor to nuclear preparation and DNA–cellulose was observed with about 30 mM-molybdate. At higher concentrations (>50 mM) molybdate completely blocked the binding of activated complexes to nuclei and DNA–cellulose. However, only a minor inhibitory effect of molybdate was seen on the receptor binding to ATP–Sephadex.

To support the finding that molybdate is effective against an activated receptor and to eliminate the possibility that the cytosol preparations used in Fig. 2(a) might have been only partially activated,

Fig. 1. Effect of molybdate on the activation of glucocorticoid-receptor complex from rat liver cytosol at 23°C

Freshly prepared [^3H]triamcinolone acetonide-receptor complexes were incubated at 23°C for 40 min with and without various concentrations of sodium molybdate. The extent of receptor-activation was measured by nuclear (O), DNA-cellulose (●) and ATP-Sepharose (▲) binding assays as described in the Materials and Methods section. Nuclear preparations (204 μg of DNA), DNA-cellulose (30 μg of DNA), and ATP-Sepharose (1 ml of suspension) were incubated with [^3H]triamcinolone acetonide-receptor complex (85 280 d.p.m.) in a final volume of 1.0 ml. A 100% binding represents 19 900, 21 500 and 28 000 d.p.m. for nuclear, DNA-cellulose, and ATP-Sepharose binding, respectively. Inset: effect of molybdate on the dissociation of [^3H]triamcinolone acetonide from the activated complexes. [^3H]Triamcinolone acetonide-receptor complexes from rat liver cytosol were heat-activated and treated with dextran-coated charcoal to remove free steroid. The contents of the assay mixture were then divided into small aliquots and incubated at 4°C for 40 min with different concentrations of sodium molybdate in a final volume of 0.5 ml. Following this incubation, dextran-coated charcoal was added to remove the free [^3H]triamcinolone acetonide dissociated from the activated complexes during incubation with molybdate. A 100% binding in the Figure equals 30 000 d.p.m.

the following experiment was performed. The cytosol preparations containing [^3H]triamcinolone acetonide-receptor complexes were fractionated with 35%-satd. $(\text{NH}_4)_2\text{SO}_4$. The receptors in such preparations have been reported to be in an activated state (Buller *et al.*, 1975) and cannot be activated further by heat (Lohmar & Toft, 1975). When $(\text{NH}_4)_2\text{SO}_4$ -fractionated receptor was incubated with different concentrations of molybdate, subsequent analysis of nuclear and DNA binding revealed patterns (Fig. 2b) similar to those seen with heat-

activated cytosol preparations (Fig. 2a). However, the molybdate concentrations required for the inhibitory effects were slightly greater than those seen in Fig. 2(a). Again, no inhibition in the binding of receptor to ATP-Sepharose was observed under these conditions (Figs. 2a and 2b).

Since the conductivity of sodium molybdate was found to be comparable to that of KCl on an equimolar basis, KCl was included in parallel series of samples. The results of Fig. 2, therefore, suggest that molybdate may be interacting with the activated glucocorticoid-receptor complex and its effects may not be due to its ionic strength. In addition, molybdate may bind to the site(s) that survive(s) the process of receptor activation.

Effect of molybdate and KCl on the extraction of acceptor-bound [^3H]triamcinolone acetonide-receptor complex

Fig. 3 shows that the majority (approx. 70%) of [^3H]triamcinolone acetonide-receptor complex bound to nuclear preparation (Fig. 3a) or DNA-cellulose (Fig. 3b) could be extracted by molybdate in a concentration-dependent manner. The receptor bound to ATP-Sepharose showed insensitivity to molybdate and could not be extracted (Fig. 3c).

Sucrose-density-gradient analysis of molybdate-extracted receptor

Aliquots of molybdate extracts from nuclear preparations and DNA-cellulose were applied onto sucrose gradients containing 0.3 M-KCl. Fig. 4 represents the sedimentation profiles of [^3H]triamcinolone acetonide-receptor complexes treated under different conditions before layering. The heat-activated and molybdate-extracted DNA-bound receptor sedimented as an approx. 4S entity whereas the receptor extracted by molybdate treatment from nuclei showed an additional smaller peak of radioactivity corresponding to about 2S.

Results similar to those presented in Fig. 3 were obtained when the nuclear [^3H]triamcinolone acetonide-receptor complexes were formed *in vivo* by administration of [^3H]triamcinolone acetonide. Nuclear preparations containing the labelled complex were incubated *in vitro* with different concentrations of sodium molybdate for the extraction of receptor (Fig. 5a). The nuclear receptor was extractable with 50 mM-molybdate whereas KCl at same concentrations was ineffective. Although nuclear receptor complexes formed both *in vivo* and *in vitro* were extractable by sodium molybdate the percent extraction differed in the two cases. Only 50% of the nuclear receptor formed *in vivo* could be extracted by molybdate whereas greater (approx. 70%) receptor content was extractable in preparations where nuclear receptor complexes were formed *in vitro*. The less efficient extraction of nuclear

Fig. 2. Effect of molybdate on the uptake of glucocorticoid-receptor complex by acceptors $[^3\text{H}]$ Triamcinolone acetonide-receptor complexes from rat liver cytosol activated by incubation at 23°C for 40 min (53 500 d.p.m.) (a), or by treatment with $(\text{NH}_4)_2\text{SO}_4$ (350 000 d.p.m.) (b), were incubated with nuclei (204 μg of DNA, $\circ$), DNA-cellulose (15 μg of DNA, $\bullet$), or ATP-Sepharose (1.0 ml of suspension, $\blacktriangle$) with and without different concentrations of sodium molybdate, in a total volume of 1.0 ml. The binding to acceptors was measured as described in the Materials and methods section. A 100% binding in Fig. 2(a) equals 5000, 4500 and 5250 d.p.m. for nuclear, DNA-cellulose, and ATP-Sepharose binding, respectively, and 63 000, 83 000 and 80 000 d.p.m. for Fig. 2(b).

Fig. 3. Effect of molybdate and KCl concentrations on the extraction of nuclear-, DNA-cellulose- and ATP-Sepharose-bound $[^3\text{H}]$ triamcinolone acetonide-receptor complexes

Aliquots (1 ml) containing nuclear suspension (a, 300 μg of DNA), DNA-cellulose (b, 20 μg of DNA) and ATP-Sepharose (c) were incubated at 4°C for 40 min with heat-activated complexes (104 880 d.p.m.). Following their uptake, $[^3\text{H}]$ triamcinolone acetonide-receptor complexes were extracted by incubation of acceptors with 1 ml of buffer G containing different concentrations of molybdate or KCl. The total binding of $[^3\text{H}]$ triamcinolone acetonide-receptor complexes to each acceptor was expressed as 100% and equals 37 369, 31 102 and 40 982 d.p.m. for nuclei, DNA-cellulose, and ATP-Sepharose, respectively. $\bullet$ — $\bullet$, KCl-extracted; $\bullet$ — $\bullet$, molybdate-extracted. Inset (d): Determination of conductivity of sodium molybdate and KCl. Different solutions containing various concentrations of sodium molybdate and KCl were prepared in buffer G. The conductivity was determined by using a conductivity bridge (Yellow Springs Instrument Co., model 31). $\bullet$ — $\bullet$, KCl; $\bullet$ — $\bullet$, sodium molybdate.

Fig. 4. Sucrose-gradient analysis of the molybdate-extracted $[^3\text{H}]$ triamcinolone acetonide-receptor complexes from nuclei and DNA-cellulose

DNA-cellulose ($300\mu\text{g}$ of DNA) and nuclear preparation (1mg of DNA) were incubated with heat-activated $[^3\text{H}]$ triamcinolone acetonide-receptor complex at 4°C for 40 min as described in the Materials and methods section. The nuclear- and DNA-cellulose-bound $[^3\text{H}]$ triamcinolone acetonide-receptor complexes were then extracted by incubation with buffer G containing 75mM -molybdate. Aliquots (0.2ml) containing extracted receptor were layered onto $5\text{--}20\%$ linear sucrose gradients (4.4ml) containing 0.3M -KCl and centrifuged at $150000g$ for 16 h. $[^{14}\text{C}]$ Ovalbumin was layered onto a separate gradient and served as a sedimentation standard (3.7S). Fractions (0.2ml) were collected by piercing the bottom of the gradient tubes. ●—●, nuclear extract; x—x, DNA-cellulose extract; ○—○, heat-activated complex.

$[^3\text{H}]$ triamcinolone acetonide-receptor complex formed *in vivo* may represent the processing of the complexes within the nuclei. The results shown in Fig. 5(b) demonstrate that the nuclear receptor formed *in vivo* had same sedimentation characteristics as those exhibited by nuclear receptor which was formed *in vitro*.

Effects of phosphatase inhibitors and other compounds on the activated receptor

Molybdate is a well-known inhibitor of phosphatases. A number of phosphatase inhibitors, including oxy compounds of transition metals of groups 5B and 6B, were tested to determine their comparative effectiveness in blocking the uptake of receptor by isolated nuclei. Also included were some of the salts of ionic buffers like NaHCO_3 and $\text{Na}_2\text{B}_4\text{O}_7$ to determine if the effects of molybdate were similar to those exhibited by these compounds. All the compounds were added to the activated

Table 1. Effect of various compounds on the nuclear uptake of activated $[^3\text{H}]$ triamcinolone acetonide-receptor complexes

All compounds were tested at 30mM .

Compound	Nuclear-bound $[^3\text{H}]$ triamcinolone acetonide-receptor complex (%)
None	100
Levamisole	100
NaF	100
NaHCO_3	91
$\text{Na}_2\text{B}_4\text{O}_7$	200
KCl	100
K_2HPO_4	80
Na_2HAsO_4	79
Na_2MoO_4	50
Na_2WO_4	0
Na_2CrO_4	27
$\text{Na}_2\text{Cr}_2\text{O}_7$	10
NaVO_3	15

Table 2. Effects of dialysis on the reversibility of molybdate action

$(\text{NH}_4)_2\text{SO}_4$ -fractionated $[^3\text{H}]$ triamcinolone acetonide-receptor complexes were incubated with buffer (10mM -Tris/HCl/ 12mM -monothioglycerol, $\text{pH } 8.0$) or 50mM -sodium molybdate at 4°C for 30 min. Identical portions were then dialysed against buffer A for 3 h at 4°C . After this treatment, the samples containing receptor complexes were incubated at 0°C or 23°C for 40 min. The extent of nuclear uptake was determined by incubating the nuclear suspension ($204\mu\text{g}$ of DNA) with 0.4ml of $[^3\text{H}]$ triamcinolone acetonide-receptor complex and 0.3ml of the above buffer at 4°C for 40 min.

Sample	Nuclear-bound $[^3\text{H}]$ triamcinolone acetonide (d.p.m./tube)	$[^3\text{H}]$ Triamcinolone acetonide complex added (d.p.m./tube)	Nuclear bound $[^3\text{H}]$ triamcinolone acetonide complex (%)
Control, dialysis			
+50mM-sodium	50955 ± 515	246360	20.7
molybdate, dialysis	47310 ± 2419	234480	20.2

Fig. 5. Effects of molybdate on the extraction *in vitro* of nuclear $[^3\text{H}]$ triamcinolone acetonide administered *in vivo*. Nuclear suspension was prepared from the liver of a rat given $15\mu\text{Ci}$ of $[^3\text{H}]$ triamcinolone acetonide for 1 h as described in the Materials and methods section. (a) The nuclear preparations ($300\mu\text{g}$ of DNA) were incubated at 4°C for 30 min with buffer G containing different concentrations of sodium molybdate (●---●) or KCl (●—●) in a final volume of 1.0 ml. A 100% extraction equals 8090 d.p.m. (b) Sedimentation rate analysis of molybdate-extracted $[^3\text{H}]$ triamcinolone acetonide-receptor complexes from the nuclear complex formed *in vivo*. The $[^3\text{H}]$ triamcinolone acetonide-receptor complexes from the nuclear preparation were extracted with 75 mM-molybdate and 0.2 ml aliquots (2700 d.p.m.) were layered on 5–20% linear sucrose gradients containing 0.3 M-KCl. The gradients were centrifuged at $150000g$ for 16 h and 0.2 ml fractions were collected. The entire fractions were used to measure the radioactivity.

receptor preparations at 30 mM concentration and their effects were determined by a nuclear binding assay. As seen in Table 1, molybdate and related compounds of groups 5B and 6B were most effective in blocking the nuclear uptake of activated receptors whereas NaF, levamisole, KCl and NaHCO_3 had no effect at these conditions. Other known inhibitors of phosphatases, K_2HPO_4 and Na_2HASO_4 , also caused noticeable inhibition. Interestingly, borate increased the nuclear uptake of glucocorticoid-receptor complexes two-fold.

Reversibility of molybdate effects

The results of attempts to determine the reversibility of molybdate effects on an already activated glucocorticoid receptor are summarized in Table 2. Aliquots of $(\text{NH}_4)_2\text{SO}_4$ -fractionated receptor complexes were incubated with 50 mM-sodium molybdate at 4°C for 30 min. The control samples received only buffer G. Both groups were dialysed for 3 h at 4°C . The resulting receptor preparations were further kept at 0°C or 23°C to determine whether molybdate treatment converted the activated receptor to a non-activated form which could be reactivated. The results of Table 2 indicate that the inhibitory

effects of molybdate on an activated receptor are completely reversed upon dialysis. In addition it is clear that molybdate-treated receptor remains in an activated form.

Ability of $[^3\text{H}]$ triamcinolone acetonide-receptor complex to rebound to acceptors after extraction with molybdate

Since the effects of molybdate were found to be reversible, whether the extraction of nuclear- or DNA-cellulose-bound receptors by molybdate affected the integrity of the receptor was examined. Receptor preparations fractionated with $(\text{NH}_4)_2\text{SO}_4$ were incubated with nuclei and DNA-cellulose and the acceptor-bound complexes were extracted with 75 mM-sodium molybdate. After a thorough dialysis, samples of the complexes were incubated with nuclei or DNA-cellulose. Not only was the effect of molybdate completely reversed, but the entire $[^3\text{H}]$ triamcinolone acetonide-receptor complex extracted with molybdate retained its capacity to bind to nuclear sites or DNA-cellulose (Table 3). These observations indicate that the extraction of receptor by molybdate does not destroy its capacity to bind to nuclei or DNA.

Table 3. *Effect of dialysis on the capacity of molybdate-extracted glucocorticoid-receptor complex to rebind the acceptors*
 Samples of $(\text{NH}_4)_2\text{SO}_4$ -fractionated $[\text{^3H}]$ triamcinolone acetone-receptor complex were used for nuclear (1.5 mg of DNA) or DNA-cellulose (240 μg of DNA) binding. Following their binding to these acceptors, the receptor complexes were extracted with 75 mM-sodium molybdate by incubation at 4°C for 30 min. The extracts from nuclei and DNA-cellulose were dialysed against buffer A for 3 h at 4°C. Equal portions of $(\text{NH}_4)_2\text{SO}_4$ -fractionated receptor complexes were also dialysed and served as controls. Following this, nuclear uptake (A,A') and DNA-cellulose (B,B') binding assays were performed. The values from control or untreated samples (A,B) were taken at 100% and compared with nuclear and DNA binding of samples extracted by molybdate (A',B').

Sample	Bound $[\text{^3H}]$ triamcinolone acetone (d.p.m./tube)	$[\text{^3H}]$ Triamcinolone acetone complex added (d.p.m./tube)	Acceptor-bound $[\text{^3H}]$ triamcinolone acetone complex (%)
A	17 500	85 000	20.6
A'	14 500	85 000	17.1
B	29 000	85 000	34.1
B'	26 500	85 000	31.2

Discussion

Studies from various laboratories have established that molybdate blocks the process of receptor activation and has no effect on the binding of activated receptor to nuclei, DNA-cellulose, phosphocellulose and ATP-Sepharose (Toft & Nishigori, 1979; Leach *et al.*, 1979; Nishigori & Toft, 1980). In these studies the concentration of molybdate used to observe its inhibitory effects was limited to 10–20 mM. Results of the present studies indicate that molybdate is, in fact, active against both non-activated and activated glucocorticoid receptor. The effects of molybdate on the process of activation (Fig. 1) are in agreement with those from other studies (Toft & Nishigori, 1979; Leach *et al.*, 1979; Nishigori & Toft, 1980). However, much higher concentrations of molybdate were required to block the binding of activated receptor to acceptors (Fig. 2). Although the reasons why higher concentrations of molybdate are required to inhibit activated receptor are not clear, the activated receptor may have undergone a change in conformation that is not sensitive to lower concentrations of molybdate. Furthermore, the inhibition in the binding of activated receptor to acceptors in the presence of molybdate is not due to an incomplete activation of cytosol preparations, since $(\text{NH}_4)_2\text{SO}_4$ -fractionated receptor also shows sensitivity to the presence of molybdate.

Previous studies have suggested that phosphorylation mechanisms may be involved in determining the level of active receptor in the cell (Nielsen *et al.*, 1977b; Leach *et al.*, 1979). Based on the effects of molybdate and other phosphatase inhibitors on the process of receptor activation, dephosphorylation of receptor protein has been suggested as a requirement for activation (Leach *et al.*, 1979; Barnett *et al.*, 1980). Studies presented in this paper suggest that molybdate is effective against the receptor in both its non-activated and activated forms (Figs. 1–3). The effects of molybdate may

not be indirect or due entirely to inhibition of phosphatases.

The activated receptor complexes bound to DNA-cellulose or nuclear sites are extracted by molybdate in a dose-dependent manner (Fig. 4). Furthermore, the molybdate-extracted receptor retains its ability to bind to DNA or nuclear sites (Table 2), although upon nuclear binding, it undergoes a change which results in the appearance of a smaller (2S) form of glucocorticoid receptor. It is not clear whether the smaller form is a subunit or a dissociated region of glucocorticoid-receptor protein, or merely an artifact.

At present, the mode of action of molybdate is not clear. This paper presents new evidence that molybdate is effective against both non-activated and activated glucocorticoid receptor. At low concentrations (<20 mM) molybdate is effective against only non-activated receptor, whereas at higher concentrations (>20 mM) its effects can be observed on the activated receptor as well. It is also possible that at higher concentrations molybdate may exert some ionic effects which may contribute to its ability to block acceptor binding and extract glucocorticoid-receptor complexes. However, equimolar concentrations of KCl and other ionic buffers seem ineffective in causing molybdate-like effects. Nishigori & Toft (1980) have shown that molybdate inhibition of avian progesterone receptor activation is pH-dependent, molybdate being ten times more effective at pH 7.0. The present studies were performed at pH 8.0, and it is possible that at a lower pH molybdate may be more effective against activated receptor at much lower concentrations. There is a good probability that molybdate causes its effects by binding to the receptor via a site which is retained after activation. Molybdate does not appear to convert the activated receptor into an inactivated state in an irreversible manner, since the molybdate-pretreated or molybdate-extracted receptor is able to bind nuclei and DNA-cellulose after dialysis. The

present studies advance our knowledge about molybdate action, but the exact mode of its operation must await future investigations.

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References

- Alberts, B. & Herrick, G. (1971) *Methods Enzymol.* **21**, 198–217
- Barnett, C. A., Schmidt, T. J. & Litwack, G. (1980) *Biochemistry* **19**, 5446–5455
- Baxter, J. D. (1972) *Proc. Natl. Acad. Sci. U.S.A.* **69**, 1892–1896
- Buller, R. E., Toft, D. O., Schrader, W. T. & O'Malley, B. W. (1975) *J. Biol. Chem.* **250**, 801–808
- Burton, K. (1956) *Biochem. J.* **62**, 315–323
- Higgins, S. J., Rousseau, G. G., Baxter, J. D. & Tomkins, G. M. (1973) *J. Biol. Chem.* **248**, 5866–5872
- John, J. K. & Moudgil, V. K. (1979) *Biochem. Biophys. Res. Commun.* **90**, 1242–1248
- King, E. G. (1932) *Biochem. J.* **26**, 292–297
- Leach, K. L., Dahmer, M. K., Hammond, N. D., Sando, J. J. & Pratt, W. B. (1979) *J. Biol. Chem.* **254**, 1184–1190
- Lohmar, P. H. & Toft, D. O. (1975) *Biochem. Biophys. Res. Commun.* **61**, 8–15
- Martin, R. G. & Ames, B. N. (1961) *J. Biol. Chem.* **236**, 1372–1379
- Milgrom, E., Atger, M. & Baulieu, E. E. (1973) *Biochemistry* **12**, 5198–5205
- Miller, J. B. & Toft, D. O. (1978) *Biochemistry* **17**, 173–177
- Moudgil, V. K. & Eessalu, T. E. (1980) *FEBS Lett.* **122**, 189–192
- Moudgil, V. K. & John, J. K. (1980a) *Biochem. J.* **190**, 799–808
- Moudgil, V. K. & John, J. K. (1980b) *Biochem. J.* **190**, 809–818
- Moudgil, V. K. & Toft, D. O. (1975) *Proc. Natl. Acad. Sci. U.S.A.* **72**, 901–905
- Moudgil, V. K. & Toft, D. O. (1977) *Biochim. Biophys. Acta* **490**, 477–488
- Moudgil, V. K., Nishigori, H., Eessalu, T. E. & Toft, D. O. (1980) in *Gene Regulation by Steroid Hormones* (Roy, A. K. & Clark, J. H., eds.), pp. 103–119, Springer-Verlag
- Nielsen, C. J., Sando, J. J., Vogel, W. M. & Pratt, W. B. (1977a) *J. Biol. Chem.* **252**, 7568–7578
- Nielsen, C. J., Sando, J. J. & Pratt, W. B. (1977b) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 1398–1402
- Nishigori, H. & Toft, D. O. (1980) *Biochemistry* **19**, 77–83
- Noma, K., Nakao, K., Sato, B., Nishizawa, Y., Matsumoto, K. & Yamamura, Y. (1980) *Endocrinology* **107**, 1205–1211
- Rice, R. H. & Means, G. E. (1971) *J. Biol. Chem.* **246**, 831–832
- Shyamala, G. & Leonard, L. (1980) *J. Biol. Chem.* **255**, 6028–6031
- Spelsberg, T. C., Knowler, J. T. & Moses, H. L. (1974) *Methods Enzymol.* **31**, 263–279
- Toft, D. O. (1972) *J. Steroid Biochem.* **3**, 515–522
- Toft, D. O. & Nishigori, H. (1979) *J. Steroid Biochem.* **11**, 413–416
- Yamamoto, K. R. & Alberts, B. M. (1972) *Proc. Natl. Acad. Sci. U.S.A.* **69**, 2105–2109