Interaction of rat liver glucocorticoid receptor with sodium tungstate

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Effects of sodium tungstate on various properties of rat liver glucocorticoid receptor were examined at pH 7 and pH 8. At pH 7, [^3H]triamcinolone acetonide binding in rat liver cytosol preparations was completely blocked in the presence of 10–20 mM-sodium tungstate at 4°C, whereas at 37°C a 30 min incubation of cytosol receptor preparation with 1 mM-sodium tungstate reduced the loss of unoccupied receptor by 50%. At pH 8.0, tungstate presence during the 37°C incubation maintained the steroid-binding capacity of unoccupied glucocorticoid receptor at control (4°C) levels. In addition, heat-activation of cytosolic glucocorticoid–receptor complex was blocked by 1 mM- and 10 mM-sodium tungstate at pH 7 and pH 8 respectively. The DNA–cellulose binding by activated receptor was also inhibited completely and irreversibly by 5 mM-tungstate at pH 7, whereas at pH 8 no significant effect was observed with up to 20 mM-tungstate. The entire DNA–cellulose-bound glucocorticoid–receptor complex from control samples could be extracted by incubation with 1 mM- and 20 mM-tungstate at pH 7 and pH 8 respectively, and appeared to sediment as a 4.3–4.6 S molecule, both in 0.01 M- and 0.3 M-KCl-containing sucrose gradients. Tungstate effects are, therefore, pH-dependent and appear to involve an interaction with both the non-activated and the activated forms of the glucocorticoid receptor.

A variety of chemical agents have been identified that block interaction of steroid receptors with nuclei, DNA–cellulose, or ATP–Sepharose (Lohmar & Toft, 1975; Nishigori & Toft, 1979, 1980; Moudgil *et al.*, 1980). Some of the compounds, rifamycin AF/013, *o*-phenanthroline, aurintricarboxylic acid and pyridoxal 5'-phosphate, have been reported to block the binding of activated receptor complex to the 'acceptors' directly. More recently, another compound, Na_2MoO_4 , has been identified as a stabilizing agent for steroid receptors (Nielsen *et al.*, 1977, 1979; Nishigori & Toft, 1980). Subsequent studies have revealed that the addition of molybdate, vanadate or tungstate blocks the activation process of steroid receptors (Toft & Nishigori, 1979; Leach *et al.*, 1979; Nishigori & Toft, 1980; Nishigori *et al.*, 1980), but these compounds were believed to have no effect on activated steroid–receptor complexes. Since molybdate and related compounds are inhibitors of phosphatases, the process of dephosphorylation has been suggested as a requirement for receptor activation.

The effects of molybdate on the activation of avian progesterone–receptor complex have been shown to be pH-dependent (Nishigori & Toft, 1980).

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molybdate being 10 times more effective at pH 7 than at pH 8. Barnett *et al.* (1980) observed that, at pH 8, tungstate presence up to 10 mM had no effect on the DNA–cellulose binding of glucocorticoid receptor. In the present studies, effects of tungstate on several properties of rat liver glucocorticoid receptor were examined at pH 7 and pH 8. Whereas at pH 8 the effect of tungstate appeared mild, at pH 7 tungstate (5 mM) inhibited the steroid binding, activation and DNA-binding of glucocorticoid receptor. In addition, tungstate was able to extract the DNA–cellulose-bound receptor in a dose-dependent manner. These data suggest that tungstate effects on receptor are direct and perhaps through its interaction with both non-activated and activated glucocorticoid receptor.

Experimental procedures

All reagents used were of analytical grade. [$6,7(\text{n})$ - ^3H]Triamcinolone acetonide (sp. radioactivity, 30–35 Ci/mmol) was obtained from New England Nuclear; Na_2WO_4 , NaVO_3 , Na_2CrO_4 and $\text{Na}_2\text{Cr}_2\text{O}_7$ were from Fisher Co.; NaF, Na_2MoO_4 and Na_2HAsO_4 were from Merck; levamisole hydrochloride was from Aldrich Chemical Co.; NaH_2PO_4 and Na_2HPO_4 were from Baker Chemical

Co.; Dextran T-70 was from Pharmacia; cellulose powder (Cellex-410) was from Bio-Rad; the other reagents were purchased from Sigma Chemical Co.

Buffers

The following buffers were prepared at 4°C at both pH 7 and pH 8 and were maintained at 4°C: buffer A, 50 mM-Tris/HCl/12 mM-monothioglycerol/10% glycerol; buffer B, 50 mM-Tris/HCl/12 mM-monothioglycerol/10% glycerol/10 mM-KCl; buffer C, 50 mM-Tris/HCl/12 mM-monothioglycerol/10% glycerol/1 M-KCl; buffer D, 50 mM-Tris/HCl/12 mM-monothioglycerol.

Preparation of DNA-cellulose

DNA-cellulose was prepared by the methods of Alberts & Herrick (1971). Calf thymus DNA (type II) was linked to Cellex-410 and these preparations contained 2 mg of DNA/g of dry DNA-cellulose as quantified by the method of Burton (1956).

Preparation of glucocorticoid receptor

Cytosol fraction containing glucocorticoid receptor was obtained from the livers of rats by methods described previously (Murakami & Moudgil, 1981a). The cytosol was used either to form a steroid-receptor complex or to determine the effect of Na_2WO_4 on the unoccupied receptor.

Effects of Na_2WO_4 on the unoccupied glucocorticoid receptor

Portions of freshly prepared cytosol preparations (0.2–0.25 ml) were mixed with 10 nM-[^3H]triamcinolone acetonide in a final volume of 0.5 ml. The mixtures were incubated at 0°C for 4 h with and without various concentrations of tungstate. The free steroid was removed by adding a portion (0.5 ml) of charcoal suspension (0.5% Norit A/0.05% Dextran T-70 made in buffer D). After 5 min, the mixtures were centrifuged twice at 1000 g for 10 min and the resultant supernatants were transferred to counting vials directly to measure the total steroid binding. In some experiments, cytosol was pre-incubated with tungstate for 30 min before the addition of the labelled steroid.

To investigate tungstate effects on the stability of unoccupied glucocorticoid receptor, cytosol (0.2–0.25 ml) was incubated at 37°C either for 30 min with various concentrations of Na_2WO_4 or for different time periods with a single tungstate concentration in a final volume of 0.5 ml. In both cases, tungstate was incubated with receptor preparations for 30 min at 0°C before the heat treatment. Contents of the tubes were cooled on ice for 15 min before the addition of 10 nM-[^3H]triamcinolone acetonide. The extent of steroid binding was measured after a 4 h incubation at 0–4°C. The amount of non-specific steroid binding was de-

termined by including duplicate tubes containing a 1000-fold excess of unlabelled steroid added before the addition of [^3H]triamcinolone acetonide. The specific steroid binding was calculated by subtracting the non-specific binding from the total steroid binding.

Effects of Na_2WO_4 on the occupied glucocorticoid receptor

Portions (0.2–0.25 ml) containing [^3H]triamcinolone acetonide-receptor complexes were incubated at 4°C for 60 min with various concentrations of Na_2WO_4 (0–20 mM) in a final volume of 0.5 ml. The complexes were also incubated at 37°C in the presence of tungstate to determine the effects on their stability. The dissociated or free steroid from the mixtures was removed by treatment with charcoal.

Activation of cytosol glucocorticoid-receptor complex

The freshly prepared cytosol glucocorticoid-receptor complexes were heat-activated by incubation at 23°C for 40 min (pH 7.5 and 8) and 60 min (pH 7) and then chilled for 15 min on ice. The preparations were then mixed with pelleted Dextran-coated charcoal (obtained by centrifugation of equal volumes of charcoal suspension) for 5 min to avoid dilution. The activated receptor complexes were recovered by centrifugation twice at 1000 g for 10 min. The cytosol [^3H]triamcinolone acetonide-receptor complexes were also activated by fractionation with $(\text{NH}_4)_2\text{SO}_4$ at 35% saturation. The resultant precipitate from the 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 the centrifugation at 12000 g for 20 min, the supernatant was used as the $(\text{NH}_4)_2\text{SO}_4$ -activated complex. The heat- and $(\text{NH}_4)_2\text{SO}_4$ -activated complexes were used for DNA-cellulose binding assay under various conditions.

To determine the effects of tungstate on the heat-activation process of glucocorticoid-receptor complex, portions of [^3H]triamcinolone acetonide-receptor complexes formed at 0°C for 4 h were directly combined with different concentrations of tungstate (0–10 mM) in a final volume of 2 ml. The mixtures were incubated at 23°C for 40 min and chilled on ice for 15 min and free steroid was removed by mixing the preparation with pelleted charcoal. The effect of tungstate on the rate of activation was also measured by incubating the [^3H]triamcinolone acetonide-receptor complex at 23°C for various time periods with and without 1 mM-tungstate at pH 7. The extent of activation was then measured by determining the amount of receptor binding to DNA-cellulose.

Reversibility of tungstate effects

In some experiments, tungstate (5 mM) was added to the activated [^3H]triamcinolone acetone–receptor complexes. After a 30 min incubation at 4°C, the preparations were mixed with 0.5 mM-KCl and dialysed against buffer A for 4–24 h at 4°C. After centrifugation at 10000 *g* for 20 min, the resultant supernatant was used for determining the DNA–cellulose binding.

DNA–cellulose binding of glucocorticoid–receptor complex

DNA–cellulose binding of cytosol- or $(\text{NH}_4)_2\text{SO}_4$ -fractionated receptor was accomplished as detailed previously (Murakami & Moudgil, 1981b). The DNA–cellulose-bound radioactivity was extracted with buffer C by an incubation at 4°C for 30 min. Specificity of receptor binding to DNA–cellulose was determined by using cellulose (Cellex-410; 10 mg/ml in buffer B) in a parallel experiment. The non-specific cellulose binding was subtracted from total receptor binding to DNA–cellulose to obtain specific DNA binding. The non-specific DNA binding was observed to be within 5% of total [^3H]triamcinolone acetone–receptor complex binding to DNA–cellulose.

The phosphatase inhibitors were dissolved in buffer D and were included in the mixtures containing glucocorticoid–receptor complexes to determine their effect on the DNA-binding.

Extraction of glucocorticoid–receptor complexes from DNA–cellulose

Preparations containing DNA–cellulose-bound glucocorticoid–receptor complexes were incubated with different concentrations of Na_2WO_4 at 4°C for 30 min with gentle shaking. In parallel experiments, the complexes were extracted using buffer C. The values obtained by extraction of complexes with buffer C (1 M-KCl) were considered to represent 100% extraction of resin-bound complexes. After centrifugation at 1000 *g* for 10 min, the radioactivity in the supernatant was measured directly.

To determine whether or not the tungstate-extracted receptor retained its ability to rebinding DNA–cellulose, the following protocol was used. $(\text{NH}_4)_2\text{SO}_4$ -fractionated glucocorticoid–receptor complexes were first incubated with DNA–cellulose and subsequently extracted by tungstate treatment. The extracted receptor was mixed with 0.5 M-KCl and dialysed against buffer A. The DNA–cellulose binding assay was then performed to determine the extent of reversibility of the tungstate effect.

Sucrose-density-gradient analysis

Linear 5–20% sucrose gradients (4.4 ml) were prepared in a buffer containing 10 mM-Tris/HCl, 12 mM-monothiolglycerol and either 10 mM-KCl

(low-salt) or 300 mM-KCl (high-salt), pH 7.0, by using a Beckman gradient former. The receptor samples, which contained 10% glycerol, were diluted with buffer D to decrease the glycerol concentration before layering on the gradient. Ovalbumin, egg-white lysozyme, bovine serum albumin, alcohol dehydrogenase, aldolase and catalase were layered on separate gradients to serve as standards for sedimentation coefficient. The gradients were centrifuged at either 100000 *g* for 12 h (low-salt) or 150000 *g* for 16 h (high-salt) on a Beckman L5-75 ultracentrifuge. Fractions (0.2 ml) were collected by piercing the bottom of each tube and the radioactivity was measured as described previously (Murakami & Moudgil, 1981a).

Results

Effect of tungstate on the steroid binding

Tungstate did not affect the dissociation of steroid from the [^3H]triamcinolone acetone–receptor complex at either pH 7 or pH 8. Although tungstate did not inhibit the steroid binding at pH 8, it did affect the binding at pH 7, with half-maximal inhibition (I.D._{50}) at 8 mM- and 4 mM-tungstate dependent on whether tungstate was added simultaneously or before [^3H]triamcinolone acetone addition (not shown). These tungstate effects can be attributed to alterations in the affinity of the receptor protein for the hormone or in the number of steroid-binding sites. A competition between the steroid and tungstate for the steroid-binding site may also be occurring.

Effects of tungstate on the stability of receptor at 37°C

Freshly prepared cytosol from rat liver was pre-incubated with different concentrations of tungstate or with buffer D at 0°C for 30 min and heated at 37°C for 30 min (Fig. 1a). Tungstate treatment prevented the loss of steroid-binding capacity of unoccupied receptor at both pH 7 and pH 8. During the 37°C incubation period, the presence of 1 mM-tungstate at pH 7 allowed 60% of the total steroid binding observed with the non-heated receptor. At higher concentrations (>1 mM) the stabilizing effects of tungstate at pH 7 appeared to decrease. The protective effect of tungstate was, however, more pronounced at pH 8 conditions, where the samples incubated at 37°C with 10 mM-tungstate showed a steroid-binding capacity equivalent to non-heated samples. At higher concentrations, the stabilizing effects of tungstate appeared to decrease both at pH 7 and pH 8 (Fig. 1a). At pH 7, the decrease may be due to loss of steroid-binding capacity but at pH 8 it does not appear to be the case.

Unoccupied receptors without tungstate were

Fig. 1. Effects of tungstate on the steroid-binding properties of glucocorticoid receptors

(a) Shows effects of tungstate concentration on the stability of unoccupied glucocorticoid receptor at 37°C . Portions of cytosol (0.2 ml) were mixed with various concentrations of tungstate in a final volume of 0.5 ml. After 30 min at 4°C , the contents of the mixture were heated at 37°C for 30 min and chilled by incubation at 4°C for 15 min. The mixtures were then complexed with 10 nM - $[^3\text{H}]$ triamcinolone acetone. A 100% specific ($[^3\text{H}]$ triamcinolone acetone binding (total - non-specific) represents 15000 c.p.m. and 6400 c.p.m. for pH 7 (○) and pH 8 (●) respectively. (b) Shows a time course for the effects of tungstate on the stability of unoccupied receptor at 37°C . Portions of cytosol (0.2 ml) were mixed with either 1 mM- (pH 7) or 10 mM-tungstate (pH 8) in a final volume of 0.5 ml. After a 30 min incubation at 4°C , the cytosol mixtures were heated at 37°C for various time periods. Samples without tungstate served as controls (----). A 100% specific binding represents 2300 c.p.m. and 4320 c.p.m. for pH 7 (○) and pH 8 (●) respectively. (c) Shows effects of tungstate concentrations on the stability of glucocorticoid-receptor complex. Portions (0.2 ml) of cytosol preparations containing $[^3\text{H}]$ triamcinolone acetone-receptor complexes were mixed with different concentrations of tungstate in a final volume of 0.5 ml. After 30 min at 4°C , the incubation tubes were heated at 37°C for 30 min. An equal volume of a charcoal suspension was added to each tube to remove free steroid resulting from the dissociation of the $[^3\text{H}]$ triamcinolone acetone-receptor complex. After centrifugation at $1000g$ for 10 min, the supernatant containing the receptor-bound $[^3\text{H}]$ triamcinolone acetone was used to measure radioactivity. The non-specific binding was determined by heating the complexes at 60°C for 20 min in the presence of $10\mu\text{M}$ unlabelled triamcinolone acetone. A 100% specific $[^3\text{H}]$ triamcinolone acetone-receptor complex represents 12000 c.p.m. and 2960 c.p.m. for pH 7 (○) and pH 8 (●) respectively. (d) Shows a time course of tungstate effects on the stability of glucocorticoid-receptor complex. Portions of cytosol (0.25 ml) containing $[^3\text{H}]$ triamcinolone acetone-receptor complexes were mixed with 1 mM- (pH 7) and 10 mM-tungstate (pH 8) for 30 min at 4°C . The tubes without tungstate addition served as controls (----). The tubes were heated at 37°C for various time periods. The contents of the tubes were then brought back to 4°C and mixed with equal volume (0.5 ml) of charcoal suspension. A 100% specific binding equals 10000 c.p.m. and 5200 c.p.m. for pH 7 (○) and pH 8 (●) respectively.

almost immediately inactivated at 37°C within 10 min (Fig. 1b). At pH 7, 1 mM-tungstate stabilized the receptor throughout the 60 min incubation at 37°C. At pH 8, there was no loss of binding capacity of receptor heated at 37°C in the presence of 10 mM-tungstate.

To determine the effect of tungstate on the stability of glucocorticoid-receptor complex at 37°C, various concentrations of tungstate were incubated with the complexes for 30 min at 4°C followed by heating for 30 min at 37°C. After removing the free [³H]triamcinolone acetonide by charcoal treatment, the macromolecular-bound [³H]triamcinolone acetonide was measured (Fig. 1c). At pH 7, tungstate (1–5 mM) prevented the dissociation of the complex at 37°C, keeping the amount of available complexes within 60% of that of non-heated samples. At higher concentrations (5–10 mM), tungstate may be involved in an exchange resulting in the release of the free steroid from the complex. In the presence of excess free steroid during the incubation of complexes at 37°C, a higher percentage (70–100%) of the complexes were retained, although the profile of tungstate effects remains unchanged (results not shown). Interestingly, contrary to its effect on unoccupied receptor, tungstate had no stabilizing influence on the glucocorticoid-receptor complex at pH 8. When tested for its ability to stabilize the complexes at pH 7 and pH 8 as a function of duration of heat treatment, tungstate at pH 7 appeared to stabilize the complexes that were heated for 0–60 min at 37°C at values well above those obtained without tungstate treatment (Fig. 1d). No significant effects of tungstate were observed on the stability of complexes heated at 37°C at pH 8.

Activation of glucocorticoid-receptor complex

The extent of receptor activation was measured by determining the DNA-cellulose binding by the [³H]triamcinolone acetonide-receptor complexes. Tungstate presence inhibited the heat activation of [³H]triamcinolone acetonide-receptor complexes with I.D.₅₀ values of 0.2 mM- and 1.5 mM-tungstate at pH 7 and pH 8 respectively (results not shown).

The effect of tungstate on the rate of activation was examined by incubating cytosol [³H]triamcinolone acetonide-receptor complexes at 23°C for different time periods in the presence of 1 mM-tungstate or buffer D (control). The DNA-cellulose binding of the complexes from the control samples increased gradually, showing maximum values at 90 min (results not shown). Presence of tungstate completely inhibited the receptor activation throughout the incubation period at 23°C, suggesting that tungstate inhibits the extent of receptor activation rather than the rate of activation.

Effects of tungstate on the DNA-cellulose binding of activated glucocorticoid-receptor complexes

To examine the effects of tungstate on the activated glucocorticoid-receptor complexes, cytosol [³H]triamcinolone acetonide-receptor complexes prepared at different pH values were heat-activated by incubation first at 23°C for 40 min (pH 7.5 and pH 8) or for 60 min (pH 7). The activated receptor was incubated with various concentrations of Na₂WO₄ and its DNA-binding capacity was determined. As reported previously (Barnett *et al.*, 1980), at pH 8 tungstate had only minor inhibitory effects on DNA-cellulose binding of glucocorticoid-receptor complexes (Fig. 2a). At lower pH values, tungstate blocked DNA binding in a concentration-dependent manner, showing complete inhibition at 5 mM and 10 mM for pH 7 and pH 7.5 respectively. The inhibitory effects of tungstate were not due to its complexing with DNA-cellulose, since a pretreatment of DNA-cellulose with up to 50 mM-tungstate was found to have no effects on the binding of activated complexes (results not shown).

Since cytosol used in the above experiments might have contained only partially activated receptor, the cytosol [³H]triamcinolone acetonide-receptor complexes fractionated by (NH₄)₂SO₄ were also used (Fig. 2b). The steroid receptors in such preparations are reported to be fully activated and cannot be activated any further by heat (Lohmar & Toft, 1975; Buller *et al.*, 1975). Not only did the tungstate-treated salt-activated complexes fail to bind to DNA-cellulose, but they also exhibited a sensitivity to the compound five times greater than the heat-activated complexes (Fig. 2b). Incubation of tungstate (3 mM) with the (NH₄)₂SO₄-fractionated receptor completely blocked the DNA binding even at pH 8. Therefore, not only are the effects of tungstate on the DNA binding pH-dependent, but they may also depend on some factors whose concentration may be altered on (NH₄)₂SO₄-fractionation. Finally, the inhibition in the DNA binding of activated complexes does not appear to be a result of a dissociation of [³H]triamcinolone acetonide from the complex.

Since tungstate is a very effective inhibitor of phosphatases, other compounds either structurally similar to tungstate (metals of element-group 5 and 6B) or with demonstrable ability to inhibit phosphatases were tested to determine the specificity of their effects on DNA-cellulose binding. The compounds were dissolved in buffer D at pH 7 and were incubated at 4°C for 30 min at 5 mM with the cytosol preparations containing activated [³H]triamcinolone acetonide-receptor complexes. Whereas monochromate and dichromate inhibited (35–55%) the binding of [³H]triamcinolone acetonide-receptor-complex to DNA-cellulose, levamisole, arsen-

Fig. 2. *Effects of tungstate on the DNA-cellulose binding of activated glucocorticoid-receptor complex*
 (a) Cytosol was prepared fresh at pH 7, pH 7.5 and pH 8, and complexed with 10 nM [³H]triamcinolone acetonide for 4 h at 4°C followed by a 40 min incubation at 23°C. Portions of activated cytosol complexes at pH 7 (24 015 c.p.m.), pH 7.5 (27 905 c.p.m.) and pH 8 (13 624 c.p.m.) were incubated at 4°C for 40 min with DNA-cellulose suspensions (40 µg of DNA) in the presence of various concentrations of tungstate in a total volume of 1 ml. The DNA-cellulose-bound complexes were then extracted with buffer C maintained at the respective pH values. DNA binding by receptor without tungstate treatment was considered as 100% and represented 10 629 c.p.m. for pH 7 (○—○), 10 530 c.p.m. for pH 7.5 (●—●) and 5 410 c.p.m. for pH 8 (●). (b) Cytosol portions containing [³H]triamcinolone acetonide-receptor complexes were fractionated with 35% saturation of (NH₄)₂SO₄ at pH 7 (14 970 c.p.m.) and at pH 8 (25 400 c.p.m.). The precipitated receptor was dissolved in buffer A of the same pH and small portions (14 970 c.p.m., pH 7; 25 400 c.p.m., pH 8) incubated in a series of tubes with tungstate or buffer D. The receptor samples without tungstate treatment represented controls and their DNA binding as 100%, which corresponds to 5 990 for pH 7 (○) and 11 500 c.p.m. for pH 8 (●).

ate, fluoride, phosphate, molybdate and vanadate had no effects on this process at 5 mM concentration (results not shown).

Sedimentation analysis of receptor after tungstate treatment

Fig. 3(a) illustrates that the non-activated and heat- or salt-activated [³H]triamcinolone acetonide-receptor complexes sedimented in the 8–9S region in low-salt gradients. A 30 min treatment with tungstate at 4°C altered the sedimentation rate of both non-activated and activated complexes. The tungstate-treated non-activated complex sedimented in two peaks, one with a smaller size (approx. 7S) and the other with a larger aggregate of approx. 11S. The activated complex sedimented in a single peak of approx. 8S but shifted to approx. 6S after tungstate treatment. Tungstate at pH 7 appears to have effects that cause both deaggregation (non-activated and activated) and aggregation (non-activated) of glucocorticoid-receptor complexes at pH 7. Interestingly, both non-activated and activated receptor forms sedimented at the same position at pH 7 (Fig. 3a). Under high-salt conditions (Fig. 3b), tungstate treatment caused aggregation of the activated

receptor yielding a 4.6S form. The sedimentation profiles of glucocorticoid receptors at pH 8 appear different and involve aggregation of receptor (Murakami & Moudgil, 1981a).

Extraction of DNA-cellulose-bound glucocorticoid-receptor complex

The heat-activated cytosol complexes were adsorbed on to DNA-cellulose and the resin was washed extensively with buffer B containing 0.01 M-KCl. The preparations were then mixed with buffer D containing different concentrations of Na₂WO₄. Fig. 4 illustrates that at pH 7 conditions, more than 60% of the radioactivity could be extracted from DNA-cellulose with 50 µM-tungstate. The entire DNA-cellulose-bound radioactivity could be routinely extracted with 1–2 mM-Na₂WO₄. The greater sensitivity of DNA-cellulose-bound receptor to lower concentrations of tungstate may be due to a removal of some cytosol proteins or factors that may limit tungstate availability to receptor. Again, at pH 8, higher (100 times) concentrations were required to extract the DNA-cellulose-bound receptor.

To examine specificity among the phosphatase inhibitors and group 5- and 6B-element-containing

Fig. 3. Effects of tungstate on the sedimentation rate of glucocorticoid-receptor complex

Portions of cytosol containing glucocorticoid-receptor complexes prepared at pH 7 were incubated with either buffer D (control) or 5 mM-tungstate. Portions (0.2 ml) of the above incubation were applied on the 5–20% sucrose gradients containing 0.01 M-KCl (a) and centrifuged at 105 000 *g* for 12 h. Symbols: ○—○, non-activated; ●—●, non-activated + 5 mM- Na_2WO_4 ; △—△, heat-activated; ▲—▲, heat-activated + 5 mM- Na_2WO_4 . (b) Shows the effect of tungstate on the sedimentation of heat-activated glucocorticoid-receptor complex in sucrose gradients containing 0.3 M-KCl (150 000 *g* for 16 h). Symbols: △, activated complex; ▲, activated complex + 5 mM- Na_2WO_4 .

Fig. 4. Extraction of DNA-cellulose-bound $[^3\text{H}]$ triamcinolone acetanide-receptor complex by tungstate. Heat-activated complexes were adsorbed on to DNA-cellulose as described in the Experimental procedures section. The resin-bound complexes were then extracted by incubation with buffer D containing either 1 M-KCl or different concentrations of tungstate. All solutions, including cytosol, were made at either pH 7 or pH 8.0. A 100% extraction refers to the amount of complex extracted by 1 M-KCl and corresponds to 15 390 c.p.m. Symbols: ○, pH 7; ●, pH 8.

compounds in releasing radioactivity from DNA-cellulose incubated with $[^3\text{H}]$ triamcinolone acetanide-receptor complexes, the fractions were mixed with the compounds (Na_2WO_4 , NaVO_3 , Na_2CrO_4 , $\text{Na}_2\text{Cr}_2\text{O}_7$, Na_2HAsO_4 , NaH_2PO_4 , Na_2HPO_4 , levamisole) prepared in buffer D. Only tungstate (1 mM) was able to extract the entire DNA-cellulose-bound glucocorticoid-receptor complex at pH 7, suggesting that at pH 7 tungstate interacts with the glucocorticoid-receptor complex in a specific manner. Although chromate appeared to inhibit DNA binding (35–55%), it was ineffective in extracting radioactivity from DNA-cellulose.

Sedimentation analysis of DNA-extracted receptor

Tungstate-extracted complex sedimented approximately as a 4.3–4.6 S entity in both 0.01 M- and 0.3 M-KCl-containing gradients (Figs. 5a and 5b). This is in agreement with the similar observations made by Dolan *et al.* (1980), who reported a 4S glucocorticoid-receptor complex form extracted from the DNA-cellulose by pyridoxal 5'-phosphate or NaCl. It is, therefore, clear that tungstate-released radioactivity is macromolecule-associated and does not result from the dissociation of glucocorticoid-receptor complex.

Fig. 5. Sedimentation analysis of the tungstate-extracted glucocorticoid-receptor complex. Portions (0.15 ml) of the complexes extracted from DNA-cellulose by incubation with 5 mM-tungstate were applied on 5–20% linear sucrose gradients containing 0.3 M-KCl (a) or 0.01 M-KCl (b). The gradients were centrifuged at 150 000 *g* for 16 h (a) or 105 000 *g* for 12 h (b). Symbols: ●, Na_2WO_4 extract; △, heat-activated glucocorticoid-receptor complex. All solutions were at pH 7.

Table 1. Effects of dialysis on the DNA-cellulose binding by tungstate-treated glucocorticoid-receptor complex

The cytosol preparations containing [^3H]triamcinolone acetone-receptor complexes were fractionated by 35% saturation of $(\text{NH}_4)_2\text{SO}_4$, redissolved in buffer A and applied (1.0 ml) to DNA-cellulose (200 μg of DNA). The resin-bound complexes were then recovered by treatment with 5 mM- Na_2WO_4 . The extracted complexes were added with 0.5 M-KCl and thoroughly dialysed against buffer A at 4°C for 4 h. Portions of $(\text{NH}_4)_2\text{SO}_4$ -fractionated receptor were also treated separately with 5 mM-tungstate mixed with 0.5 M-KCl and dialysed in the same way.

Treatment	Total complex added/tube (d.p.m.)	[^3H]Triamcinolone acetone bound to DNA-cellulose/tube (d.p.m.)	DNA binding (%)
$(\text{NH}_4)_2\text{SO}_4$ -fractionated complex	54 760	34 000 $\pm$ 1860	62.1
Na_2WO_4 added	61 290	1430 $\pm$ 160	2.3
Na_2WO_4 extract from DNA-cellulose	49 560	4550 $\pm$ 1010	9.2

Reversibility of tungstate effect

Previous studies have shown that the effects of molybdate and vanadate are reversible and can be eliminated by a thorough dialysis (Nishigori & Toft, 1980; Leach *et al.*, 1979; Nishigori *et al.*, 1980). In our studies, the addition of tungstate to the activated complexes and the extraction of complexes

from DNA-cellulose by tungstate both caused an irreversible change in the properties of glucocorticoid-receptor complex resulting in a loss of DNA-cellulose-binding capacity (Table 1). In this experiment, approx. 60% of the total complexes were bound to DNA-cellulose as opposed to 20–40% of the complexes that bound DNA routinely in heat-activated cytosol preparations. This increased DNA binding may be due to a removal of some inhibitor(s) of DNA binding in crude preparations.

Discussion

The observations that phosphatase inhibitors like molybdate, tungstate and vanadate stabilize the steroid receptors and block the transformation of cytosol receptor complexes and that the rate of activation of glucocorticoid-receptor complexes is stimulated by incubation with calf intestine alkaline phosphatase (Barnett *et al.*, 1980) are significant and suggested that the activation involves a dephosphorylation of the receptor itself or of some regulatory component(s) (Leach *et al.*, 1979; Barnett *et al.*, 1980). However, the following lines of evidence indicate that mechanisms other than simple dephosphorylation of receptor or its regulatory component(s) may be involved in the process of activation. (a) Although Na_2MoO_4 and Na_2WO_4 block the process of receptor activation, other phosphatase inhibitors, such as levamisole, fluoride and arsenate, which are specific inhibitors of alkaline phosphatases (Fernley, 1971), do not prevent the

activation process (Nishigori & Toft, 1980; Barnett *et al.*, 1980). (b) Relatively high non-physiological concentrations of the exogenous calf intestinal alkaline phosphatase are required to stimulate activation (Barnett *et al.*, 1980). (c) Several phosphorylated compounds, including ATP, have also been shown to increase the extent of activation of glucocorticoid receptors (John & Moudgil, 1979; Moudgil & John, 1980; Barnett *et al.*, 1980; Moudgil *et al.*, 1981).

The results presented in this paper suggest that tungstate effects are due to its interaction with the glucocorticoid receptor. Although the actions of tungstate on glucocorticoid receptor may be somewhat similar to those of molybdate and vanadate, it seems that tungstate at pH 7 is more potent and highly specific. Therefore, these compounds either may be interacting in a pH-dependent manner with different sites or may possess altered affinity for the same site on the receptor. The following observations made earlier in the present paper support the argument in favour of a direct tungstate action on the receptor. (1) Addition of tungstate to unoccupied receptor at 4°C results in decreased steroid binding, whereas at 37°C, it stabilizes, and prevents inactivation of, both unoccupied and occupied receptors. (2) Tungstate blocks the process of receptor activation and reduces specifically the DNA-binding capacity of activated receptors (Fig. 2). (3) The addition of tungstate causes a conformational change in the receptor in both its non-activated and activated forms, which results in a change in its sedimentation properties under both low- and high-salt conditions (Fig. 3). (4) Finally, low concentrations of tungstate are able to specifically extract the DNA-cellulose-bound complexes (Fig. 4). It is not clear how tungstate exerts its effects. It could either bind to a DNA-binding site of the receptor or act in an allosteric manner.

Since low concentrations are required to observe its effects, tungstate interaction with the receptor may be very strong and may involve an irreversible change in the conformation of receptor, rendering it incapable of rebinding to DNA-cellulose (Table 1). This is another line of difference between molybdate, vanadate and tungstate actions, since the effects of the former two agents are known to be reversible (Leach *et al.*, 1979; Nishigori & Toft, 1980; Nishigori *et al.*, 1980).

The mechanism by which tungstate exerts its effects on glucocorticoid-receptor complex is not clearly understood at present. It is a potent phosphatase inhibitor and has been reported to be capable of forming complexes with P_i at acidic pH (Cotton & Wilkinson, 1972). Studies by Leach *et al.* (1979) suggest that molybdate may block activation by binding thiol or phosphate moieties on the receptor protein. It is, therefore, possible that

tungstate may bind to phosphate groups within the receptor molecule and thus block a dephosphorylation essential for activation of the complex. However, addition of excess phosphate in the incubation mixtures did not alter the tungstate effects on activation, DNA-cellulose binding or extraction of glucocorticoid-receptor complexes (results not shown).

Nishigori & Toft (1980) have suggested the possibility that molybdate or tungstate may exert their effects via an interaction with polypeptide amino acid residues. The thiol group of cysteine and the imidazole group of histidine have been shown to interact with molybdate (Weathers *et al.*, 1979). Another possibility is that tungstate may influence receptor function through its interaction with heavy-metal ions. Paigen (1958) has postulated that molybdate may inhibit the activities of particulate phosphoprotein phosphatase from mouse liver by interacting with heavy metals. This postulation would be further strengthened by the observations of Shyamala (1975) and Lohmar & Toft (1975), which suggest that steroid receptors may be metallo-proteins.

Finally, tungstate can be used as a chemical probe in the studies on the analysis of the processes that precede and follow receptor activation. The present studies represent the tungstate effects on both non-activated and activated glucocorticoid-receptor complexes. The discrepancies between present results and previous studies on the effects of tungstate (Nishigori & Toft, 1980; Leach *et al.*, 1979; Barnett *et al.*, 1980) may have arisen from the conditions of the assay. Obviously, the site(s) that interact with tungstate may become exposed or altered at neutral pH in a manner that favours interaction between tungstate and the receptor protein. The effects of tungstate reported in the present paper appear to represent a general phenomenon. Our preliminary results with avian progesterone-receptor complex also indicate that tungstate is able to block the process of activation and DNA-cellulose binding by activated receptor complexes. This is consistent with the similar effects of molybdate on glucocorticoid receptor previously reported from this laboratory (Murakami & Moudgil, 1981b). Therefore, the use of tungstate and other similar inhibitors may provide important insight into the regions of steroid receptors involved in various functions.

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