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Arsenite and Cadmium(II) as Probes of Glucocorticoid Receptor Structure and Function*

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Low concentrations of arsenite, but not arsenate, and Cd^{2+} blocked steroid binding to the glucocorticoid receptors of HTC cells. Inhibition by arsenite was faster and occurred at lower concentrations than for Cd^{2+}. Half-maximal inhibition of [^{3}H]dexamethasone binding was seen after a 30-min preincubation with ~7 μM arsenite. The effect of arsenite and of Cd^{2+} appears to be mediated by a reaction with vicinal dithiols of the receptor as shown by (*a*) the reversal of arsenite inhibition by much lower concentrations of dithiothreitol (~0.1 mM) than of β-mercaptoethanol (~10 mM); (*b*) the ability of both arsenite and Cd^{2+} to block [^{3}H]dexamethasone 21-mesylate labeling of receptors but not of other thiol-containing proteins; and (*c*) the known selectivity of arsenite and of Cd^{2+} for reactions with vicinal dithiols. Arsenite forms a tight complex with these vicinal dithiols since the removal of loosely associated arsenite by gel exclusion chromatography did not reverse the inhibition of steroid binding. The effect of other ions on steroid binding was also examined. Half-maximal inhibition of binding occurred with ~5 μM selenite, whereas up to 300 μM Zn^{2+} was without effect.

Much higher concentrations of arsenite were required for effects on unactivated and activated complexes. Arsenite slowly induced a loss of unactivated complexes but rapidly inhibited a portion of the DNA binding of activated complexes. Any effect on activation occurred at arsenite concentrations equal to or higher than those that inhibited DNA binding. In contrast, Cd^{2+} concentrations similar to those that block steroid binding caused a biphasic loss of unactivated complexes and a marginal loss of activated complexes.

This is the first report of effects of arsenite on glucocorticoid receptors. These results confirm directly our earlier hypothesis that steroid binding to rat glucocorticoid receptors involves a vicinal dithiol (Miller, N. R., and Simons, S. S., Jr. (1988) *J. Biol. Chem.* 263, 15217–15225) and show that arsenite is a potent new reagent for probing receptor structure and function.

Glucocorticoid receptors participate in several reactions in the course of mediating steroid regulation of specific gene expression. They must selectively bind the appropriate steroids with high affinity, undergo activation to a form of receptor-steroid complex that has increased affinity for DNA and nuclei, translocate into the nuclei, and associate with the biologically active glucocorticoid regulatory elements of responsive genes. Dramatic progress has been achieved recently in understanding these steps, but the relationships between protein structure and function are only vaguely understood. The primary amino acid sequence of the receptor in several species (1–3) has been determined, but the various functional elements that have been defined by molecular biology techniques appear to be modular (4–7), thus giving rise to a very plastic model of the receptor protein. In order to obtain more information about the structure and function of the native receptor, we have complemented these molecular biology approaches with specific antibodies (8) and chemical reagents such as affinity labels (9–11), proteases (12–14), and thiol-specific reagents (15). Thiol-specific reagents are particularly attractive because (*a*) thiols are the most reactive nucleophiles commonly found in biological systems, (*b*) there are 20 thiols in the glucocorticoid receptor, and (*c*) thiols have been found to be involved in steroid binding (11, 15, 16), DNA binding (15, 17–19), and possibly activation (18, 20).

In our search for other thiol-specific reagents (10, 15), we were attracted by arsenite (arsenic(III)) and divalent cadmium. Arsenic is best known as a poison. However, arsenic toxicity is mediated by the affinity of arsenic(III) (21–24), and possibly a reduced form arsenic(I) (25), for the thiol groups of proteins. The stability of arsenic(III) adducts with thiols is greatly enhanced if cyclic dithioarsenites can be formed (26). Therefore, the ability of low concentrations of arsenic(III) to inhibit the biological activity of a given protein has been taken as evidence that the integrity of two closely spaced thiols (or vicinal dithiols) is required for biological activity (21, 24, 27–29). Similarly, Cd^{2+} has been found to be an efficient inhibitor of enzymes containing essential dithiols (27–29).

It has long been appreciated that the binding of steroids to their cognate receptor proteins is inhibited by thiol reagents (30–33), and the tacit assumption has been that a single reduced thiol is involved. However, we have recently presented evidence that steroid binding to rat glucocorticoid receptors involves two thiol groups that are close enough to each other to be able to form an intramolecular disulfide (15). In this paper, we have used arsenite and Cd^{2+} as two new chemical probes of the importance of vicinal dithiols in receptor structure and function. We report that sodium arsenite is a potent inhibitor of steroid binding to rat glucocorticoid receptors. These data firmly establish the role of vicinal dithiols in steroid binding. We have also looked at selenite and Zn^{2+}, which are reported to influence ligand binding to receptors (34, 35). Sodium arsenite and sodium selenite were found to be more potent than any other nonsteroidal reagent in inhibiting steroid binding. Divalent cadmium and zinc were increasingly less potent in blocking steroid binding. Both

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arsenite and divalent cadmium were found to have moderate effects on the behavior of unactivated and activated receptor-steroid complexes.

MATERIALS AND METHODS

Unless otherwise indicated, all operations were performed at 0 °C.

Chemicals—[^{1}H]Dexamethasone (Sigma), [^{3}H]dexamethasone (40 or 47 Ci/mmol, Amersham Corp.), and [^{3}H]dexamethasone 21-mesylate (37.3 or 49.9 Ci/mmol, Du Pont-New England Nuclear) were commercially available. TAPS[1] (Ultrol grade) was purchased from Behring Diagnostics; sodium arsenite was from J. T. Baker Chemical Co., and zinc chloride and sodium selenite pentahydrate were from Fluka. Methyl methanethiolsulfonate (MMTS; stored at 0 °C) and anhydrous cadmium chloride were used as received from Aldrich. Reagents for SDS-polyacrylamide gel electrophoresis, including Coomassie Blue R-250, were from Bio-Rad. Fluorescent Ult-Emit autoradiography marker was from Du Pont-New England Nuclear.

All ^{3}H-labeled samples were counted in Hydrofluor (National Diagnostics) at 40–55% counting efficiency in a Beckman 5801 liquid scintillation counter with automatic cpm-to-dpm conversion.

Buffers and Solutions—TAPS buffer was composed of 25 mM TAPS, 1 mM EDTA, and 10% glycerol. The pH of the TAPS buffer was adjusted to 8.2, 8.8, or 9.5 at 0 °C with sodium hydroxide. Two-fold concentrated SDS sample buffer contained 0.6 M Tris (pH 8.85), 2% SDS, 0.2 M dithiothreitol, 20% glycerol, and bromphenol blue.

Cells and Preparation and Labeling of Receptors—The growth of HTC cells in spinner and monolayer cultures in S77 medium supplemented with 5% fetal and 5% newborn bovine serum (Biofluids) and 0.03% glutamine has been described (36). HTC cell cytosol containing the steroid-free receptors was prepared, stored in liquid N_2, and labeled at 0 °C with [^{3}H]dexamethasone or [^{3}H]dexamethasone 21-mesylate ± excess [^{1}H]dexamethasone ± 20 mM Na_2MoO_4 (12, 13). Free steroid was removed by adding a 10% dextran-coated charcoal suspension in pH 8.8 TAPS buffer (added volume = 20% of reaction volume). Nonspecific binding/labeling equaled that seen with excess [^{1}H]dexamethasone. Covalent [^{3}H]dexamethasone 21-mesylate-labeled receptors were quantitated on SDS-polyacrylamide gels (12, 15).

The toxicity of arsenite or Cd^{2+} was determined by incubating monolayer cultures of cells in 60-mm Petri dishes with medium containing various concentrations of each agent for 17 h at 37 °C in a 5% CO_2 incubator. The cells were then gently rinsed with the existing medium to dislodge loosely attached cells. The unattached cells were diluted 1:12 into fresh untreated medium in a T75 flask while 3 ml of fresh untreated medium was added to the attached cells. Both cultures were incubated in a CO_2 incubator for 48 h, during which time the morphology and growth of the cells were monitored.

DNA Binding of Receptor-Steroid Complexes on Minicolumns—The procedure, which is a modification of that described by Holbrook *et al.* (37), has been described elsewhere (19). Briefly, two columns in 1.0-ml syringes (top column = 0.3 ml of DNA-cellulose; bottom column = 0.3 ml of DEAE-cellulose) are equilibrated with pH 8.8 TAPS buffer. Samples (100 μl) are loaded onto each pair of minicolumns and rapidly chromatographed with 7 ml of pH 8.8 TAPS buffer. Air is forced through the minicolumns to remove all liquid, and the radioactivity retained by each matrix is quantitated by scintillation counting. The specifically bound complexes were calculated by subtracting the binding of receptor solutions containing excess competing [^{1}H]dexamethasone from that of uncompeted receptor solutions containing only [^{3}H]dexamethasone.

Polyacrylamide Gel Electrophoresis—The preparation of samples for gels and the procedures for electrophoresis are as described (10). Constant percentage acrylamide gels (10.5–11% with a 1:40 ratio of bisacrylamide to acrylamide) were run in water-cooled (15 °C) Protean II slab gel apparatus (Bio-Rad) at 30 mA/gel (20 mA/gel while in the stacking gel). Gels were fixed and stained in 50% methanol, 7.5% acetic acid containing 0.01% Coomassie Blue R-250, destained in 10% methanol, 7.5% acetic acid, incubated for 1 h in Enhance (Du Pont-New England Nuclear) and 30–60 min in 10% Carbowax PEG 8000 (formerly Peg 6000; Fisher) with constant shaking at room temperature, dried on a Bio-Rad model 443 slab gel drier at 60 °C with a sheet of dialysis membrane backing (Bio-Rad) directly over the gel to prevent cracking, marked with Ult-Emit at the positions of the molecular weight markers, and fluorographed for 7–12 days with Kodak X-Omat XAR-5 film.

[1] The abbreviations used are: TAPS, 3-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino}-1-propanesulfonic acid; MMTS, methyl methanethiolsulfonate; SDS, sodium dodecyl sulfate; DTT, dithiothreitol.

RESULTS

Effect of Arsenite on Steroid Binding to Glucocorticoid Receptors—Steroid binding to HTC cell receptors was totally inhibited by a 30-min preincubation with very low concentrations of sodium arsenite (arsenic(III)) at 0 °C in the presence (Fig. 1) or absence (data not shown) of 20 mM Na_2MoO_4. The kinetics of arsenite inhibition were rapid. There was no increased inhibition of [^{3}H]dexamethasone binding when the preincubation period was extended to 2.5 h, and greater than 95% of the final inhibition was achieved with co-incubation of arsenite and ^{3}H-steroid (data not shown). This is in contrast to the biphasic time-dependent dose-response curve for inhibition of steroid binding by the thiol-specific reagent MMTS, which also inhibits steroid binding at concentrations below the ~0.8 mM thiol content of crude HTC cell receptor solutions (15). However, sodium arsenite is about 5 times more potent than MMTS. Thus, the elimination of 50% of steroid-binding activity requires ~7 μM arsenite (Fig. 1) *versus* ~40 μM MMTS (15).

Three lines of evidence indicate that the effects of arsenite are mediated by reaction with thiols. First, it is known that arsenite, but not arsenate (arsenic (V)), reacts with thiol groups (21). Similarly, although 40 μM arsenite blocked all subsequent steroid binding (Fig. 1), the preincubation of steroid-free receptors with 250-fold higher concentrations (*i.e.* 10 mM) of sodium arsenate for up to 2.5 h at 0 °C caused less than an 8% loss of steroid binding activity (data not shown). Second, added dithiothreitol (DTT) or β-mercaptoethanol was able to reverse completely the effects of arsenite preincubation (Fig. 2). Third, preincubation of cytosol with arsenite completely blocked the covalent labeling of the 98-kDa receptor by [^{3}H]dexamethasone 21-mesylate (Fig. 3*A*). We have shown previously that the only amino acid of the receptor which is labeled to any extent by dexamethasone 21-mesylate is Cys^{656} of the rat receptor (11). The concentration of arsenite

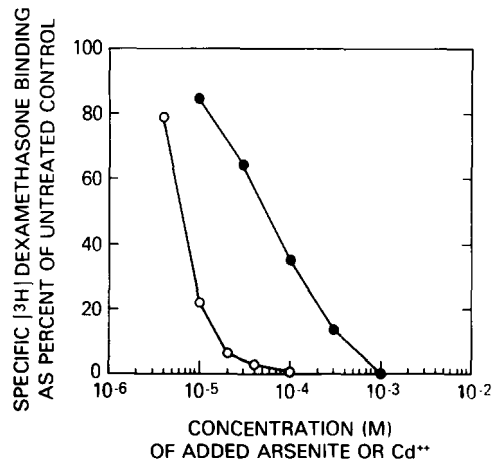

FIG. 1. **Effect of arsenite and Cd^{2+} preincubation on [^{3}H] dexamethasone binding to steroid-free receptors.** Steroid-free receptors containing 21 mM Na_2MoO_4 were treated with various concentrations of 100 × (in pH 8.8 TAPS buffer) sodium arsenite for 30 min or cadmium chloride for 2.5 h. The binding activity of these receptors was then assayed by incubating with [^{3}H]dexamethasone ± excess [^{1}H]dexamethasone for 2.5 h and removing free steroid with added dextran-coated charcoal as described under "Materials and Methods." The amount of specific binding of [^{3}H]dexamethasone after arsenite (○) and Cd^{2+} (●) preincubation was expressed as a percent of the untreated control.

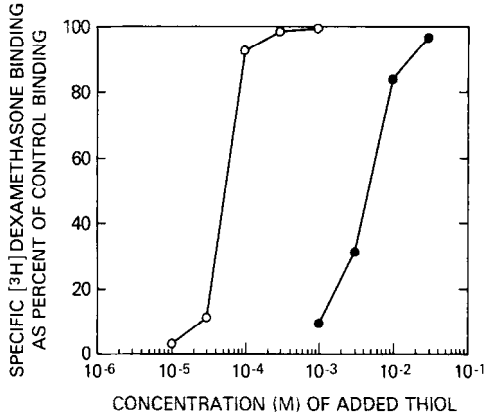

FIG. 2. **Reversal of arsenite inhibition of steroid binding to steroid-free receptor by DTT and β-mercaptoethanol.** Steroid-free receptors in pH 8.8 TAPS buffer containing 21 mM Na_2MoO_4 were preincubated with 40 μM arsenite for 30 min and then adjusted to various concentrations of DTT or β-mercaptoethanol with 100 × solutions of each thiol in pH 8.8 TAPS buffer. After a further 30-min preincubation, the binding activity of these receptors was assayed by incubating with [^{3}H]dexamethasone ± excess [^{1}H]dexamethasone for 2.5 h and removing free steroid with added dextran-coated charcoal as described under "Materials and Methods." The amount of specific binding of [^{3}H]dexamethasone after DTT (○) and β-mercaptoethanol (●) preincubation was expressed as a percent of the untreated control. Longer preincubation times (*i.e.* 2.5 h) with each thiol gave the same results (data not shown).

required for total inhibition of dexamethasone 21-mesylate labeling of Cys^{656} (~40 μM) was the same as that needed to block dexamethasone binding (Fig. 3*A versus* Fig. 1). Furthermore, as with the inhibition of [^{3}H]dexamethasone binding, the rate of arsenite blockage of affinity labeling was fast. Co-incubation of arsenite and [^{3}H]dexamethasone 21-mesylate yielded negligible amounts of labeled receptors (Fig. 3*B*). Finally, arsenite inhibition of receptor labeling by [^{3}H]dexamethasone 21-mesylate was rapidly reversed both by DTT and by β-mercaptoethanol (Fig. 3*C*), and up to 10 mM sodium arsenate had no effect on the labeling of receptors (data not shown). It should be noted that this reaction of arsenite with thiol groups occurs even in the presence of 20 mM Na_2MoO_4, which is known to complex thiol groups (38–41). A similar inability of Na_2MoO_4 to prevent the reaction of several other reagents with thiols has also been reported (15).

Several lines of evidence argue that this reaction of arsenite with thiols is actually with only a small class of thiols, *i.e.* two proximal thiols that are spatially equivalent to vicinal dithiols. First, it is well known that arsenite specifically reacts with vicinal dithiols (23–29). Second, [^{3}H]dexamethasone 21-mesylate labeling of the thiol groups of nonreceptor proteins (10, 15) was only marginally reduced, if at all, by arsenite (Fig. 3). We have presented evidence previously that glucocorticoid receptors contain two closely spaced thiols that are involved in steroid binding (15). Although these closely spaced thiols might be expected to form a cyclic complex with arsenite, such a disposition of cysteines in proteins is statistically rare. In fact, arsenite inhibits the activity of relatively few enzymes (25, 27–29, 42) and should block the labeling by dexamethasone 21-mesylate of only a small fraction of the total thiol groups. Thus, the fact that arsenite minimally inhibited the labeling of nonreceptor proteins (Fig. 3) is consistent with only a small subpopulation of thiols (such as vicinal dithiols and including Cys^{656} of the receptor) being affected. For comparison, it should be noted that MMTS reacts with all thiols and blocks [^{3}H]dexamethasone 21-mesylate labeling of all cytosolic proteins, including the receptor (15). Third, DTT was ~100 times more effective than β-mercaptoethanol in reversing arsenite inhibition of [^{3}H]dexamethasone binding to free receptors (Fig. 2). The greater potency of dithiols such as DTT in reversing the effects of arsenite has generally been cited as evidence for the involvement of a cyclic arsenite complex, which is most efficiently disrupted by those thiols that can give new cyclic arsenite complexes (23, 27, 29).

DTT is thought to be more potent than β-mercaptoethanol in reversing the effects of arsenite because DTT is bidentate and can form a very stable cyclic complex with arsenite. This suggested that the vicinal dithiols involved in arsenite inhibition of steroid binding should give a similar, very stable complex. This was confirmed by the observation that steroid-free receptors that had been pretreated with 10^{-4} M arsenite and then chromatographed on Sephadex G-10 columns still could not bind steroid unless DTT was added to cleave the vicinal dithiol-arsenite complexes (Table I).

Effect of Cd^{2+} on Steroid Binding to Glucocorticoid Receptors—Cd^{2+} is another inorganic ion that specifically reacts with vicinal dithiols (27–29). Cd^{2+} did block dexamethasone binding to steroid-free receptors but was much less effective than arsenite. Complete inhibition of dexamethasone binding required 30 min of preincubation with 1×10^{-3} M Cd^{2+} *versus* ~4×10^{-5} M arsenite; with lower concentrations of Cd^{2+}, the amount of inhibition increased with preincubation time (Fig. 1 and data not shown). Half-maximal inhibition was seen after a 2.5-h preincubation with ~60 μM $CdCl_2$. However, the reaction of Cd^{2+} with receptors does appear to involve vicinal dithiols since [^{3}H]dexamethasone 21-mesylate labeling of receptors was totally prevented by 1 mM Cd^{2+}, at which point the labeling of most other nonreceptor proteins was unaffected (data not shown). The inhibition of dexamethasone binding and of [^{3}H]dexamethasone 21-mesylate labeling caused by preincubation with 1 mM Cd^{2+} was reversed, but only partially, by a second, 2.5-h preincubation at 0 °C with 10 mM DTT (data not shown). This partial reversal is presumably due to the high concentrations of Cd^{2+} which were used.

Effect of Selenite and Zn^{2+} on Steroid Binding to Glucocorticoid Receptors—It has been reported recently that sodium selenite (Na_2SeO_3) is extremely effective in blocking steroid binding to glucocorticoid receptors. Thus, preincubation of approximately 10-fold purified rat liver receptors with 3 μM sodium selenite for 5 min at 20 °C caused a 50% inhibition of [^{3}H]dexamethasone binding (34). We have extended these studies and found that selenite was also very effective in blocking steroid binding to crude receptors at reduced temperatures. In particular, a 50% inhibition of [^{3}H]dexamethasone binding to crude HTC cell receptors was achieved by a 30-min preincubation at 0 °C with 5 μM selenite (Fig. 4). Thus, selenite and arsenite appear to be of approximately equal potency in blocking steroid binding to glucocorticoid receptors.

Very low concentrations (1–3 μM) of divalent zinc (Zn^{2+}) have been found recently to inhibit thyroid hormone binding to its nuclear receptor (35). Given the homologies between the T_3 and glucocorticoid receptors (and the other receptors in the superfamily of DNA binding proteins (43)) and the anomalous ability of increasing Zn^{2+} to increase and then decrease the DNA binding of the DNA-binding domain fragment of the glucocorticoid receptor (44), we examined the effects of Zn^{2+} on steroid binding to glucocorticoid receptors. Unlike the T_3 receptor, the glucocorticoid receptor showed no change in ligand-binding behavior with Zn^{2+} concentrations up to 300 μM (Fig. 4). Steroid binding was eliminated at higher

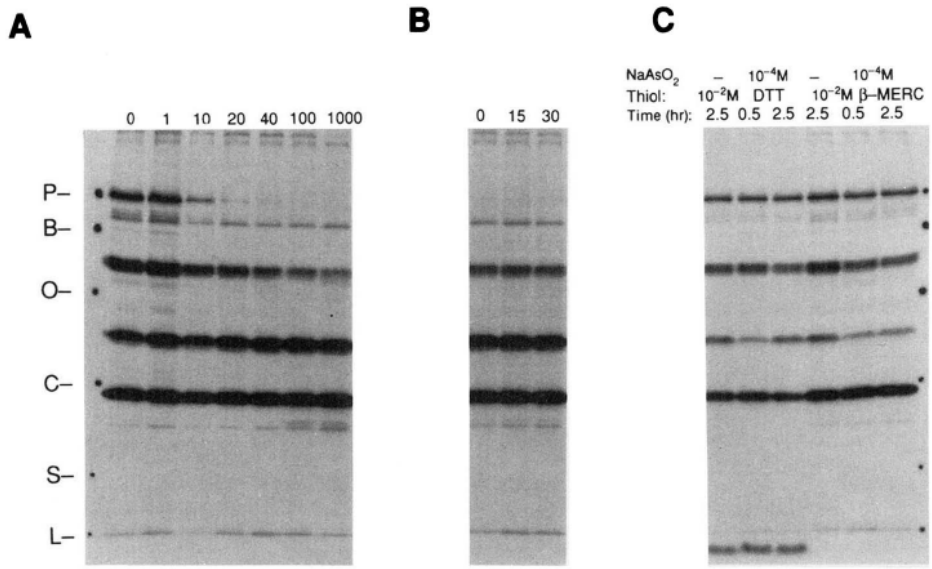

FIG. 3. **Effect of sodium arsenite ± thiols on [^{3}H]dexamethasone 21-mesylate labeling of HTC cell receptors.** *A*, fluorograph showing arsenite inhibition of [^{3}H]dexamethasone 21-mesylate labeling of receptors. Steroid-free receptors containing 21 mM Na_2MoO_4 were treated with various concentrations of 100 × (in pH 8.8 TAPS buffer) sodium arsenite for 30 min before being labeled by 1.5×10^{-7} M [^{3}H]dexamethasone 21-mesylate for 2.5 h. The covalently labeled receptors were electrophoresed on a denaturing 9% polyacrylamide gel and visualized by fluorography as described under "Materials and Methods." *B*, fluorograph showing kinetics of arsenite inhibition of [^{3}H]dexamethasone 21-mesylate labeling of receptors. Steroid-free receptors were pretreated with 10^{-4} M arsenite for 0, 15, or 30 min as in *A* before being treated with [^{3}H]dexamethasone 21-mesylate. Analysis of the labeled solutions was as in *A*. *C*, fluorograph showing reversal of arsenite inhibition of [^{3}H]dexamethasone 21-mesylate labeling of receptors by added thiols. Steroid-free receptors were preincubated for 30 min with buffer ± arsenite (final concentration, 10^{-4} M) as above in *A* and then treated for 0.5 or 2.5 h with 10 mM DTT or β-mercaptoethanol before being labeled with 1.5×10^{-7} M [^{3}H]dexamethasone 21-mesylate for 2.5 h. The covalently labeled receptors were electrophoresed on a denaturing 9% polyacrylamide gel and visualized by fluorography as described under "Materials and Methods." The molecular mass standards (obtained from Pharmacia LKB Biotechnology Inc.) are: *P*, phosphorylase *b*, (97,400); *B*, bovine serum albumin (66,300); *O*, ovalbumin (45,000); *C*, carbonic anhydrase (30,600); *S*, soybean trypsin inhibitor (21,500); L, α-lactalbumin (14,400). Intact 98-kDa receptors appear at the same position as phosphorylase *b*; an 82-kDa receptor fragment migrates between *P* and *B* (12).

Zn^{2+} concentrations, but this may be related to the apparent precipitation of $Zn(OH)_2$ in our 10 × stock solution.

Effect of Arsenite on Unactivated Receptor-Steroid Complexes—Short term incubations with arsenite had little effect on unactivated[2] complexes. Of the complexes initially detected by either activated charcoal assay or by binding to DNA/DEAE minicolumns, 22 ± 3% ($n = 3$) were lost after a 30-min incubation at 0 °C with 10 mM arsenite either in the presence or absence of 20 mM Na_2MoO_4 (data not shown). However, with longer incubations of 2.5 h, the recovery of unactivated complexes in the presence of 10 mM arsenite decreased by 58 ± 1% ($n = 3$) (Table II). With lower arsenite concentrations (*i.e.* 1 mM), the extended incubation times afforded a proportionally smaller decrease (*i.e.* ~25%). Thus longer incubations of low arsenite concentrations do not appear to afford a biphasic dose-response curve, as was seen for low concentrations of MMTS (15). This time-dependent response of unactivated complexes occurs at much higher arsenite concentrations than are required for the faster reaction with steroid-free receptors (see Fig. 1). Nevertheless, the arsenite-induced loss of unactivated complexes does involve a reaction with free thiols since the loss was completely prevented by co-incubation with 10 mM MMTS (Table II).

[2] The terms "activate" and "activation" are used in this paper to describe the currently unknown mechanism by which initially formed receptor-steroid complexes, with little or no affinity for DNA or nuclei, are converted by manipulations such as heat, dilution, increased salt, or increase pH to complexes with relatively high affinity for DNA or nuclei.

TABLE I

Binding capacity of receptors ± arsenite after Sephadex G-10 chromatography

Receptors were treated either with buffer or with [^{3}H]dexamethasone ± excess [^{1}H]dexamethasone for 2.5 h. $NaAsO_2$ was added to a final concentration of 100 μM from a 100 × stock, and the mixture was incubated for an additional 30 min. An aliquot (500 μl) was then chromatographed on a 9-ml Sephadex G-10 column and the receptor eluted in the void volume by the addition of pH 8.8 TAPS buffer. Where indicated, 100 × DTT was added to the chromatographed receptor to a final concentration of 1 mM and incubated for an additional 20 min. Finally, each sample was adjusted to a final concentration of 5×10^{-8} M [^{3}H]dexamethasone ± excess [^{1}H]dexamethasone. After 2.5 h, dextran-coated charcoal was added to remove free steroid, and the specific binding was determined as described under "Materials and Methods." The 100% binding was 90,860 dpm for steroid-bound receptors and 43,780 dpm for steroid-free receptors.

Post-Sephadex G-10 treatment of	$-NaAsO_2$	$+NaAsO_2$
	% of control	
Steroid-bound receptors		
[^{3}H]Dexamethasone	100	85
[^{3}H]Dexamethasone + DTT	101	82
Steroid-free receptors		
[^{3}H]Dexamethasone	100	4
[^{3}H]Dexamethasone + DTT	113	131

This concentration of MMTS is known not to disrupt unactivated complexes (15).

Two observations argue that the time-dependent loss of unactivated complexes is not due simply to a reaction of arsenite with those steroid-free receptors that are formed by the dissociation of steroid-bound receptors. First, inactivation

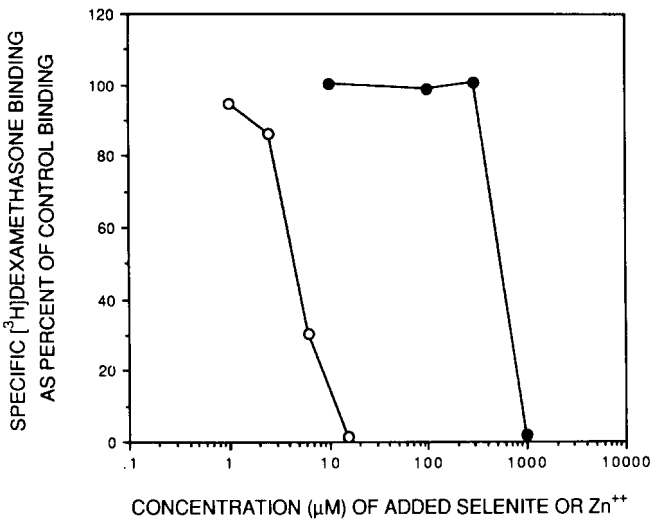

FIG. 4. **Effect of selenite and Zn^{2+} preincubation on [^{3}H] dexamethasone binding to steroid-free receptors.** Steroid-free receptors containing 21 mM Na_2MoO_4 were treated with various concentrations of 10 × (at 0 °C in pH 8.8 TAPS buffer) sodium selenite or zinc chloride for 30 min. The binding activity of these receptors was then assayed by incubating with [^{3}H]dexamethasone ± excess [^{1}H]dexamethasone for 2.5 h and removing free steroid with added dextran-coated charcoal as described under "Materials and Methods." The amount of specific binding of [^{3}H]dexamethasone after selenite (○) and Zn^{2+} (●) preincubation was expressed as a percent of the untreated control.

TABLE II

Time-dependent loss of unactivated receptor-steroid complexes during incubation with arsenite

Receptors bound with [^{3}H]dexamethasone ± excess [^{1}H]dexamethasone in the presence of 20 mM Na_2MoO_4 were prepared as described under "Materials and Methods." At time zero, aliquots were treated with pH 8.8 TAPS buffer ± enough [^{1}H]dexamethasone (200 × EtOH solution), MMTS (50 × buffer solution), and/or arsenite (33 × buffer solution) to give the indicated final concentrations. After the designated incubation times, dextran-coated charcoal was added to remove the free steroid, and the amount of specific binding was determined and expressed as the percent of buffer-treated complexes remaining after 0.5 h.

Treatment	Time of incubation	Initial complexes remaining
	h	%
None	0.5	100
None	2.5	103
2×10^{-5} M Dexamethasone	2.5	96
10 mM AsO_2^-	0.5	79
10 mM AsO_2^-	2.5	42
10 mM MMTS	2.5	98
10 mM AsO_2^- + 10 mM MMTS	0.5	93
10 mM AsO_2^- + 10 mM MMTS	2.5	93

of dissociated complexes should not require arsenite concentrations of >1 mM since <0.1 mM arsenite can totally block steroid binding to steroid-free receptors (see Fig. 1). Second, the dissociation of [^{3}H]dexamethasone-bound receptors, in the presence of excess [^{1}H]dexamethasone to prevent the rebinding of [^{3}H]dexamethasone, was much slower than the arsenite-induced loss of binding (Table II). Thus, arsenite actively decreases the amount of unactivated complexes, perhaps by inducing a conformational change that results in steroid dissociation, as opposed to simply inactivating dissociated receptors.

Effect of Arsenite on Activation and the DNA Binding of Activated Complexes—Earlier reports indicated that dextran-coated charcoal cannot be used to detect all of the receptor-steroid complexes in activated cytosol solutions (45). However, these studies undoubtedly were complicated by receptor proteolysis in these solutions (9, 12, 46). Using preparations of intact 98-kDa receptors (see Fig. 3*A*), we found that dextran-coated charcoal routinely detected 82 ± 6% (n = 7) of the total complexes, as determined by binding to either DNA/DEAE or double DEAE minicolumns (data not shown). Therefore, all of our studies were conducted with DNA/DEAE minicolumns.

When activated complexes were subsequently incubated with arsenite for 30 min at 0 °C, there was a concentration-dependent inhibition of DNA-binding capacity (Fig. 5). This inhibition was only partial, but higher concentrations (*i.e.* 25 mM) caused no further inhibition (data not shown and Ref. 19). The effects of arsenite were rapid. Incubation of activated complexes with 10 mM arsenite for 10, 30, and 60 min gave identical results (data not shown). This decrease was not a nonspecific salt effect because up to 10 mM NaCl (which has the same ionic strength as 10 mM arsenite) caused no decrease in DNA binding in this assay (data not shown). All of the DNA-binding data were expressed as percent of the total complexes detected on DNA/DEAE minicolumns. This was done to minimize the influence of the ~25% loss of total initial complexes which was seen with 10 mM arsenite, presumably due to a time-dependent loss of complexes as noted above for unactivated complexes. Among those receptors remaining, the proportion that would bind to DNA was reduced by 40 ± 7% (n = 4). Thus, as with unactivated complexes, the concentration of arsenite required for any appreciable effect on activated complexes is much higher than that seen for steroid-free receptors.

In order to assess the effects of arsenite on the activation

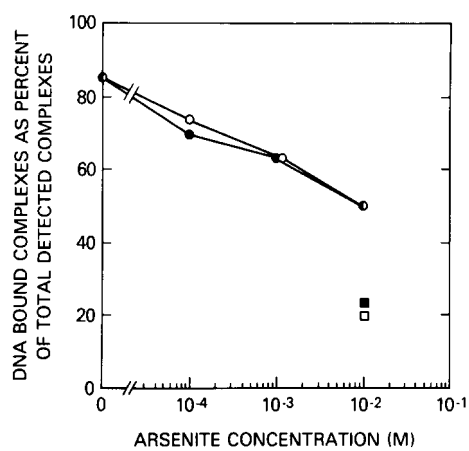

FIG. 5. **Effect of arsenite on activation and DNA binding of receptor-[^{3}H]dexamethasone complexes.** Steroid-free receptors (60% crude cytosol in pH 9.5 TAPS buffer) were bound with 5×10^{-8} M [^{3}H]dexamethasone ± 2.3×10^{-5} M [^{1}H]dexamethasone for 2.5 h. Each solution was split into two groups, diluted with an equal volume of pH 8.8 TAPS buffer, adjusted to the indicated final arsenite concentrations with 100 × stock solutions in pH 8.8 TAPS buffer, and incubated for 0.5 h either before or after activation at 20 °C for 20 min. Duplicate aliquots were analyzed on DNA/DEAE minicolumns, and the amount of receptor-steroid complexes that bound to DNA, and the total complexes, were determined as described under "Materials and Methods." The amount of complexes that bound to DNA, expressed as a percentage of the total detected complexes, was plotted against the concentration of arsenite present either before (○) or after (●) activation. In a similar but separate experiment in which buffer ± 10 mM arsenite gave levels of DNA binding identical to the above experiment (buffer; 88%; 10 mM arsenite, 53%), the DNA binding of samples treated with 10 mM MMTS ± 10 mM arsenite either before (□) or after (■) activation was determined and plotted. The data points for MMTS ± arsenite were superimposable.

of receptor-steroid complexes, increasing concentrations of arsenite were added immediately prior to activation, which was accomplished by dilution and heating at 20 °C for 20 min. The analysis of the subsequent binding on minicolumns was complicated by the fact that the above noted time-dependent loss of unactivated complexes in the presence of arsenite would be expected to be greater with the elevated temperatures of activation. However, when normalized to the total amount of receptors detected on the DNA/DEAE minicolumns, there was again a moderate effect of arsenite which was indistinguishable from that seen for arsenite added after activation (Fig. 5). Therefore, the data do not permit one to distinguish between whether arsenite causes inhibition of activation at the same, or higher, concentration as required for inhibition of DNA binding or whether arsenite is without effect on activation.

MMTS, like arsenite, displayed the same dose-response curves for inhibition of DNA binding whether the MMTS was added before or after the activation step (data not shown). Although MMTS was more effective in reducing the amount of DNA binding of activated complexes than was arsenite, ~25% of the initial DNA-binding capacity remained even in the presence of 3–30 mM MMTS (Fig. 5; Refs. 15 and 19). The effects of MMTS and arsenite were not, however, additive. When both 10 mM MMTS and 10 mM arsenite were added either before or after activation, the residual DNA binding was the same as that seen with 10 mM MMTS alone (Fig. 5).

Effect of Cd^{2+} on Unactivated and Activated Receptor-Steroid Complexes—The loss of unactivated complexes in the presence of 1 mM Cd^{2+} exhibited biphasic kinetics, whereas activated complexes were much less affected (Fig. 6). The influence of Cd^{2+} on activation was difficult to assess due to the Cd^{2+}-induced loss of unactivated complexes. Thus, most of the [^{3}H]dexamethasone-bound complexes were lost when 1 mM Cd^{2+} was present during activation; lower concentrations (30–300 μM Cd^{2+}) had no significant effect (data not shown).

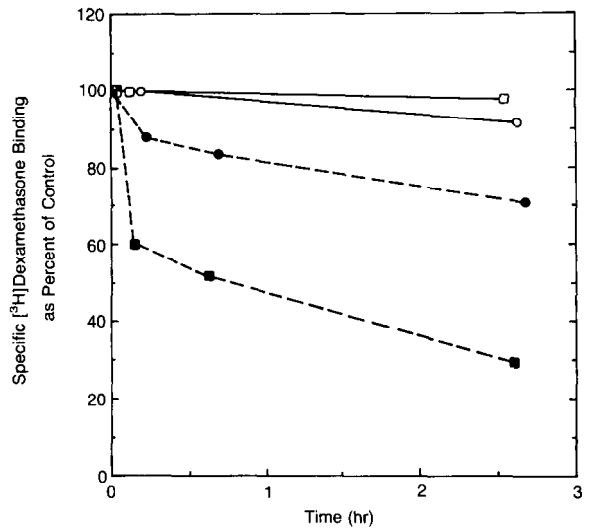

FIG. 6. **Effect of Cd^{2+} on unactivated and activated receptor-steroid complexes.** Activated complexes were bound with steroid and activated by dilution as in Fig. 5. For unactivated complexes, 3 volumes of crude receptors, 2 volumes of pH 9.5 TAPS buffer, and 5 volumes of pH 8.8 TAPS buffer were incubated in the presence of 20 mM Na_2MoO_4 and 5×10^{-8} M [^{3}H]dexamethasone ± 2.3×10^{-5} M [^{1}H]dexamethasone for 2.5 h. Aliquots of activated and unactivated complexes were then treated with buffer or adjusted to 1 mM $CdCl_2$ with a 20 × stock in pH 8.8 TAPS buffer. After the indicated incubation times, free steroid was removed by the addition of dextran-coated charcoal. The specific binding remaining in the activated (●---●) and unactivated (■---■) cytosols was determined as described under "Materials and Methods" and expressed as percent of the value of the activated (○——○) and unactivated (□——□) controls at ≤0.15 h, which was assumed to be unchanged from the zero time value.

Preliminary results indicate that 1 mM Cd^{2+} added after activation causes a partial loss of complexes and of DNA-binding capacity among the remaining complexes (data not shown).

Toxicity of Arsenite and Cd^{2+} in HTC Cells—Arsenic and cadmium are notoriously toxic to cells. Since arsenic toxicity is mediated by arsenite (21–24), the toxicity of arsenite and Cd^{2+} to HTC cells was investigated. After 17 h at 37 °C, >99% of the cells treated with 100 μM arsenite were nonviable. The LD_{50} was about 40 μM arsenite, whereas 10 μM had little if any effect. The LD_{50} for Cd^{2+} was about 1 mM; 100 μM Cd^{2+} had little if any effect (data not shown).

DISCUSSION

The present studies show that arsenite and Cd^{2+} are two new reagents for modifying glucocorticoid receptor function. Low concentrations of either reagent block steroid binding (Fig. 1), cause the dissociation of unactivated receptor-steroid complexes (Table I and Fig. 6), and partially inhibit the DNA binding of activated complexes (Fig. 5). Arsenite is particularly useful in that it forms a tight complex (Table I) that blocks steroid binding to receptors at a concentration that is ~10 fold lower than that needed with Cd^{2+} and ~20-fold lower than the ~0.8 mM thiol concentration of the receptor solutions (15). Selenite has been described recently as a very effective inhibitor of steroid binding to partially purified rat liver receptors at pH 7.6 at 20 °C (34). We have extended these results with the observation that micromolar concentrations of selenite at 0 °C block steroid binding to crude receptors at pH 8.8 at 0 °C (Fig. 4). Thus, to the best of our knowledge, arsenite and selenite are the most potent thiol reagents for modifying glucocorticoid receptors other than the affinity label dexamethasone 21-mesylate (9, 47).

The inhibitory activities of arsenite and Cd^{2+} are especially useful because they offer a possible correlation with receptor structure. Both reagents are known to have a high affinity and selectivity for vicinal (*i.e.* spatially proximal) dithiol groups (23–29). Several observations support our conclusion that the inhibition of steroid binding to receptors by arsenite or by Cd^{2+} is mediated by reactions with vicinal dithiols. First, the ability of added thiols to reverse the effects of arsenite (*cf.* Figs. 2 and 3 *C*) and of Cd^{2+} proves that the inhibition of steroid binding proceeds via reactions with thiol groups. Second, the effects of arsenite on steroid-free receptors are much more efficiently reversed by those dithiols that can form cyclic complexes with arsenite (Fig. 2). Such reversal by low concentrations of dithiols but not by monothiols is considered diagnostic for the involvement of a vicinal dithiol (23, 27, 29). Third, since the concentration of vicinal dithiols that are appropriately situated for reaction with arsenite (or Cd^{2+}) is much less than the total concentration, the complete inhibition of steroid binding by arsenite concentrations below that of the total thiols (Fig. 1) is the expected result. Fourth, the ability of arsenite (or Cd^{2+}) to prevent [^{3}H]dexamethasone 21-mesylate labeling of receptors at concentrations lower than those required to block covalent labeling of all cytosolic thiol groups (Fig. 3*A*) is consistent with complexation with the lower concentrations of vicinal dithiols. Fifth, a tighter binding of Cd^{2+} than Zn^{2+} to proteins is diagnostic of Cd^{2+} binding to vicinal dithiols (48). Such a tighter binding to the receptor should be reflected in a greater potency to inhibit steroid binding, which was observed in Fig. 1 *versus* Fig. 4. Collec-

tively, these data argue that arsenite and Cd^{2+} inhibit steroid binding via complexation with vicinal dithiols. Therefore, these results establish firmly the role of at least one vicinal dithiol group in the binding of steroid to the rat glucocorticoid receptor (15). These data plus the inability of Sephadex chromatography to reverse the effects of arsenite also indicate that arsenite forms a tight complex with the receptor. Thus, the reaction of arsenite with receptor may prove useful for the introduction of a heavy atom into receptor preparations to be examined by x-ray crystallography.

In contrast to the precise structural conclusions about the reactions of arsenite, much less can be said about the reactions of selenite. Selenite is a reasonably strong oxidant and reacts rapidly with a total of four thiols according to Equation 1

$$4\ RSH + H_2SeO_3 \rightarrow \underset{(I)}{RS\text{-}Se\text{-}SR} + RS\text{-}SR + 3\ H_2O \qquad (1)$$

to yield both disulfides and selenotrisulfides (I) (49). However, this reaction is complicated further by the ability of I to undergo exchange reactions with other selenotrisulfides and with thiols (50) and to be reduced further to free elemental selenium in the presence of excess thiols (51). Thus, it will be very difficult to relate the selenite-induced inhibition of steroid binding with a structural feature(s) of the receptor.

Thioredoxin reductase is required for generating and maintaining the steroid-binding activity of glucocorticoid receptors (52). Any effect of arsenite or Cd^{2+} on thioredoxin reductase can be ruled out, however, since this enzyme is inhibited by 10^4 lower concentrations of Cd^{2+} than arsenite (*i.e.* 5×10^{-7} *versus* 5×10^{-3} M; Ref. 27), whereas receptor binding activity is more sensitive to arsenite than to Cd^{2+} (Fig. 1). In fact, for many dithiol-containing enzymes, Cd^{2+} is a more potent inhibitor than arsenite (27, 42, 48). We have not yet determined the reason for the greater potency of arsenite (AsO_2^-) than Cd^{2+} on steroid-free receptors. However, it may reflect a conformational change in the receptor which is induced by the dithiol-metal adduct formation. This proposal is consistent with the slow formation of an intramolecular disulfide with MMTS (15) and the much lower affinity of Cd^{2+} for those disulfides that are reorganized to a vicinal dithiol arrangement (48). Similarly, it is not clear why Zn^{2+} and Cd^{2+}, both of the same group in the periodic table, are so different in their ability to block steroid binding. Certainly, the disparate size and charge of the various ions could be an important factor in interactions with the receptor. Differences in bond length, bond angle, bond strength, and coordination geometry of the arsenic-, cadmium-, and zinc-sulfur complexes are other possible causes for unequal effects. Understanding these differences should provide valuable information concerning receptor structure and function.

Similar concentrations of Cd^{2+} modify the properties of steroid-free receptors and unactivated and activated complexes. Whether or not the biphasic loss of unactivated complexes in the presence of 1 mM Cd^{2+} (Fig. 6) can be attributed to receptor heterogeneity (19) has not yet been resolved. The fact that those concentrations of arsenite which affect steroid-free receptors have no effect on unactivated or activated complexes suggests that arsenite reactions with receptor-steroid complexes involve individual —SH groups. Alternatively, the spatial organization of the reacting vicinal dithiols of the receptor-steroid complexes may have been altered by the binding of steroid and are no longer optimal for cyclization with arsenite. In fact, we (14) and others (53) have found evidence that steroid binding to glucocorticoid and estrogen (54–56) receptors is accompanied by a conformational change. This explanation is supported further by the observation that MMTS, which rapidly reacts with isolated —SH groups (10, 15), does not affect unactivated complexes but does block the arsenite-induced dissociation of unactivated complexes (Table I). Thus, arsenite may slowly react with vicinal dithiols of unactivated complexes to cause a conformational change resulting in the dissociation of bound steroid.

The effect of arsenite on the DNA binding of activated complexes and on activation is much less than that of MMTS (Fig. 5). However, this is partially because arsenite and MMTS each selectively inhibits the DNA binding of separate subpopulations of activated complexes (19). The effects of MMTS and arsenite are not additive (Fig. 5), as would be expected since MMTS reaction with all thiols would prevent arsenite reaction with either vicinal dithiols or single thiols. In view of the report that those thiol groups in the native form of the progesterone receptor which are involved in DNA binding are not accessible to DTT (57), it is perhaps surprising that other small molecules such as MMTS and arsenite are able to interact with the thiol groups of the native glucocorticoid receptor to block DNA binding. With regard to activation of the complexes, any effect that arsenite or MMTS may have requires the same or higher concentrations of reagent as needed to decrease the DNA binding of activated complexes. These results with MMTS confirm those of Bodwell *et al.* (17) and of Tienrungroj *et al.* (18), who found recently that MMTS blocks DNA binding but not activation.

There are no reports of arsenite effects and very few reports of Cd^{2+} effects on glucocorticoid receptors. Metallothioneine I mRNA is known to be induced by 30 μM Cd^{2+} and by 0.1 μM dexamethasone in cadmium-sensitive S180 mouse sarcoma cells, whereas the combination of the two inducers gave no increased induction over that seen just with Cd^{2+} (58). This lack of additive effects was proposed to result from the ability of Cd^{2+} to decrease the amount of dexamethasone-bound receptors found in the nucleus. More recently, low concentrations of Cd^{2+} were found to be able to replace Zn^{2+} in restoring the DNA binding of truncated aporeceptors, but paradoxically, Cd^{2+} concentrations above 0.1 mM gave no DNA binding (44). Our results show that Cd^{2+} affects multiple receptor functions. Cd^{2+} reacts with thiol groups to block steroid binding to receptors (Fig. 1), destroys existing complexes (Fig. 6), and may decrease the DNA binding of activated complexes (data not shown), each of which would decrease the number of nuclear bound receptor-steroid complexes.

It is generally considered that arsenic toxicity results from the formation of stable cyclic complexes between arsenite and those enzymes, and enzyme co-factors, which contain vicinal dithiols (22, 24). Our data indicate that arsenite forms such stable complexes with cell-free glucocorticoid receptors at concentrations that are similar to those causing whole cell toxicity. Thus, it is appropriate to speculate that glucocorticoid receptor inactivation may be a component of arsenite toxicity.

The binding of agonists but not competitive antagonists to the acetylcholine-binding site of nicotinic receptors appears to cause a local conformational change of the disulfide between Cys^{192} and Cys^{193} (59). Reduction of this disulfide to a vicinal dithiol dramatically alters the binding specificity of the receptors (59). In contrast, the vicinal dithiols that we have described in the glucocorticoid receptor are both reduced in the native receptor. This receptor does not bind steroid when the vicinal dithiols are oxidized to an intramolecular disulfide and does not display uniformly altered binding of agonists or antagonists when the vicinal dithiols are converted to mixed disulfides by reaction with MMTS (15). Neverthe-

less, these results do demonstrate the potential importance of vicinal dithiol-disulfide equilibria in enabling receptors to convert the structural information of a ligand into the observed biological responses.

In conclusion, Cd^{2+} and especially arsenite are new, chemically specific reagents for the modification of glucocorticoid receptor activity. As far as we are aware, this is the first report of an effect of these substances on the function of cell-free glucocorticoid receptors. That the effects of arsenite appear to involve reaction with vicinal dithiols to give a very stable complex should be particularly useful in further studies of glucocorticoid receptor structure and function.

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