Sulfhydryl-Modifying Reagents Reversibly Inhibit Binding of Glucocorticoid–Receptor Complexes to DNA–Cellulose†

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ABSTRACT: Glucocorticoid–receptor complexes from intact rat thymus cells incubated with [^{3}H]dexamethasone at 0 °C are in the nonactivated form and do not bind to DNA–cellulose. Upon being warmed, they are transformed to activated complexes that bind to DNA–cellulose at 0 °C. We have found that treatment of dexamethasone–receptor complexes with the sulfhydryl-modifying reagents methyl methanethiosulfonate (MMTS) and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), either before or after the warming, inhibits subsequent binding to DNA–cellulose. The effects of these reagents can be reversed at 0 °C by dithioerythritol and other sulfhydryl-containing compounds. These results provide the first clear evidence that sulfhydryl-modifying reagents inhibit the binding of activated dexamethasone–receptor complexes to DNA–cellulose and suggest that sulfhydryl groups may be located in or near the DNA binding domain of the rat thymus glucocorticoid–receptor complex. Furthermore, addition of dithioerythritol at 0 °C to nonactivated receptor complexes that have been treated with MMTS or DTNB produces a substantial increase in the capacity of these complexes to bind to DNA–cellulose, raising the possibility that sulfhydryl groups may be associated with a region on the receptor that plays a critical role in the activation process.

Glucocorticoids enter the cytoplasm of target cells and associate with specific receptors to form glucocorticoid–receptor complexes. At physiological temperatures these complexes rapidly undergo a process termed "activation" by which they acquire affinity for nuclei and DNA–cellulose (Munck & Foley, 1979). The activated glucocorticoid–receptor complex can also associate with other polyanions such as carboxymethylcellulose (Milgrom et al., 1973) and phosphocellulose (Kalimi et al., 1975). Activation of the glucocorticoid–receptor complex under cell-free conditions can be produced by heat (Munck et al., 1972; Baxter et al., 1972), high ionic strength (Higgins et al., 1973), dilution, and gel filtration (Goidl et al., 1977).

Treatment of unbound glucocorticoid receptors with sulfhydryl-modifying reagents has been shown to prevent the receptor from interacting with the steroid to form steroid–receptor complexes (Schaumburg, 1972; Kobolinski et al., 1972; Rees & Bell, 1975). However, treatment of the glucocorticoid–receptor complex with reagents that alkylate sulfhydryl groups has little effect on the complex (Young et al., 1975; Kalimi & Love, 1980). This is not true for all sulfhydryl-modifying reagents, as organomercurials completely dissociate the hormone–receptor complex (Banerji & Kalimi, 1981).

We shall use the term "sulfhydryl-modifying reagent" to refer to those reagents that can react with the glucocorticoid–receptor complex and oxidize the sulfhydryl group (Rec–SH) of cysteine residues to either a disulfide (Rec–S–S–X; X is from the sulfhydryl-modifying reagent) or a thioether (Rec–SX). Compounds containing a sulfhydryl group (C–SH) will be referred to as such or as reducing agents and, in the context of this paper, will be used to reduce the disulfides formed by the sulfhydryl-modifying reagents.

Few studies have examined the effects of sulfhydryl-modifying reagents on activation and DNA binding of the glucocorticoid–receptor complex. Young et al. (1975) reported that iodoacetamide inhibited DNA binding of glucocorticoid–receptor complexes, but because iodoacetamide was present during the activation procedure, it was not possible to determine whether the reagent inhibited activation or binding of the complexes to DNA or both. Kalimi & Love (1980), using iodoacetamide and *N*-ethylmaleimide, reexamined this question and concluded that the effect of these two reagents was on activation and not DNA binding.

Both iodoacetamide and *N*-ethylmaleimide have been shown to react not only with the sulfhydryl group of cysteine but also with the amine group of lysine (Brewer & Riehm, 1967; Smyth et al., 1964; Wallenfels & Eisele, 1968). This is a group believed to participate in binding of the glucocorticoid–receptor complex to DNA–cellulose (Cake et al., 1978). In order to reduce the possibility of this unwanted side reaction, we have employed two different sulfhydryl reagents, methyl methanethiosulfonate (MMTS)[1] and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), which are very specific for the sulfhydryl group and whose effects can be reversed by sulfhydryl-containing compounds through thio–disulfide interchange (Brocklehurst, 1979).

We demonstrate that both MMTS and DTNB interfere with binding of dexamethasone–receptor complexes to DNA–cellulose. Furthermore, sequential oxidation and reduction of the sulfhydryl groups of nonactivated cytosols significantly increases the capacity of dexamethasone–receptor complexes to bind to DNA–cellulose.

Experimental Procedures

Materials. [6,7-^{3}H(N)]Dexamethasone (50 Ci/mmol) was purchased from New England Nuclear, Boston, MA. Unlabeled dexamethasone was purchased from Steraloids, Wilton, NH. Cellulose CF11 was obtained from Whatman, Clifton, NJ. P-60 (100–200 mesh) gel-filtration matrix and HAP (Bio-Gel HT) were obtained from Bio-Rad Laboratories,

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[1] Abbreviations: HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; MMTS, methyl methanethiosulfonate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTNA, 6,6′-dithiobis(nicotinic acid); CPD, bis(2-carboxyphenyl) disulfide; DTEA, dithiobis(ethylamine); IAcN, iodoacetamide; NEM, *N*-ethylmaleimide; GSSG, oxidized glutathione; HAP, hydroxylapatite; DTE, dithioerythritol.

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Richmond, CA. Calf thymus DNA was purchased from P-L Biochemicals, Milwaukee, WI. All other reagents were obtained from Sigma Chemicals, St. Louis, MO.

Animals. Male Sprague-Dawley (CD strain) rats (Charles River Breeding Laboratories, Wilmington, MA) were bilaterally adrenalectomized at least 4 days prior to their use and maintained on 0.9% NaCl and lab chow ad libitum.

Methods. (*A*) *Preparation of [^{3}H]Dexamethasone–Receptor Complexes.* Rats were decapitated, and the thymuses were removed and placed at room temperature in 5–10 mL of modified Krebs–Ringer bicarbonate solution (the bicarbonate concentration was reduced from 25 mM to 10 mM; HEPES buffer was added to a final concentration of 10 mM, the pH was adjusted to 7.35–7.4, and the normal gassing step was omitted). The thymuses were minced and gently homogenized by hand in a conical glass homogenizer (Kontes Glass Co.) and filtered through a 200-mesh nylon screen (nitex gauze, Tetco, Elmsford, NY). The cells were made up to 50 mL with modified Krebs–Ringer solution and centrifuged for 2.5 min at 450*g*. The supernatant was removed, and the cells were resuspended in 5–10 mL of modified Krebs–Ringer bicarbonate solution and then filtered, washed, and centrifuged as in the previous step. Cells were adjusted to a cytocrit of 0.3–0.4 mL of packed cells/mL of cell suspension, and the suspension was cooled for 15 min on ice. All remaining steps were carried out at 0–3 °C unless otherwise indicated. The cell suspension was then added to a solution, in modified Krebs–Ringer buffer, of [^{3}H]dexamethasone at 6 times the final concentration of 20–40 nM and incubated for 2 h at 0 °C. To obtain cytosol containing [^{3}H]dexamethasone–receptor complexes, cells were lysed by adding the cell suspension to 5 volumes of 1.5 mM $MgCl_2$ containing dextran-coated charcoal (Munck & Wira, 1975; Munck & Foley, 1976); after 15 min, charcoal and cellular debris were removed by centrifugation at 10000*g* for 2 min. The protein concentration of the resulting cytosol supernatants used in this study was approximately 1 mg/mL as determined by the dye binding method of Bradford (1976) with bovine serum albumin as a standard.

In experiments involving treatment of nonactivated complexes with sulfhydryl-modifying reagents, EGTA (200 mM, pH 8.3) was added to the cytosol immediately after centrifugation to give a final concentration of 4 mM and pH 7.4. EGTA was found to stabilize these complexes from proteolytic degradation during the subsequent warming step. However, in those experiments where cytosols were treated after being warmed (i.e., activated complexes), EGTA decreased the inhibition in DNA–cellulose binding caused by the sulfhydryl-modifying reagents. Therefore, EGTA was not added to those cytosols.

(*B*) *Activation Procedure.* Nonactivated [^{3}H]dexamethasone–receptor complexes obtained from the above procedure were activated by being warmed at 25 °C for 15 min. We have found that these conditions result in maximum activation (50–75% of total complexes) for a given preparation as determined by DNA–cellulose chromatography.

(*C*) *HAP Chromatography.* Receptor-bound steroid was assayed by binding of [^{3}H]dexamethasone–receptor complexes to HAP (Erdös et al., 1970). Small columns (0.3-mL bed volume) were packed from a 50% slurry (v/v) of HAP in 5 mM phosphate buffer (pH 7.4) into a 3-mL (0.35-cm inside diameter) plastic syringe (B-D Dickerson) with a porous 70-μm polypropylene disk (Bolabs, Lake Havasu City, AZ) fitted into the bottom. Columns were fitted to a vacuum box, which had provisions for controlling vacuum pressure and for collecting eluates from the columns. Samples of cytosols (50 μL) containing [^{3}H]dexamethasone–receptor complexes were applied to the columns and allowed to enter the bed for 1 min. Phosphate buffer (5 mM, 2 mL, pH 7.4) was applied to the column, and enough vacuum was applied to draw the liquid through the column in approximately 2 min. The vacuum was left on for an additional minute to remove as much eluate as possible from the HAP. The HAP column bed was placed in a vial with scintillation fluid and the radioactivity determined to give the amount of receptor-bound [^{3}H]dexamethasone. The entire eluate was counted to determine free steroid.

(*D*) *DNA–Cellulose Chromatography.* DNA–cellulose, prepared according to the method of Alberts & Herrick (1971), contained approximately 350 μg of calf thymus DNA/mL of hydrated packed matrix.

DNA–cellulose columns were prepared and chromatographed exactly as those described for HAP chromatography except that the 2 mL of phosphate buffer was allowed to pass through the column by gravity; this required about 2 min. Vacuum was used for 1 min after the columns had drained to remove as much residual liquid as possible from the matrix. The DNA–cellulose column bed and eluate were assayed separately for radioactivity. The former gave DNA binding complexes; the latter represents complexes that did not bind to DNA–cellulose plus free steroid.

(*E*) *Gel Filtration.* In many experiments, P-60 gel-filtration columns (equilibrated in 4 mM EGTA–50 mM HEPES buffer, pH 7.6) were used to remove excess sulfhydryl-modifying reagents. Aliquots (0.9 mL) of cytosol containing [^{3}H]dexamethasone–receptor complexes were applied to a 2.5-mL (0.8 × 4.2 cm) column of P-60 and allowed to enter the matrix. Complexes were eluted from the column by application of 1.1 mL of HEPES buffer to the top of the column and by allowing it to fully enter the matrix. Gel filtration on 5.0-mL (1.2 × 4.3) columns of P-60 was performed in the same fashion except that the sample size was 1.8 mL and the elution volume was 2.2 mL. At least 99% of the sulfhydryl reagents were removed by these protocols as judged by experiments using IAcN-treated bovine serum albumin as a marker for the complexes and DTNB as the sulfhydryl-modifying reagent (data not shown).

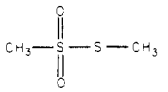
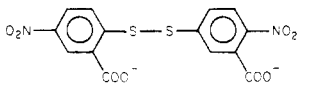
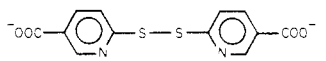
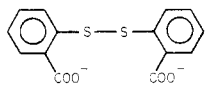
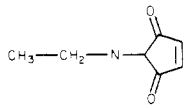
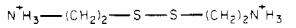
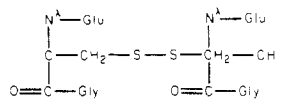
(*F*) *Measurement of Radioactivity.* All radioisotopic measurements were performed on a Packard Tri-Carb liquid scintillation counter with an efficiency for ^{3}H of 35%. All data were corrected for background and quenching.

(*G*) *Specific Binding.* Specific binding was determined by incubation with 20–40 nM [^{3}H]dexamethasone. Nonsaturable binding, measured in the presence of 2.6 μM unlabeled dexamethasone under various conditions studied, in particular with MMTS- and DTNB-treated cytosols, yielded values that were only 3–5% of the amount of [^{3}H]dexamethasone bound on DNA–cellulose or HAP in the absence of unlabeled dexamethasone. Therefore, no correction has been applied for nonsaturable binding to the data presented here.

Results and Discussion

Effect of Sulfhydryl-Modifying Reagents on [^{3}H]Dexamethasone–Receptor Complexes prior to Warming. To determine how the sulfhydryl reagents listed in Table I affect the binding of steroid to the nonactivated dexamethasone–receptor complex, nonactivated complexes were treated with reagents for 30 min at 0 °C. At this time aliquots were removed for assay of receptor-bound [^{3}H]dexamethasone and free steroid by HAP chromatography. The results are given in column A of Table I. The capacity of the treated complexes

Table I: Treatment of [^{3}H]Dexamethasone-Receptor Complexes with Sulfhydryl-Modifying Reagents prior to Warming[a]

compd	structure	receptor-bound [^{3}H]dexamethasone: (A) HAP bound before gel filtration (cpm)	after gel filtration and warming: (B) HAP bound (cpm)	DNA-cellulose bound: (C) cpm	DNA-cellulose bound: (D) % of HAP
control		1826	1078	760	71
MMTS (0.25 mM)		1900	1073	324	30
DTNB (0.25 mM)		1634	758	226	28
DTNA (0.25 mM)		1091	367	102	28
CPD (0.25 mM)		1960	913	501	55
IAcN (8 mM)		1894	1033	601	58
NEM (5 mM)		1436	789	647	85
DTEA (5 mM)		1960	995	551	55
GSSG (5 mM)		1881	1037	751	72

[a] Cytosols containing nonactivated [^{3}H]dexamethasone-receptor complexes were treated with sulfhydryl-modifying reagents from stock solutions at 20 times the final concentration in 1 M HEPES buffer, pH 8.0. The final pH of the control and sulfhydryl-treated cytosols was 7.9–8.0 at 0 °C. After 30 min at 0 °C, aliquots (50 μL) were removed to determine receptor-bound steroid by HAP chromatography. The remaining cytosol was subjected to gel filtration to remove the sulfhydryl-modifying reagent and then warmed to 25 °C for 15 min to activate the complexes. After being cooled to 0 °C, aliquots (50 μL) were assayed for receptor-bound steroid as well as for DNA binding complexes (DNA-cellulose chromatography). Aliquots were applied to the columns as rapidly as possible, generating some variability in the actual sample size. However, the fraction of cpm retained on the column matrix (HAP or DNA-cellulose) to the total cpm applied was very consistent; the range varied less than 3.1% from the mean of duplicate columns. The results in column A have been normalized to a constant applied sample size by multiplying the fraction of column bound cpm times 2200 cpm. The values for columns B and C were obtained as for those of column A, but the results were normalized to the average cpm applied to the duplicate HAP and DNA-cellulose columns for each treatment. The normalization in columns B and C was not done to a uniform sample size because the extent of dissociation that occurred with the different treatments and during the warming slightly altered the amount of steroid-bound receptor. Corrections have been made for the dilution of complexes by the gel-filtration step. Column D is the percentage that column C is of column B. The extent of DNA-cellulose binding before the warming was not determined, but the results from several previous experiments showed that the control and most of the sulfhydryl-modifying reagents had DNA-cellulose binding equal to 10–15% but that MMTS and DTNB had binding approximately equal to their final value (30 and 28%, respectively).

to become activated was then investigated by removing the sulfhydryl-modifying reagents by gel filtration and warming to 25 °C for 15 min. Aliquots were assayed for total dexamethasone–receptor complex and free steroid by HAP chromatography (column B) and for DNA-binding complexes by DNA–cellulose chromatography (columns C and D).

Most sulfhydryl-modifying reagents had little effect on receptor-bound steroid (column A in Table I). Only DTNA and NEM produced a substantial decrease, indicating dissociation of the steroid–receptor complex during the 30-min incubation with these reagents. At the concentrations used in this paper, the sulfhydryl-modifying reagents themselves had little direct effect on either HAP or DNA–cellulose binding since the same extent of binding (for either nonactivated or activated complexes) was observed after the reagents had been removed by gel filtration (data not shown).

The values for HAP binding in column B are lower than those in column A as a consequence of receptor losses (approximately 10%) during gel filtration and the dissociation of steroid from the complexes during warming. In comparison with the reduction in receptor-bound steroid for the control, the DTNA value (367 cpm) is approximately 40% lower, probably due to dissociation of the complex during the warming procedure.

Columns C and D (Table I) show the effect of sulfhydryl-modifying reagents on binding of the dexamethasone–receptor complex to DNA–cellulose. In control cytosols, 71% of the total receptor complexes bound to DNA–cellulose after being warmed to 25 °C, indicating substantial activation. The sulfhydryl reagents MMTS, DTNB, and DTNA produced a marked reduction in DNA–cellulose binding, to 30% or less, despite the fact that the reagents were removed before the activation step. CPD, IACN, and DTEA gave a moderate reduction in DNA–cellulose binding while GSSG was without effect. The large increase in DNA–cellulose binding caused by NEM in this experiment is not a consistent result; in three similar experiments treated cytosol gave only 3–8% more DNA–cellulose binding than the control.

For the experiment in Table I as for many of the subsequent experiments, gel filtration was used to separate low molecular

Table II: Treatment of [^{3}H]Dexamethasone-Receptor Complex with Sulfhydryl-Modifying Reagents after Warming[a]

compd	(A) HAP bound (cpm)	DNA-cellulose bound (B) cpm	DNA-cellulose bound (C) % of HAP bound
control	1080	559	52
MMTS (0.25 mM)	1161	132	11
DTNB (0.5 mM)	1055	264	25
DTNA (0.5 mM)	807	176	22
CPD (0.5 mM)	1126	333	30
IAcN (8 mM)	1135	432	38
NEM (5 mM)	887	517	58
DTEA (5 mM)	1131	683	60
GSSG (5 mM)	1140	617	54

[a] Cytosol containing [^{3}H]dexamethasone-receptor complexes was activated by a warming to 25 °C for 15 min, then cooled to 0 °C, and divided into nine portions. Each portion was treated for 60 min with a sulfhydryl reagent or buffer from the concentrated stock solutions described in Table I. Aliquots (50 μL) were applied to duplicate columns of HAP and DNA-cellulose. Column A, the amount of receptor-bound steroid, and column B, representing DNA binding forms of the complex, were normalized to an applied sample containing 1250 cpm as described in Table I. Column C is the percentage that column B is of column A. The range of values for both HAP and DNA-cellulose was within 3.0% of the mean of duplicate columns.

weight reagents from the receptor complexes. Gel filtration has been reported to activate glucocorticoid–receptor complexes from rat liver (Goidl et al., 1977) and from a mouse pituitary cell line AtT-20 (Vedeckis, 1981). However, we have not detected any significant activation after gel filtration with rat thymus cytosols. Whether these differences reflect differences between thymus and other tissue or methods of cytosol preparation is unclear.

The reduction in DNA–cellulose binding caused by the sulfhydryl-modifying reagents could be due to either blocking of activation or inhibition of DNA–cellulose binding. In order to determine the effects of sulfhydryl-modifying reagents on activated complexes, the following experiments were performed.

Treatment of [^{3}H]Dexamethasone–Receptor Complexes with Sulfhydryl-Modifying Reagents after Warming. [^{3}H]-Dexamethasone–receptor complexes were first warmed to 25 °C for 15 min, treated for 60 min at 0 °C with a sulfhydryl reagent, and assayed for binding to HAP and DNA–cellulose. Results in Table II are representative of three experiments with similar results. The sulfhydryl reagents had little effect on HAP binding (column A). Only DTNA and NEM caused any dissociation of complexes. In contrast, a wide range of effects was seen on DNA–cellulose (columns B and C). The greatest effect was observed with MMTS, which reduced DNA–cellulose binding to 11%, compared to 52% for the control. DTNB, DTNA, and CPD also reduced DNA–cellulose binding substantially, and IAcN reduced it slightly. The other reagents if anything increased DNA binding.

These results show clearly that some of the sulfhydryl-modifying reagents are capable of decreasing DNA–cellulose binding of complexes that are already activated and suggest that these reagents react with a sulfhydryl group in or near the DNA binding domain of the complex. However, since the same reagents decrease DNA–cellulose binding when used to treat complexes before the warming (Table I), they may also effect the activation process itself.

Kalimi & Love (1980) demonstrated that treatment of rat liver cytosol for 30 min at 0 °C with 8 mM IAcN or 5 mM NEM, prior to a warming for 30 min at 25 °C, substantially inhibited nuclear binding. These reagents were not removed before the warming. Neither IAcN nor NEM treatment altered nuclear binding of already warmed complexes.

In our experiments (Tables I and II), iodoacetamide decreased DNA–cellulose binding when used before or after the warming. NEM caused no decrease in DNA–cellulose binding, under either condition, a result we have repeated with two different lots of reagent. If we omit the gel-filtration step when treatment is before the warming, so that NEM is present during the warming procedure, there is substantial reduction in DNA–cellulose binding (from 60 to 35%), with little effect on hormone dissociation.

The reason for the discrepancies between our results and those of Kalimi & Love is not known but may be related to differences in tissue (liver vs. thymus), tissue preparation (homogenization vs. hypotonic lysis), or the method used to monitor activation (nuclei vs. DNA–cellulose binding).

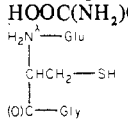
MMTS and DTNB were used to further examine the effects of sulfhydryl-modifying reagents on nonactivated and activated complexes. These reagents have characteristics that make them particularly useful for probing sulfhydryl-containing proteins. MMTS, a thio disulfoxide sulfhydryl-modifying reagent, reacts rapidly with sulfhydryl groups, leaving an unchanged thiomethyl group on the protein (Smith et al., 1975). DTNB, a well-known reagent for the quantitation of sulfhydryl groups (Elman, 1958), reacts leaving a relatively bulky, negatively charged thionitrobenzoate group on the protein. Reactions with both reagents can be reversed by compounds possessing a sulfhydryl group.

Time Course of Inhibition of DNA–Cellulose Binding by MMTS and DTNB. Figure 1 shows the time course for the effect of MMTS and DTNB on DNA–cellulose binding with treatment either before or after the warming to 25 °C for 15 min. In cytosols treated before the warming, the indicated time is from when the reagent was added to the cytosol to the time the sample was applied to the gel-filtration column. Because of the 6–8 min required to complete the gel filtration, it was impossible to determine the exact time course of reaction of these reagents with the nonactivated complexes. Within the 6–8 min it took to complete gel filtration, essentially maximum reduction of DNA–cellulose binding was obtained.

When cytosols were treated after the warming, the time included that from when the reagent was added until samples were assayed for binding by DNA–cellulose and HAP chromatography. In contrast to the rapid effect on DNA–cellulose binding seen with DTNB before the warming, its inhibiting effect on DNA–cellulose binding of activated complexes proceeded at a much slower rate, even though the concentration used was 3-fold higher than that used for treatment before the warming. The rate at which MMTS inhibited DNA–cellulose binding of cytosols treated after the warming was rapid enough compared with treatment before the warming so that any differences in rate would not have been observed because of the 6–8-min lag period imposed by the gel-filtration step.

The difference in the rate at which DTNB alters DNA–cellulose binding of complexes treated before and after the warming may be due to conformational and ionic changes known to accompany activation of the glucocorticoid–receptor complex (Atger et al., 1976; Holbrook et al., 1983a; Vedeckis, 1981). These changes may alter the reactivity or accessibility of critical sulfhydryls to a charged, relatively large sulfhydryl-modifying reagent like DTNB but not to a neutral and smaller molecule like MMTS. Alternatively, the two reagents may be reacting with different sulfhydryl groups on nonac-

Table III: Reversal of the Effects of MMTS and DTNB on [^{3}H]Dexamethasone-Receptor Complexes by Compounds Containing Sulfhydryls[a]

sulfhydryl-containing compd	structure	DNA-cellulose bound as a percent of total receptor-bound steroid applied					
		treatment before warming			treatment after warming		
		control	MMTS	DTNB	control	MMTS	DTNB
none		59 ± 1	26 ± 1	34 ± 1	65 ± 3	40 ± 1	36 ± 2
mercaptoethanol	$HOCH_2CH_2-SH$	69 ± 4	30 ± 2	51 ± 1	72 ± 0	45 ± 2	45 ± 0
mercaptoethylamine	$H_2NCH_2CH_2-SH$	72 ± 1	44 ± 0	62 ± 1	74 ± 0	64 ± 1	55 ± 6
thioglycerol	$HOCH_2CH(OH)CH_2-SH$	65 ± 2	32 ± 0	53 ± 3	68 ± 1	45 ± 3	49 ± 2
dithioerythritol	$HS-CH_2(CHOH)_2CH_2-SH$	67 ± 2	40 ± 1	75 ± 1	69 ± 1	56 ± 2	72 ± 4
cysteine	$HOOC(NH_2)CHCH_2-SH$	71 ± 1	42 ± 2	61 ± 1	74 ± 2	57 ± 1	59 ± 3
glutathione		68 ± 2	39 ± 3	59 ± 1	78 ± 1	53 ± 5	64 ± 3

[a] Nonactivated [^{3}H]dexamethasone-receptor complexes were treated for 30 min with MMTS (0.25 mM), DTNB (0.25 mM), or buffer with the concentrated stock solutions described in Table I, then subjected to gel filtration, and warmed to 25 °C for 15 min. Sulfhydryl-containing compounds (0.5 M stock solutions in 50 mM HEPES buffer, pH 7.6) were added to give final concentrations of 50 mM. After 60 min (0 °C), binding to HAP and DNA-cellulose was determined. When treatment was after the warming, activated [^{3}H]dexamethasone-receptor complexes were treated for 60 min with MMTS (0.25 mM), DTNB (0.75 mM), or buffer with the concentrated stock solutions described in Table I. Following gel filtration, sulfhydryl-containing compounds were added as described above and allowed to incubate for 60 min (0 °C) before determining binding to HAP and DNA-cellulose. Results are expressed as the mean of values obtained from duplicate columns of HAP and DNA-cellulose as described for columns B-D in Table I. Variability is expressed as half of the range of values. Control cytosols bound approximately 700 cpm (65% of total cpm applied) to HAP when sulfhydryl-modifying reagent treatment was before the warming and approximately 800 cpm (75% of total) when treatment was after the warming. MMTS-treated cytosols had slightly greater (1-4%) HAP binding while DTNB had slightly lower (4-7%) binding.

tivated and activated complexes. These groups may have different reactivities and decrease DNA–cellulose binding by different pathways, i.e., direct inhibition of DNA–cellulose binding and prevention of activation.

Concentration Dependence of MMTS and DTNB Treatment on [^{3}H]Dexamethasone–Receptor Complexes. Figure 2 shows the concentration dependence of the MMTS and DTNB effects on DNA–cellulose binding for cytosols treated either before or after the warming to 25 °C for 15 min. Although the extent of DNA–cellulose binding for the controls (0 concentration point) was different if treatment was before or after the warming, the concentration-dependence curves for each reagent were similar regardless of whether cytosols were treated before or after activation. However, there was a considerable difference in the shape of the curves for the two reagents: DTNB-treatment gave a flatter concentration dependence than did MMTS treatment. Furthermore, the two agents differed in potency. MMTS causes a 50% reduction in DNA–cellulose binding at about 0.125–0.25 mM while DTNB produced its half-maximum effect at a lower concentration (0.03–0.06 mM).

Reversal of the Effects of MMTS and DTNB on [^{3}H]-Dexamethasone–Receptor Complexes by Sulfhydryl-Containing Compounds. If MMTS and DTNB alter binding of complexes to DNA–cellulose by oxidizing critical sulfhydryl groups, their effect should be reversed by the addition of a reducing agent such as DTE. Figure 3 shows that DNA–cellulose binding can be restored in MMTS- and DTNB-treated cytosols by addition of 50 mM DTE, whether treatment with the reagent was before or after the warming. Interestingly, DTE restores DNA–cellulose binding of complexes treated with DTNB much faster than those treated with MMTS. Other reducing agents acted similarly to DTE when cytosols were treated with sulfhydryl-modifying reagents before the warming (Table III). All of the reducing agents restored DNA–cellulose binding of complexes in DTNB-treated cytosols to a substantially greater extent than in MMTS-treated cytosols, consistent with what was found for DTE in Figure 3. However, when cytosols were treated with MMTS and DTNB after the warming, mercaptoethylamine and mercaptoethanol (Table III) restored DNA–cellulose binding for both

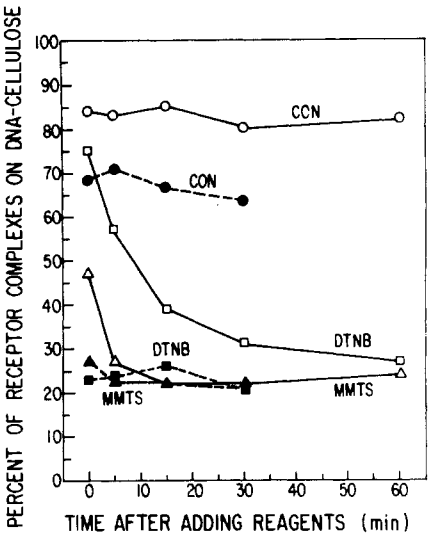


FIGURE 1: Time course of the inhibition of DNA–cellulose binding by MMTS and DTNB. Treatment before warming (– –). MMTS [final concentration 0.25 mM (▲)], DTNB [0.25 mM (■)], or buffer (●) was added from the concentrated stock solution described in Table I to cytosols containing nonactivated [^{3}H]dexamethasone–receptor complexes. The cytosols were mixed, left at 0 °C, and applied to a P-60 gel-filtration column after the time indicated. The initial time point was at 30 s. After completion of the gel filtration, which took 6–8 min, the complexes were warmed for 15 min at 25 °C, cooled, and assayed for binding to HAP and DNA–cellulose. Treatment after warming (—). MMTS [final concentration 0.5 mM (△)], DTNB [0.75 mM (□)], or buffer (○) was added from the concentrated stock solutions described in Table I to cytosols containing activated [^{3}H]dexamethasone–receptor complexes at 0 °C. The cytosols were mixed, left at 0 °C, and assayed for binding to HAP and DNA–cellulose at the indicated times. The initial time point was at 30 s. When treatment by sulfhydryl-modifying reagents was before the warming, the results are expressed as the mean of values obtained from duplicate columns of HAP and DNA–cellulose as described for columns B–D in Table I. Values for HAP binding were similar to those for Table III. The range of values was less than the width of the symbols. When treatment was after the warming, data points were obtained from single columns of HAP and DNA–cellulose.

MMTS- and DTNB-treated complexes.

Generally, DTE appears to be the best reducing agent, perhaps because each molecule of DTE has two sulfhydryl

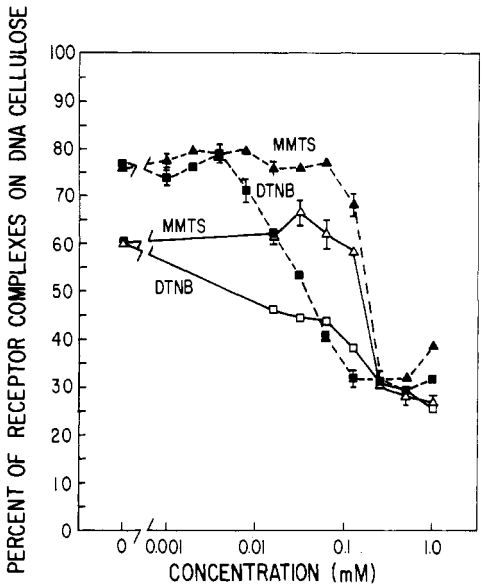


FIGURE 2: Concentration dependence of the MMTS- and DTNB-mediated inhibition of DNA–cellulose binding. Treatment before warming (– –). Cytosols containing nonactivated [^{3}H]dexamethasone–receptor complexes were treated for 30 min with the appropriate concentration of MMTS (▲) or DTNB (■) from 20-fold concentrated stock solutions in 1 M HEPES buffer, pH 8 (final pH 7.9–8.0 at 0 °C). Complexes were subjected to gel filtration, warmed to 25 °C for 15 min, and assayed for binding to HAP and DNA–cellulose. Treatment after warming (—). Cytosols containing activated [^{3}H]dexamethasone–receptor complexes were treated with the appropriate concentration of MMTS (△) or DTNB (□) as described above. After 60 min, the complexes were assayed for binding to HAP and DNA–cellulose. Values for HAP binding were similar to those for Table III. Results are expressed as the mean and range (error bars when larger than the symbol) of values obtained from duplicate columns of HAP and DNA–cellulose as described for columns B–D in Table I.

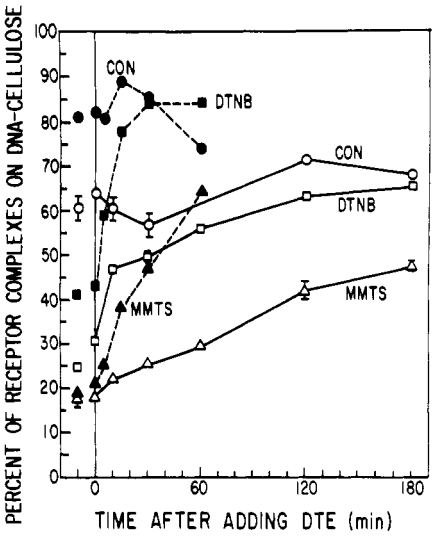


FIGURE 3: Reversal by DTE of the MMTS- and DTNB-mediated inhibition of DNA–cellulose binding. Treatment with sulfhydryl reagents before warming (—). [^{3}H]Dexamethasone–receptor complexes were treated for 30 min with 0.25 mM MMTS (△), 0.25 mM DTNB (□), or buffer [control (○)] from the concentrated stock solutions described in Table I. The complexes were subjected to gel filtration, warmed for 15 min at 25 °C, and then allowed to cool on ice for 5 min. DTE was added to a final concentration of 50 mM from a 0.5 M stock solution in 50 mM HEPES buffer, pH 7.6. At the time of mixing (zero time point) and at the times indicated, aliquots were removed to determine the extent of binding to HAP and DNA–cellulose. Values plotted before the zero time point were determined less then 10 min prior to the addition of DTE. Treatment with sulfhydryl reagents after warming (– –). [^{3}H]Dexamethasone–receptor complexes were warmed to 25 °C for 15 min, cooled on ice for 5 min, and then treated for 60 min with 0.25 mM MMTS (▲), 0.75 DTNB (■), or buffer [control (●)] from the stock solutions described in Table I. DTE (final concentration 50 mM) was added, and aliquots were removed for assay as described above. Values for HAP binding were similar to those for Table III. Results are expressed as the mean and range (error bars when larger than the symbol) of values obtained from duplicate columns of HAP and DNA–cellulose as described for columns B–D in Table I.

groups and, therefore, has greater reducing power per molecule than the other reducing agents. Aside from DTE, the charged reducing agents (mercaptoethylamine, cysteine, and glutathione) were better than the uncharged agents (mercaptoethanol and thioglycerol).

DTE Induces DNA–Cellulose Binding of the MMTS- and DTNB-Treated [^{3}H]Dexamethasone–Receptor Complex at 0 °C. We find that substantial DNA–cellulose binding is induced by addition of DTE at 0 °C to the MMTS- and DTNB-treated dexamethasone–receptor complex without the complexes ever having been warmed. This is shown in Figure 4 where nonactivated [^{3}H]dexamethasone–receptor complexes were treated with the two sulfhydryl-modifying reagents (or buffer) and subjected to gel filtration. Half of the cytosols from each treatment was kept on ice while the other half was warmed to 25 °C for 15 min. The addition of DTE (0 °C) at this point caused a significant increase in DNA–cellulose binding only with complexes that had been treated with sulfhydryl-modifying reagents. The rate of increase in DNA–cellulose binding was the same as that for MMTS-treated cytosols whether or not they had been warmed. However, warming causes a significant increase (approximately 100% greater at 180 min) in DNA–cellulose binding for the DTNB-treated complex.

The relationship between sulfhydryl groups and activation is unclear, but the data from Figures 3 and 4 show that oxidation followed by reduction of the nonactivated GRC leads to its transformation to an activated form at 0 °C, circumventing the normal warming step. It is clear that there is also another component to this process, as the DTNB-treated GRC acquires greater DNA–cellulose binding if it is warmed before the reduction step. Although the mechanism of activation of the GRC by oxidation/reduction is unknown, it can be added to a substantial list of reagents known to activate the GRC (i.e., high ionic strength, ATP and other nucleotide tri- and diphosphates, pyrophosphate, etc.; Holbrook et al., 1983b).

The nonactivated complex is a large, perhaps tetrameric, form, whereas the activated complex appears to be a monomer (Vedeckis, 1983; Holbrook et al., 1983a). Apparently, the nonactivated complex is unstable so that perturbation with heat and many reagents will transform it to the presumably more stable activated form. Oxidation of the nonactivated GRC by sulfhydryl-modifying reagents may also sufficiently perturb the conformation so that subsequent reduction allows the GRC to activate (dissociate) instead of returning to its original conformation.

Vedeckis (1983) has reported that an 18-h treatment of mouse AtT-20 pituitary tumor cells with thioglycerol or mercaptoethanol, but not DTE, increased the amount of activated complexes. This finding agrees with our results in that DTE caused no significant increase in activated complexes without pretreatment with a sulfhydryl reagent. Vedeckis, however, was limited to 10 mM dithiothreitol because of substantial losses in receptor-bound steroid. This is a problem that we have not encountered with rat thymocytes and short incubation times.

Conclusions

From the results described, we cannot conclude whether

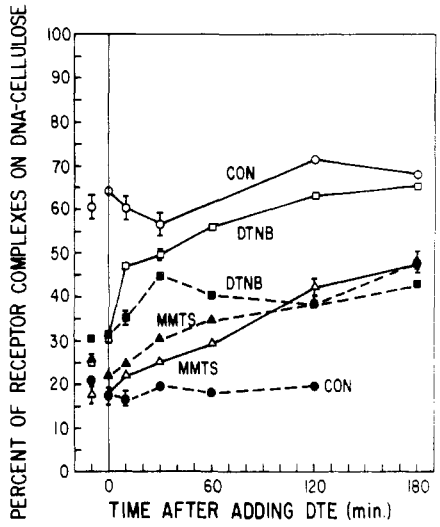


FIGURE 4: Induction of DNA–cellulose binding at 0 °C by the addition of DTE to the MMTS- and DTNB-treated nonactivated [^{3}H]dexamethasone–receptor complex. [^{3}H]Dexamethasone–receptor complexes were treated for 30 min with 0.25 mM MMTS (△, ▲), 0.25 mM DTNB (□, ■), or buffer (○, ●) as in Figure 3. Cytosol from each treatment was divided into equal portions, and half was warmed (—) for 15 min at 25 °C and then put on ice to cool. The remaining half was kept on ice (– –) the entire time. DTE (final concentration 50 mM) was added as described in Figure 3. At the time of mixing (zero time point) and at the times indicated, aliquots were removed to determine the extent of binding to HAP and DNA–cellulose. Values plotted before the zero time point were determined less then 10 min prior to the addition of DTE. Values for HAP binding were similar to those for Table III. Results are expressed as the mean and range (error bars when larger than the symbol) of values obtained from duplicate columns of HAP and DNA–cellulose as described for columns B–D in Table I.

MMTS and DTNB treatment of nonactivated complexes prior to warming interferes with the activation process, with DNA–cellulose binding, or with both these steps. However, it is clear from the results in Table II that oxidation of sulfhydryl groups greatly diminishes binding of the activated dexamethasone–receptor complex to DNA–cellulose. This indicates the probable existence of a cysteine residue in or near the DNA binding domain of the complex.

A sulfhydryl group in or near the steroid binding domain has been well documented. Whether or not this is (these are) the same sulfhydryl(s) that is (are) associated with the DNA binding domain remains an open question. Indirect evidence suggests that there may be distinct sulfhydryls in each binding domain. Simons et al. (1983) demonstrated that glucocorticoid–receptor complexes from both HTC and rat liver cells that were covalently labeled with dexamethasone 21-mesylate were able to undergo activation and bind to DNA–cellulose. Dexamethasone 21-mesylate is known to selectively react with thiol anions (Simons et al., 1980) and probably reacts with the sulfhydryl(s) in the steroid binding domain. If this sulfhydryl was also associated with the DNA binding domain and was necessary for the DNA–receptor interaction, the covalently coupled dexamethasone–receptor complex would not be expected to bind DNA–cellulose. We are currently carrying out experiments designed to obtain a more definitive answer to this question, and our preliminary results suggest the existence of distinct sulfhydryls in each binding domain.

Acknowledgments

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References

Alberts, B., & Herrick, C. (1971) *Methods Enzymol. 21*, 198.
Atger, M., & Milgrom, E. (1976) *Biochemistry 15*, 4298.
Banerji, A., & Kalimi, M. (1981) *Steroids 37*, 409.
Baxter, J. D., Rousseau, G. G., Benson, M. C., Garcea, R. L., Ito, J., & Tomkins, G. M. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 1892.
Bradford, M. M. (1976) *Anal. Biochem. 72*, 248.
Brewer, C. F., & Riehm, J. P. (1967) *Anal. Biochem. 18*, 248.
Brocklehurst, K. (1979) *Int. J. Biochem. 10*, 259.
Cake, M. H., DiSorbo, D. M., & Litwack, G. (1978) *J. Biol. Chem. 253*, 4886.
Eisen, H. J., Schleenbaker, R. E., & Simons, S. S., Jr. (1981) *J. Biol. Chem. 256*, 12920.
Elman, G. L. (1958) *Arch. Biochem. Biophys. 74*, 443.
Erdös, T., Best-Belpomme, M., & Bessada, R. (1970) *Anal. Biochem. 37*, 244.
Goidl, J. A., Cake, N. H., Dolan, K. P., Parchman, L. G., & Litwack, G. (1977) *Biochemistry 16*, 2125.
Higgins, S. J., Rousseau, G. C., Baxter, J. D., & Tomkins, G. M. (1973) *J. Biol. Chem. 248*, 5866.
Holbrook, N. J., Bodwell, J. E., Jeffries, M., & Munck, A. (1983a) *J. Biol. Chem. 258*, 6477.
Holbrook, N. J., Bodwell, J. E., & Munck, A. (1983b) *J. Biol. Chem. 258*, 14885.
Kalimi, M., & Love, K. (1980) *J. Biol. Chem. 255*, 4687.
Kalimi, M., Colman, P., & Feigelson, P. (1975) *J. Biol. Chem. 250*, 1080.
Kobolinski, M., Beats, M., Kalimi, M., & Feigelson, P. (1972) *J. Biol. Chem. 247*, 7897.
Milgrom, E., Atger, M., & Baulieu, E. E. (1973) *Biochemistry 12*, 5198.
Munck, A., & Wira, C. (1975) *Methods Enzymol. 36A*, 255.
Munck, A., & Foley, R. (1976) *J. Steroid Biochem. 7*, 1117.
Munck, A., & Foley, R. (1979) *Nature (London) 278*, 752.
Munck, A., Wira, C., Young, D. A., Mosher, K. M., Hallahan, C., & Bell, P. A. (1972) *J. Steroid Biochem. 3*, 567.
Rees, A. M., & Bell, P. A. (1975) *Biochim. Biophys. Acta 411*, 121.
Schaumburg, B. P. (1972) *Biochim. Biophys. Acta 261*, 219.
Simons, S. S., Jr., Pons, M., & Johnson, D. F. (1980) *J. Org. Chem. 45*, 3084.
Simons, S. S., Jr., Schleenbaker, R. E., & Eisen, H. J. (1983) *J. Biol. Chem. 258*, 2229.
Smith, D. J., Maggio, E. T., & Kenyon, G. L. (1975) *Biochemistry 14*, 766.
Smyth, D. G., Blumenfeld, O. O., & Konigsberg, W. (1964) *Biochem. J. 91*, 589.
Vedeckis, W. V. (1981) *Biochemistry 20*, 7237.
Vedeckis, W. V. (1983) *Biochemistry 22*, 1983.
Wallenfels, K., & Eisele, B. (1968) *Eur. J. Biochem. 3*, 267.
Young, H. A., Parks, W. P., & Scolnick, E. M. (1975) *Proc. Natl. Acad. Sci. (U.S.A.) 72*, 3060.