Effects of Calf Intestinal Alkaline Phosphatase, Phosphatase Inhibitors, and Phosphorylated Compounds on the Rate of Activation of Glucocorticoid–Receptor Complexes†

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ABSTRACT: The effects of (1) calf intestinal alkaline phosphatase, (2) phosphatase inhibitors, and (3) phosphorylated compounds on the rate of activation of rat liver and kidney glucocorticoid–receptor complexes have been investigated. The extent of activation has been quantitated by measuring the binding of [^{3}H]triamcinolone acetonide–receptor complexes to DNA–cellulose and visualized by chromatographic separation of unactivated and activated ^{3}H-labeled steroid–receptor complexes on DEAE-Sephadex A-50 or DEAE-cellulose. The addition of calf intestinal alkaline phosphatase stimulates the rate of activation (0 °C, 2 h) of [^{3}H]triamcinolone acetonide–receptor complexes in rat liver cytosol. Heat denaturation (90 °C, 15 min) of the exogenous enzyme or addition of the competitive inhibitor, sodium arsenate, prevents the alkaline phosphatase mediated stimulation. In addition, formation of activated [^{3}H]triamcinolone acetonide–receptor complexes (25 °C, 30 min) in rat liver and kidney cytosols is blocked by some phosphatase inhibitors (molybdate and tungstate) but not by others (vanadate, fluoride, arsenate, and levamisole). Our results with calf intestinal alkaline phosphatase and with molybdate and tungstate are consistent with the idea that activation of glucocorticoid–receptor complexes involves a dephosphorylation reaction. It is not known whether the exogenous enzyme is directly modifying the hormone receptor or whether its action is on some components(s) which regulates activation of the [^{3}H]triamcinolone acetonide–receptor complexes. The rate of activation (15 °C, 30 min) of glucocorticoid–receptor complexes is also stimulated by *p*-nitrophenol and by a variety of low molecular weight phosphoesters (*p*-nitrophenyl phosphate, α-naphthyl phosphate, ATP, AMP, α-glycerophosphate, and β-glycerophosphate). These small molecular weight compounds can be differentiated into two groups based on their ability to stimulate or inhibit the rate of heat inactivation of unbound receptors. It is not known how these compounds act to stimulate activation of hormone–receptor complexes. Possible mechanisms are discussed.

In intact cells the specific binding of glucocorticoids is an energy-dependent process (Munck & Brinck-Johnsen, 1968; Munck et al., 1972; Ishii et al., 1972). This observation led Munck et al. (1972) to propose that the receptor exists in two forms and that ATP[1] may be required to generate the steroid binding form from a nonbinding precursor. More recent reports by Nielsen et al. (1977a,b) and by Sando et al. (1979a,b) suggest that either the glucocorticoid receptor or some component regulating the receptor must be phosphorylated in order for steroid binding to occur. This hypothesis is based on three kinds of evidence. First, incubation of the 100000*g* supernatant fractions of mouse fibroblasts (L cells) and rat liver with exogenous calf intestinal alkaline phosphatase converts the unbound receptor to a form which does not bind steroid (Nielsen et al., 1977a). Enzymatic inactivation of unbound receptor, as well as enzymatic dephosphorylation of *p*-nitrophenyl phosphate, is inhibited by arsenate and is zinc dependent. The enzyme preparation was protease-free by several criteria. Second, the glucocorticoid binding activity in the 100000*g* supernatant fractions from L cells, rat liver, and rat thymocytes disappears following incubation of the cytosol at 25 °C. Heat inactivation of steroid binding activity is totally prevented by sodium molybdate while fluoride and glucose 1-phosphate inhibit the rate of this inactivation (Nielsen et al., 1977b). Third, the loss of steroid binding activity by L cell cytosol during incubation at 25 °C can be slowed by the addition of ATP (Sando et al., 1979a). Moreover, the steroid binding capacity of heat-treated cytosol from mouse L cells and rat thymocytes can be partially restored by incubation with ATP–Mg in the presence of molybdate (Sando et al., 1979a,b). These results suggest that either the receptor protein or some some regulatory component must be phosphorylated in order for steroid binding to occur.

In vitro treatment of cytosol-containing glucocorticoid–receptor complexes with heat, salt, gel filtration, dilution, and various chemicals produces a more positively charged molecular species with increased affinity for nuclei and for polyanions such as DNA–cellulose and phosphocellulose (Baxter et al., 1972; Milgrom et al., 1973; Higgins et al., 1973; Kalimi et al., 1975; Goidl et al., 1977; Cake & Litwack, 1978; Bailly et al., 1978; Litwack et al., 1980; Sakaue & Thompson, 1977). This transformation has been termed "activation". Recent studies in this laboratory (Litwack et al., 1980) and by Munck & Foley (1979) demonstrate that the activated form of the receptor which is produced in vitro is also formed in vivo and that its appearance following injection of glucocorticoids is time dependent. These studies clearly demonstrate that "activation" of glucocorticoid–receptor complexes is a physiologically relevant process.

On the basis of the observations of Nielsen et al. (1977a,b) and Sando et al. (1979a,b), we investigated the effects of exogenous calf intestinal alkaline phosphatase and phosphatase

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[1] Abbreviations used: ATP, adenosine 5′-triphosphate; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; DEAE, diethylaminoethyl; EDTA, ethylenediaminetetraacetic acid.

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inhibitors on the activation of glucocorticoid–receptor complexes. The extent of activation has been quantitated by measuring binding to DNA–cellulose and visualized by ion-exchange chromatography using either DEAE-Sephadex (Litwack et al., 1980) or DEAE-cellulose (Sakaue & Thompson, 1977). Earlier studies by Munck et al. (1972) suggested that, when the receptor was released from the nucleus, it was not competent to rebind steroid and that its transformation to a steroid binding form was an ATP-dependent process. We reasoned that if phosphorylation of the receptor was necessary for steroid binding to take place, then activation of the hormone–receptor complexes may involve dephosphorylation.

The data presented in this paper show that exogenous alkaline phosphatase can stimulate the rate of activation of glucocorticoid–receptor complexes and that the phosphatase inhibitors molybdate and tungstate prevent formation of the activated hormone–receptor complex. An unexpected finding was that a variety of small molecular weight phosphorylated compounds stimulate the rate of activation of hormone–receptor complexes. These compounds can be separated into two groups on the basis of their differential effects on the heat inactivation of unbound receptors. The possible mechanisms by which these compounds exert their effects on glucocorticoid receptors are discussed.

Experimental Procedures

Materials

[6,7-^{3}H(*N*)]Triamcinolone acetonide[2] (sp act. 37.1 Ci/mmol) was purchased from New England Nuclear Corp. DEAE-Sephadex A-50, Sephadex G-25, and Dextran T-500 were obtained from Pharmacia, and Cellex 410 was obtained from Bio–Rad Laboratories. DEAE-cellulose (Whatman DE52) was purchased from Whatman Ltd. National Diagnostics, Somerville, NJ, was the source for Liquiscint. With the exception of *p*-nitrophenol (Research Plus Laboratories, Inc., Denville, NJ) and levamisole hydrochloride[3] (Aldrich Chemical Co., Inc., Milwaukee, WI), biochemical compounds were purchased from Sigma Chemical Co. Sodium vanadate was also purchased from Sigma, but other inorganic salts were either Baker or Fisher Certified Reagents. Partially purified calf intestinal alkaline phosphatase, type VII (1075 units/mg of protein), was purchased from Sigma Chemical Co.

Methods

Preparation of Cytosol. Adrenalectomized male rats (110–140 g Sprague–Dawley strain) were used 4–14 days following surgery. Animals were killed by decapitation and tissue was perfused in situ with ice-cold 0.145 M NaCl. Tissue was homogenized with a motor driven Teflon pestle (10 strokes) in an equal volume of either TSM (0.05 M Tris-HCl, pH 8.1 at 0–4 °C, 0.25 M sucrose, and 3 mM $MgCl_2$) or BSM (0.2 M borate, pH 8.0 at 0–4 °C, 0.25 M sucrose, and 3 mM $MgCl_2$) buffer. The cytosol was obtained by centrifugation of the homogenate at 100000*g* for 1 h at 0–4 °C in a Beckman Model L5-50 ultracentrifuge. Cytosol was used immediately or stored frozen in liquid N_2 for use at a later date.

Specific Cytosolic Binding of [^{3}H]Triamcinolone Acetonide. The cytosol was incubated with [^{3}H]triamcinolone acetonide at a final concentration of either 30 or 50 nM (as indicated) in the presence or absence of a 100- or 1000-fold excess of unlabeled steroid. Nonspecific binding was 5–10% of total bound ^{3}H-labeled steroid. After incubation for 1.5–2 h at 0–4 °C, the specifically bound radioactivity was determined by using the dextran-coated charcoal technique (Beato & Feigelson, 1972). Appropriate aliquots were counted in 10 mL of Liquiscint with a counting efficiency for ^{3}H of ~35%.

Specific Binding of [^{3}H]Triamcinolone Acetonide–Receptor Complexes to DNA–Cellulose. DNA–cellulose was prepared according to the procedure of Alberts & Herrick (1971). A 100-μL aliquot of cytosol, preincubated with [^{3}H]triamcinolone acetonide, was mixed with 50 μL of packed DNA–cellulose and incubated at 0–4 °C for 45 min. At the end of the incubation, pellets were washed 4 times with 2-mL aliquots of ice-cold TE buffer (10 mM Tris-HCl, pH 8.0 at 0–4 °C, and 1 mM EDTA disodium salt). The pellet was resuspended in 0.8 mL of the same buffer, and an appropriate aliquot was assayed for radioactivity.

Ion-Exchange Chromatography. All chromatographic procedures were performed in a 4 °C cold room. DEAE-Sephadex A-50 columns (8-mL bed volume) were prepared in 10-cm^3 plastic, disposable syringes. Dextran-coated charcoal (0.5 mL) was mixed with the initial 2 mL of resin to adsorb free steroid during chromatography (Parchman & Litwack, 1977). The columns were equilibrated with 0.02 M potassium phosphate buffer (pH 6.8 at 0–4 °C). A 0.5-mL sample of [^{3}H]triamcinolone acetonide labeled cytosol was applied to the column which was then washed with 16 mL of the equilibration buffer. The wash was collected as eight 2-mL fractions. Adsorbed hormone–receptor complexes were eluted with a 0–0.5 M linear KCl gradient prepared in the equilibration buffer. Fractions (0.5 mL) were collected and analyzed for bound radioactivity, A_{280}, and KCl concentration. The KCl concentration was determined by measuring the conductivity of appropriate samples with a Markson Model 10 conductivity meter and comparison with a standard KCl curve.

DEAE-cellulose (Whatman DE 52) columns (3-mL bed volume) were prepared in 5-cm^3 plastic, disposable syringes. The columns were equilibrated with KPD buffer (5 mM potassium phosphate and 0.5 mM dithiothreitol, pH 7.6 at 0–4 °C). Prelabeled cytosol was treated with dextran-coated charcoal, and a 0.3-mL sample was applied to the column. The column was washed with 10 mL of the equilibration buffer, and the wash was discarded. Bound radioactivity was eluted with a linear 5–400 mM potassium phosphate gradient. Thirty 1-mL fractions were collected and analyzed for radioactivity and salt concentration as described above.

Preparation of Alkaline Phosphatase. Partially purified calf intestinal alkaline phosphatase type VII (1075 units/mg of protein, 37 °C, pH 10.4) was obtained from Sigma Chemical Co. as a crystalline suspension in 3.2 M $(NH_4)_2SO_4$. Preliminary experiments showed that $(NH_4)_2SO_4$ activated glucocorticoid–receptor complexes at concentrations as low as 7 mM (data not shown). Therefore, the alkaline phosphatase was desalted prior to use as follows: the suspension was redissolved with 1 mL of TSM buffer and applied to a Sephadex G-25 column (Pharmacia PD-10, prepacked columns). The alkaline phosphatase was eluted from the column in 3.0 mL of TSM, and the desalted enzyme preparation was stabilized by the addition of $MgCl_2$ (1 mM) and $ZnCl_2$ (0.1 mM).

Results

Effect of Calf Intestinal Alkaline Phosphatase on Rate of Activation of [^{3}H]Triamcinolone Acetonide–Receptor Com-

[2] Triamcinolone acetonide is the trivial name for 9-fluoro-11β,21-dihydroxy-16α,17-[(1-methylethylidene)bis(oxy)]pregna-1,4-diene-3,20-dione.

[3] Levamisole is the trivial name for L-6-phenyl-2,3,5,6-tetrahydroimidazo[2,1-*b*]thiazole.

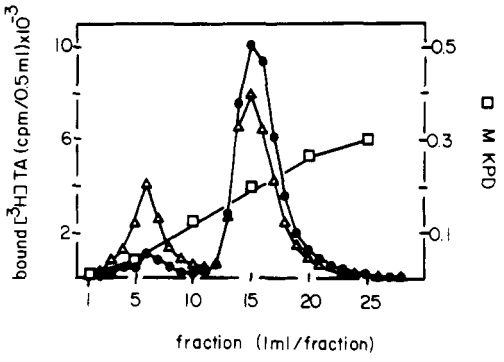

FIGURE 1: Effect of calf intestinal alkaline phosphatase on formation of activated [^{3}H]triamcinolone acetonide–receptor complexes. Liver cytosol prepared in TSM buffer was incubated with 30 nM [^{3}H]-triamcinolone acetonide at 0–4 °C. At the end of the 2-h incubation, desalted calf intestinal alkaline phosphatase was added to an aliquot of labeled cytosol at a final concentration of 452 μg/mL, and the incubation was continued for an additional 2 h at 0–4 °C. The cytosol was adjusted to 10 mM Na_2MoO_4 to prevent additional activation during chromatography and treated with dextran-coated charcoal. 0.3 mL was applied to a DEAE-cellulose column (3-mL bed volume). The columns were washed with 10 mL of KPD equilibration buffer, pH 7.6, and the wash was discarded. The bound complexes were eluted with a 5–400 mM linear potassium phosphate gradient. Thirty 1-mL fractions were collected. (●) No additions; (△) incubation with calf intestinal alkaline phosphatase.

plexes. At 0–4 °C activation of glucocorticoid–receptor complexes is minimal. The formation of activated complexes at this temperature is stimulated by incubation of liver cytosol with desalted calf intestinal alkaline phosphatase (Figure 1). Table I shows that the stimulation of activation is the result of the phosphatase activity of the desalted enzyme and is not due to a minor contaminant in the preparation. Experiments 1 and 2 demonstrate that activation by the desalted alkaline phosphatase can be prevented by 1 mM arsenate. Arsenate also inhibits the exogenous enzyme when *p*-nitrophenyl phosphate is used as the substrate (Fernley, 1971; personal observation), but arsenate alone has no effect on endogenous activation of the hormone–receptor complexes (see Table II). Experiment 3 demonstrates that heat denaturation of alkaline phosphatase activity (90 °C, 15 min; Nielsen et al., 1977a) destroys the activating capacity of the enzyme. This experiment also shows that $MgCl_2$ and $ZnCl_2$, which were added after desalting to stabilize the enzyme, do not activate glucocorticoid–receptor complexes when added alone. Since preliminary data indicated that very low concentrations of $(NH_4)_2SO_4$ (7 mM) can activate glucocorticoid–receptor complexes, a third control was included to eliminate the possibility that activation was due to residual salt. Buffer was adjusted to the same $(NH_4)_2SO_4$ concentration as the dissolved enzyme preparation (1.6 M) and desalted on a Sephadex G-25 column. When the desalted buffer was substituted for the desalted enzyme preparation, there was no activation of the [^{3}H]triamcinolone acetonide–receptor complexes. Finally, preincubation of DNA–cellulose with alkaline phosphatase had no effect on the subsequent binding of unactivated steroid–receptor complexes to DNA–cellulose (data not shown). The stimulation of the rate of activation by the desalted alkaline phosphatase is more efficient when cytosol is prepared in TSM buffer than when cytosol is prepared in BSM buffer (data not shown). This observation is consistent with the report that Tris stimulates alkaline phosphatase activity by functioning as a phosphate acceptor (Dayan & Wilson, 1964; Wilson et al., 1964) and with reports that borate inhibits calf intestinal alkaline phosphatase (Zittle & Della Monica, 1950).

The rate of activation of [^{3}H]triamcinolone acetonide–receptor complexes formed in rat kidney medulla, kidney cortex, thymus, heart, and the glucocorticoid-sensitive human lymphoid cell line (CEM) (Norman & Thompson, 1977) is also stimulated by the addition of calf intestinal alkaline phosphatase (data not shown).

Table I: Effect of Calf Intestinal Alkaline Phosphatase on Activation of [^{3}H]Triamcinolone Acetonide–Receptor Complexes[a]

treatment	[^{3}H]triamcinolone acetonide specifically bound to DNA–cellulose: cpm/100 μL of cytosol	% of total binding
expt 1		
1 mM $MgCl_2$ and 0.1 mM $ZnCl_2$	1142	2.1
133 μg/mL alkaline phosphatase	3473	6.3
133 μg/mL alkaline phosphatase plus 1 mM $Na_2HAsO_4 \cdot 7H_2O$	1696	3.1
1 mM $Na_2HAsO_4 \cdot 7H_2O$	1336	2.4
expt 2		
1 mM $MgCl_2$ and 0.1 mM $ZnCl_2$	1142	2.6
265 μg/mL alkaline phosphatase	5099	11.5
265 μg/mL alkaline phosphatase plus 1 mM $Na_2HAsO_4 \cdot 7H_2O$	2334	5.3
1 mM $Na_2HAsO_4 \cdot 7H_2O$	1128	2.6
expt 3		
TSM buffer	1259	2.9
533 μg/mL alkaline phosphatase	4108	10.1
heat-denatured alkaline phosphatase	1479	3.5
1 mM $MgCl_2$ and 0.1 mM $ZnCl_2$	1150	3.2
desalted $(NH_4)_2SO_4$ buffer	1409	3.2

[a] Liver cytosol prepared in TSM buffer was incubated with 30 nM [^{3}H]triamcinolone acetonide at 0–4 °C. Nonspecific binding was determined in the presence of a 1000-fold excess of unlabeled steroid. The indicated additions were made to the labeled cytosol, and incubation was continued at 0–4 °C for 2 h. Each value represents an average of duplicate determinations.

Effect of Phosphatase Inhibitors on Activation of [^{3}H]-Triamcinolone Acetonide–Receptor Complexes. Table II shows that heat activation (25 °C, 30 min) of the [^{3}H]triamcinolone acetonide–receptor complexes formed in liver and kidney cytosols is prevented by the phosphatase inhibitors molybdate, tungstate, and vanadate but is not blocked by fluoride, arsenate, or levamisole which are also known inhibitors of phosphatase activity (Cotton & Wilkinson, 1972; Fernley, 1971; Lopez et al., 1976; Paigen, 1958; VanEtten et al., 1974). The experiment presented in Table III suggested that molybdate and tungstate prevented conversion of the hormone–receptor complexes to the activated form, whereas vanadate prevented binding of the activated complexes to DNA–cellulose. This interpretation was based on the observation that activation, as measured by DNA–cellulose binding, was inhibited by molybdate and tungstate if the compounds were added to labeled cytosol prior to heat treatment but that they had little effect if added after 30 min at 25 °C. Vanadate inhibition was equally effective whether the compound was added before or after heat treatment. Preincubation of DNA–cellulose with vanadate followed by washing had no effect on the subsequent binding of the activated steroid–receptor complexes (data not shown). This observation suggests that vanadate interacts with the activated

Table II: Effect of Phosphatase Inhibitors on the Activation of [^{3}H]Triamcinolone Acetonide–Receptor Complexes[a]

additions	min at 25 °C	specifically bound [^{3}H]triamcinolone acetonide: DNA–cellulose binding cpm/100 μL of cytosol	% of total	total bound (cpm/100 μL of cytosol)
		Liver Cytosol		
none	0	3 304	5.2	57 714
	30	26 613	46.9	56 648
NaF, 1 mM	30	21 207	38.5	55 048
10 mM	30	22 222	40.7	54 542
Na_2HAsO_4, 1 mM	30	24 025	45.4	52 852
10 mM	30	19 343	40.7	47 425
levamisole, 1 mM	30	23 648	42.3	55 864
10 mM	30	20 261	35.6	56 879
Na_2MoO_4, 1 mM	30	19 506	30.1	64 772
10 mM	30	4 233	5.6	74 678
Na_2WO_4, 1 mM	30	14 514	22.2	65 136
10 mM	30	4 385	6.0	73 067
$NaVO_3$, 1 mM	30	12 288	18.6	66 071
10 mM	30	10 101	14.2	70 896
		Kidney Cytosol		
none	0	1 772	2.6	68 008
	30	20 963	39.6	52 886
NaF, 10 mM	30	21 726	42.0	51 709
Na_2HAsO_4, 10 mM	30	19 266	39.3	48 968
levamisole, 10 mM	30	17 414	40.3	43 116
Na_2MoO_4, 10 mM	30	3 831	6.1	62 264
Na_2WO_4, 10 mM	30	4 164	6.7	61 736
$NaVO_3$, 10 mM	30	11 681	24.4	47 764

[a] Cytosol prepared in BSM buffer was incubated with 30 nM (liver) or 50 nM (kidney) [^{3}H]triamcinolone acetonide at 0–4 °C. Nonspecific binding was determined by incubating parallel tubes with a 100-fold excess of unlabeled steroid. Inhibitors were added at the indicated concentrations, and samples were heat activated at 25 °C for 30 min. Each value represents an average of duplicate determinations.

Table III: Effect of Adding Phosphatase Inhibitors before and after Heat Activation on the Binding of [^{3}H]Triamcinolone Acetonide–Receptor Complexes to DNA–Cellulose[a]

additions	min at 25 °C: before addition	after addition	specifically bound [^{3}H]triamcinolone acetonide: DNA–cellulose binding cpm/100 μL of cytosol	% of total	total bound (cpm/100 μL of cytosol)
none	0		207	0.5	42 651
	30		21 691	40.5	53 522
	45		23 737	45.0	52 738
Na_2MoO_4,	0	45	8 628	24.4	35 341
10 mM	30	15	17 032	41.1	41 422
Na_2WO_4,	0	45	2 818	6.1	45 937
10 mM	30	15	15 047	30.7	48 876
$NaVO_3$,	0	45	5 849	13.6	42 799
10 mM	30	15	8 575	19.5	43 941

[a] Liver cytosol prepared in BSM buffer was incubated with 30 nM [^{3}H]triamcinolone acetonide at 0–4 °C. Nonspecific binding was determined by incubating parallel tubes with a 100-fold excess of unlabeled steroid. Labeled cytosol was incubated at 25 °C in the presence or absence of inhibitor as indicated. Each value represents an average of duplicate determinations.

complexes and not with the DNA–cellulose.

The differential effects of these compounds were also tested by examining the effect of molybdate, tungstate, and vanadate on the formation of activated [^{3}H]triamcinolone acetonide–

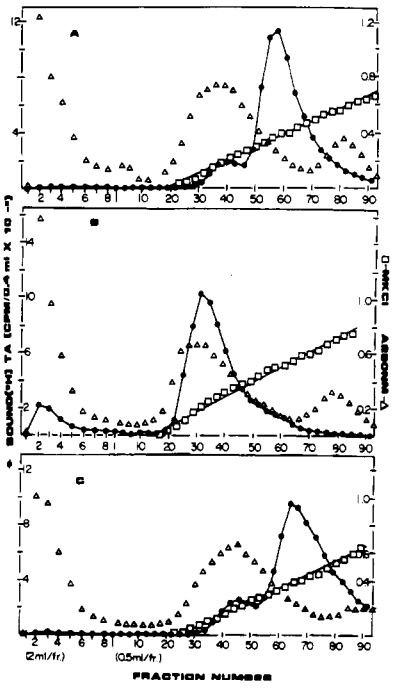

FIGURE 2: Effect of Na_2MoO_4 on formation of activated [^{3}H]-triamcinolone acetonide–receptor complexes. Liver cytosol prepared in BSM buffer was incubated with 30 nM [^{3}H]triamcinolone acetonide at 0–4 °C. Cytosol containing unactivated complexes was adjusted to 10 mM Na_2MoO_4, and 0.5 mL was applied to a DEAE-Sephadex A-50 column (panel A). An equivalent amount of labeled cytosol was incubated at 25 °C for 30 min to produce activated complexes and was adjusted to 10 mM Na_2MoO_4 prior to chromatography on DEAE-Sephadex A-50 columns (panel B). A third set of samples was adjusted to 10 mM Na_2MoO_4 and incubated at 25 °C for 30 min prior to DEAE-Sephadex A-50 chromatography (panel C). All columns were washed with 16 mL of the 0.02 M KH_2PO_4–K_2HPO_4, pH 6.8, equilibration buffer, and the wash was collected as eight 2-mL fractions. Bound complexes were eluted with a 0–0.5 M linear KCl gradient, and 0.5-mL fractions were collected. DEAE-Sephadex A-50 columns have a bed volume of 8 mL. 0.5 mL of dextran-coated charcoal was mixed with the bottom 2 mL of resin to remove unbound steroid.

receptor complexes using ion-exchange chromatography. The effect of molybdate on the formation of activated glucocorticoid–receptor complexes from liver cytosol is shown in Figure 2. Using chromatography on DEAE-Sephadex our laboratory has identified two proteins in rat liver cytosol which preferentially bind synthetic glucocorticoids (Litwack et al., 1973; Litwack & Rosenfield, 1975). Liver binder II, which binds to DEAE-Sephadex, fulfills the criteria of the glucocorticoid receptor (Litwack et al., 1973), while liver binder IB, which does not bind to DEAE-Sephadex, represents a binding protein of unknown physiological function (Litwack & Rosenfield, 1975). More recent work from this laboratory (Litwack et al., 1980) had established that the major glucocorticoid binding protein in rat kidney medulla resembles liver binder II (the glucocorticoid receptor). The activated form of the [^{3}H]triamcinolone acetonide binding protein detected in kidney cortex resembles liver binder IB in its elution position from DEAE-Sephadex and in its Stokes radius (20–25 Å) (Litwack et al., 1980). However, since it is known that glucocorticoids are capable of binding to the mineralocorticoid receptor (Pasqualini & Sumida, 1977), we cannot exclude the possibility that this peak may represent the binding of [^{3}H]-triamcinolone acetonide to the mineralocorticoid receptor. The panel of Figure 2 labeled A shows that the unactivated steriod–receptor complexes in liver cytosol elutes at a KCl concentration of ~0.4 M. A similar chromatogram is obtained

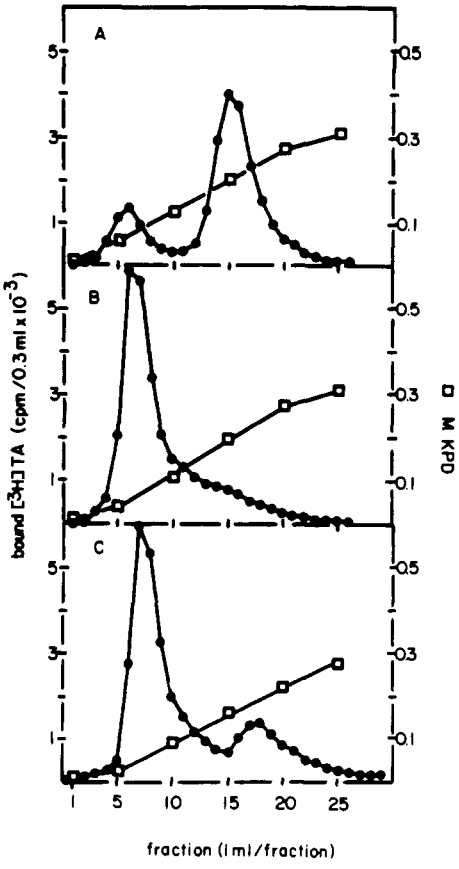

FIGURE 3: Effect of $NaVO_3$ on formation of activated [^{3}H]triamcinolone acetonide–receptor complexes. Liver cytosol prepared in BSM buffer was incubated with 30 nM [^{3}H]triamcinolone acetonide at 0–4 °C. Cytosol containing [^{3}H]triamcinolone acetonide–receptor complexes was chromatographed on DEAE-cellulose columns (3-mL bed volume). All columns were washed with 10 mL of KPD equilibration buffer, pH 7.6, and the wash was discarded. Bound complexes were eluted with a 5–400 mM potassium phosphate gradient. Thirty 1-mL fractions were collected. (A) Unactivated; (B) activated (25 °C, 30 min); (C) activated (25 °C, 30 min) in the presence of 50 mM $NaVO_3$.

for the unactivated hormone–receptor complexes formed in kidney medulla and kidney cortex (data not shown). If cytosols are heated to 25 °C for 30 min prior to chromatography on DEAE-Sephadex, the [^{3}H]triamcinolone acetonide–receptor complexes from liver (panel B) and kidney medulla elute at a KCl concentration of 0.2 M whereas the activated complexes formed in kidney cortex do not bind to the column and elute in the wash fraction (data not shown). Addition of molybdate to the cytosol prior to chromatography does not affect the elution position of either the unactivated or activated steroid–receptor complexes. Panel C shows that addition of 10 mM molybdate to the liver cytosol prior to heat treatment prevents formation of the activated complexes. Identical results were obtained for kidney medulla and kidney cortex (data not shown).

The effects of tungstate and vanadate on the formation of activated glucocorticoid–receptor complexes from liver cytosol were examined by using the DEAE-cellulose chromatography system described by Sakaue & Thompson (1977). In this system unactivated glucocorticoid–receptor complexes from liver cytosol elute at a potassium phosphate concentration of ~0.18 M, and following heat treatment the activated complexes elute at a potassium phosphate concentration of 0.05 M (Figure 3A,B). Tungstate (50 mM) prevented formation of the activated species (data not shown), whereas inclusion of 50 mM vanadate during the heat activation step has no effect (Figure 3C).

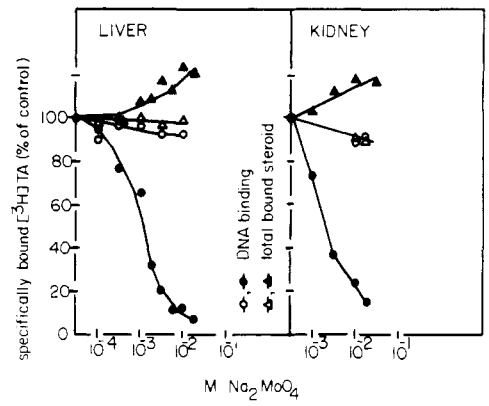

FIGURE 4: Dose–response curve for the effect of Na_2MoO_4 on activation of [^{3}H]triamcinolone acetonide at 0–4 °C. Nonspecific binding was determined in the presence of a 100-fold excess of unlabeled steroid. Cytosol containing labeled steroid–receptor complexes was incubated at 25 °C for 30 min in the presence (●, ▲) or absence (○, △) of added Na_2MoO_4. At the end of this time a set of samples (○, △) was adjusted to the appropriate concentration of Na_2MoO_4, and the incubation was continued at 25 °C for an additional 15 min. Specific binding of [^{3}H]triamcinolone acetonide to receptor (△, ▲) and specific binding of the steroid–receptor complex to DNA–cellulose (○, ●) were determined in duplicate at each concentration. Individual points represent an average of two to four separate experiments. Specifically bound steroid averaged 44 336 cpm/100 μL of liver cytosol and 48 675 cpm/100 μL of kidney cytosol for control values. Control values for hormone–receptor complexes specifically bound to DNA–cellulose average 15 030 cpm/100 μL of liver cytosol and 21 302 cpm/100 μL of kidney cytosol.

Table IV: Effect of Phosphatase Inhibitors on Activation of [^{3}H]Triamcinolone Acetonide–Receptor Complexes by Calf Intestinal Alkaline Phosphatase[a]

additions	[^{3}H]triamcinolone acetonide specifically bound to DNA-cellulose: cpm/100 μL of cytosol	% of total binding
1 mM $MgCl_2$ and 0.1 mM $ZnCl_2$	785	2.4
265 μg/mL alkaline phosphatase alone	2752	8.4
265 μg/mL alkaline phosphatase plus		
10 mM Na_2MoO_4	116	0.4
10 mM Na_2WO_4	652	2.0
10 mM NaF	2890	8.9
10 mM Na_2MoO_4	94	0.3
10 mM Na_2WO_4	664	2.0
10 mM NaF	899	2.8

[a] Liver cytosol prepared in TSM buffer was incubated with 30 nM [^{3}H]triamcinolone acetonide at 0–4 °C. Nonspecific binding was determined in the presence of a 1000-fold excess of unlabeled steroid. The indicated additions were made to the labeled cytosol, and incubation was continued at 0–4 °C for 2 h. Each value represents an average of duplicate determinations.

Dose–response curves for the effect of molybdate on the activation of [^{3}H]triamcinolone acetonide–receptor complexes from liver and kidney cytosols are presented in Figure 4. In both tissues 50% inhibition of activation is achieved between 1 and 5 mM Na_2MoO_4 and the effect is maximal at 10 mM. There is a slight but reproducible stimulation of steroid binding in the presence of molybdate which may reflect the ability of this compound to stabilize unbound glucocorticoid receptors (Nielsen et al., 1977b). Similar experiments conducted with $NaVO_3$ indicate that the effect of this compound on the binding of the activated steroid–receptor complexes to DNA–cellulose is also optimal at 10 mM (data not shown).

Table V: Effect of Phosphorylated Compounds on the Rate of Activation of [^{3}H]Triamcinolone Acetonide-Receptor Complexes[a]

additions	min at 15 °C	specifically bound [^{3}H]triamcinolone acetonide: DNA-cellulose binding cpm/100 μL of cytosol	DNA-cellulose binding % of total	total bound (cpm/100 μL of cytosol)
none	0	1112	1.6	67657
	30	3388	5.0	67376
10 mM *p*-nitrophenol	30	23166	39.5	58701
10 mM *p*-nitrophenyl phosphate	30	17604	27.6	63672
10 mM α-naphthyl phosphate	30	18408	37.8	48677
10 mM ATP	30	18396	33.1	55608
10 mM AMP	30	9539	17.0	55968
10 mM α-glycero-phosphate	30	8485	15.1	56326
10 mM β-glycero-phosphate	30	5896	10.0	58629
10 mM glucose 1-phosphate	30	3359	5.5	61326
10 mM glucose	30	3759	6.5	57100

[a] Liver cytosol prepared in TSM buffer was incubated with 30 nM [^{3}H]triamcinolone acetonide at 0–4 °C. Nonspecific binding was determined by incubating parallel tubes with a 100-fold excess of unlabeled steroid. Glucocorticoid-receptor complexes were activated by heating at 15 °C for 30 min in the presence and absence of the indicated compounds. Each value represents an average of duplicate determinations.

The experiment presented in Table IV shows that activation of liver [^{3}H]triamcinolone acetonide–receptor complexes by exogenous alkaline phosphatase is prevented by molybdate and by tungstate but is not prevented by fluoride. These data are consistent with the ability of Na_2MO_4 and Na_2WO_4 (but not NaF) to block heat-induced activation of glucocorticoid–receptor complexes (see Table II).

Effect of Phosphorylated Compounds on Rate of Activation of Glucocorticoid–Receptor Complexes. In an early series of experiments with alkaline phosphatase, we discovered that *p*-nitrophenyl phosphate, a phosphatase substrate, significantly stimulated the rate of activation of glucocorticoid–receptor complexes at 15 °C. This prompted us to investigate the effect of a variety of potential alkaline phosphatase substrates on the activation process. As shown in Table V, the most effective stimulators of activation were *p*-nitrophenyl phosphate, *p*-nitrophenol, α-naphthyl phosphate, and ATP. AMP, α-glycerophosphate, and β-glycerophosphate stimulated the rate of activation two- to threefold. Glucose 1-phosphate and glucose had no effect on the activation process.

The effect of *p*-nitrophenol on the activation of the glucocorticoid–receptor complexes was examined in more detail. The results presented in Table V suggested that *p*-nitrophenol was more potent in stimulating activation than its precursor *p*-nitrophenyl phosphate. This was confirmed by examining the dose–response curves for the two compounds. Figure 5 shows that suboptimal concentrations of *p*-nitrophenol more effectively stimulate activation of the glucocorticoid–receptor complexes than do the same concentrations of its precursor *p*-nitrophenyl phosphate. Both compounds stimulated the rate of activation equally at 15 mM. Figure 6 compares the kinetics of activation of the glucocorticoid–receptor complexes at 15 °C in the presence and absence of *p*-nitrophenol as measured

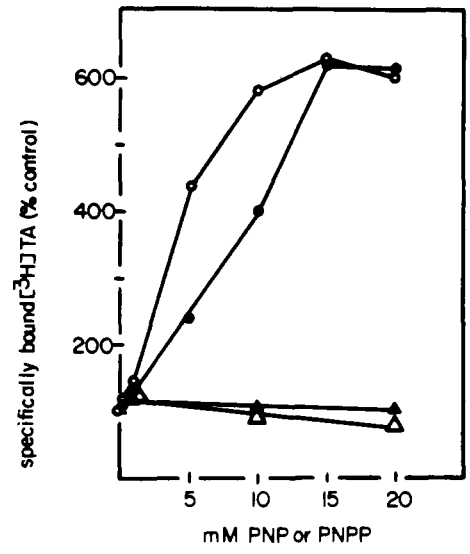

FIGURE 5: Dose–response curve for the effect of *p*-nitrophenol and *p*-nitrophenyl phosphate on activation of [^{3}H]triamcinolone acetonide–receptor complexes. Liver cytosol prepared in TSM buffer was incubated with 30 nM [^{3}H]triamcinolone acetonide in the presence and absence of a 100-fold excess of unlabeled steroid. Activation of the hormone–receptor complex in the presence of *p*-nitrophenol (○, △) or *p*-nitrophenyl phosphate (●, ▲) was examined by incubation at 15 °C for 30 min. The specific binding of [^{3}H]triamcinolone acetonide to receptor (△, ▲) and specific binding of the hormone–receptor complexes to DNA–cellulose (○, ●) were determined in duplicate. Values represent an average of two separate experiments. In control cytosol the specifically bound steroid averaged 73615 cpm/100 μL of cytosol, and the average for hormone–receptor complexes specifically bound to DNA–cellulose was 4116 cpm/100 μL of cytosol.

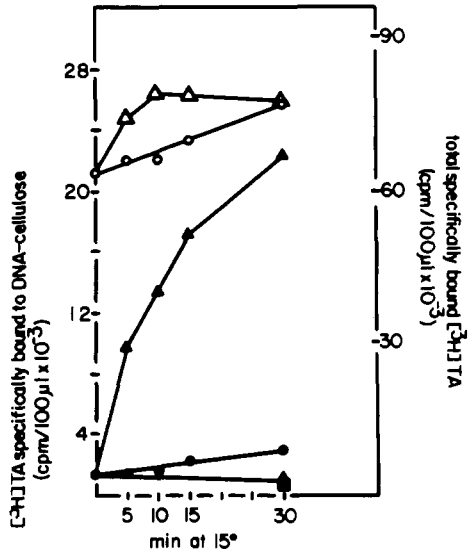

FIGURE 6: Kinetics of activation of [^{3}H]triamcinolone acetonide–receptor complexes by *p*-nitrophenol. Liver cytosol prepared in TSM buffer was incubated with [^{3}H]triamcinolone acetonide in the presence and absence of a 100-fold excess of unlabeled steroid. Following formation of the hormone–receptor complexes, samples were adjusted as follows and incubated at 15 °C: 10 mM *p*-nitrophenol (△, ▲); 10 mM Na_2MoO_4 (■); 10 mM *p*-nitrophenol plus 10 mM Na_2MoO_4 (♦); no additions (○, ●). At the indicated time periods, the specific binding of [^{3}H]triamcinolone acetonide to receptor (○, △) and the specific binding of the steroid–receptor complexes to DNA–cellulose (▲, ■, ◆, and ●) were determined in duplicate.

by DNA–cellulose binding. At this temperature there is a twofold stimulation of activation after 30 min in the absence of *p*-nitrophenol. Addition of 10 mM *p*-nitrophenol during heat treatment results in a 13-fold increase over the non-

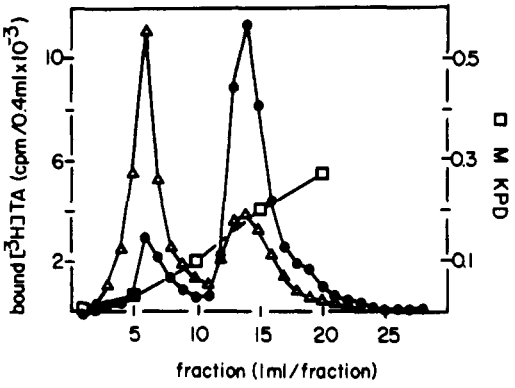

FIGURE 7: Effect of *p*-nitrophenol on formation of activated [^{3}H]-triamcinolone acetonide–receptor complexes. Liver cytosol prepared in TSM buffer was incubated with 30 nM [^{3}H]triamcinolone acetonide at 0–4 °C. The labeled complexes were activated in the absence (●) or presence (△) of 10 mM *p*-nitrophenol by incubating at 15 °C for 30 min. Labeled cytosol was treated with dextran-coated charcoal, and 0.3 mL was applied to a DEAE-cellulose column (3-mL bed volume). The columns were washed with 10 mL of KPD equilibration buffer, pH 7.6, and the wash was discarded. Bound complexes were eluted with a 5–400 mM linear potassium phosphate gradient. Thirty 1-mL fractions were collected.

heat-treated form. Activation effected in the presence of *p*-nitrophenol is completely blocked by inclusion of an equimolar concentration of either Na_2MoO_4 or Na_2WO_4 (data not shown). Figure 7 presents the DEAE-cellulose chromatogram of glucocorticoid–receptor complexes activated at 15 °C in the presence and absence of 10 mM *p*-nitrophenol. This experiment demonstrates that *p*-nitrophenol stimulates the formation of activated glucocorticoid–receptor complexes and that it is not not simply enhancing the binding of the unactivated complexes to DNA–cellulose.

The published reports that many of the treatments which affect activation of hormone–receptor complexes (heat, high salt, dilution, and molybdate) have similar effects on inactivation of unbound receptor suggested that compounds which stimulate the rate of activation of glucocorticoid–receptor complexes might also increase the rate at which steroid binding capacity is lost from unlabeled cytosol. An experiment to test this hypothesis is presented in Figure 8. At 25 °C the steroid binding capacity of unlabeled cytosol is reduced to 50% in 60 min. The addition of 10 mM *p*-nitrophenol markedly stimulates the rate of inactivation of the unbound receptor, and binding capacity is totally lost by 1 h. *p*-Nitrophenyl phosphate also increases the rate of inactivation of steroid binding capacity, but, as already shown for activation of glucocorticoid–receptor complexes, it is less effective than *p*-nitrophenol. As previously shown for the effects on activation, an equimolar concentration of Na_2MoO_4 completely blocks the effect of both compounds on the inactivation of the unbound receptor. Similar effects on heat inactivation of unbound receptor are seen with α-naphthyl phosphate and β-naphthyl phosphate. Neither α-glycerophosphate nor β-glycerophosphate has an effect on heat inactivation of unbound receptor (data not shown).

The effects of *p*-nitrophenol and *p*-nitrophenyl phosphate on activation of [^{3}H]triamcinolone acetonide–receptor complexes from whole kidney cytosol were also examined, and the results are presented in Table VI. As with liver cytosol, the rate of activation of the steroid–receptor complexes is stimulated by both compounds, and the effect is blocked by equimolar concentrations of Na_2MoO_4. Unlike the liver glucocorticoid–receptor complexes, the [^{3}H]triamcinolone acetonide–receptor complexes from kidney cytosol are markedly

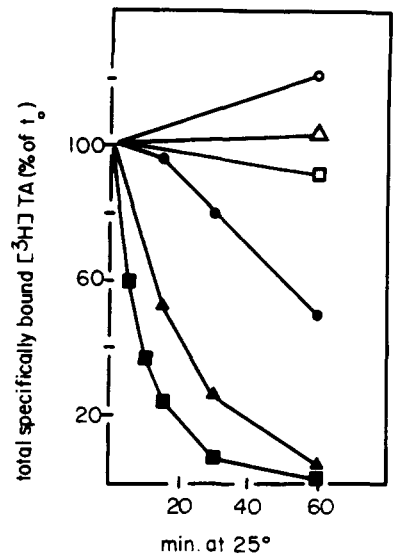

FIGURE 8: Heat denaturation of unbound glucocorticoid receptors in the presence of *p*-nitrophenyl phosphate and *p*-nitrophenol. Liver cytosol prepared in TSM buffer was adjusted as follows: no additions (●); 10 mM *p*-nitrophenyl phosphate (▲); 10 mM *p*-nitrophenol (■); 10 mM Na_2MoO_4 (○); 10 mM *p*-nitrophenyl phosphate plus 10 mM Na_2MoO_4 (△); 10 mM *p*-nitrophenol plus 10 mM Na_2MoO_4 (□). The cytosol, containing unbound receptor, was incubated at 25 °C for the indicated time periods. Steroid binding capacity of the heat-treated cytosol was determined by incubation with 30 nM [^{3}H]triamcinolone acetonide in the presence and absence of a 100-fold excess of unlabeled steriod at 0–4 °C for 1.5 h. Each value was determined in duplicate, and the data presented represent an average of two or three separate experiments. The averages for the control value for each treatment (given as cpm/100 μL of cytosol) are as follows: no addition, 58 150; 10 mM *p*-nitrophenyl phosphate, 76 169; 10 mM *p*-nitrophenol, 75 275; 10 mM Na_2MoO_4, 58 801; 10 mM *p*-nitrophenyl phosphate plus 10 mM Na_2MoO_4, 70 294; 10 mM *p*-nitrophenol plus 10 mM Na_2MoO_4, 79 155.

Table VI: Effect of *p*-Nitrophenol and *p*-Nitrophenyl Phosphate on Activation of [^{3}H]Triamcinolone Acetonide–Receptor Complexes in Kidney Cytosol[a]

		specifically bound [^{3}H]triamcinolone acetonide		
		DNA–cellulose binding		total bound
additions	min at 15 °C	cpm/100 μL of cytosol	% of total	(cpm/100 μL of cytosol)
none	0	951	3.3	28 440
	30	9 772	36.3	26 878
Na_2MoO_4, 10 mM	30	183	0.5	37 604
p-nitrophenyl phosphate, 10 mM	30	16 581	71.6	23 132
plus Na_2MoO_4, 10 mM	30	342	1.3	25 659
p-nitrophenol, 10 mM	30	18 223	88.9	20 503
plus Na_2MoO_4, 10 mM	30	1 433	5.8	24 533

[a] Kidney cytosol prepared in TSM buffer was incubated with 50 nM [^{3}H]triamcinolone acetonide at 0–4 °C. Nonspecific binding was determined by incubating parallel tubes in the presence of a 100-fold excess of unlabeled steroid. [^{3}H]Triamcinolone acetonide–receptor complexes were activated by incubating at 15 °C for 30 min in the presence and absence of the indicated compounds. Each value represents an average of duplicate determinations.

activated in 30 min at 15 °C in the absence of the added compounds. Whether this is a reflection of differences in

kidney and liver cytosol or is an inherent difference in the activation of the glucocorticoid receptor which is specific for kidney is not known.

Discussion

Activation of glucocorticoid–receptor complexes involves a change to a more positively charged macromolecule with no apparent change in molecular weight (Litwack et al., 1980). Activation of the hormone–receptor complex in in vitro is stimulated by heat, salt, dilution (gel filtration), and various chemical treatments (Baxter et al., 1972: Milgrom et al., 1973; Higgins et al., 1973; Kalimi et al., 1975; Goidl et al., 1977; Cake & Litwack, 1978; Bailly et al., 1978; Litwack et al., 1980; Sakaue & Thompson, 1977). The data presented in this paper are consistent with the idea that dephosphorylation of the glucocorticoid–receptor complex or of some regulatory component(s) is involved in the activation mechanism. Two lines of evidence support this concept. First, the rate of activation of glucocorticoid–receptor complexes is stimulated by incubation with calf intestinal alkaline phosphatase (Table I, Figure 1). Second, the formation of activated complexes is blocked by molybdate and tungstate (Table II, Table III, Figure 2, and Figure 3), both of which are potent phosphatase inhibitors (Paigen, 1958; VanEtten et al., 1974; Lopez et al., 1976; Rothstein & Meier, 1949; Nordlie & Arion, 1964; Paietta & Sands, 1978; Japundzic et al., 1978; Roberts & Bazer, 1976). Although a number of phosphorylated compounds which are potential phosphatase substrates also increase the rate of activation at 15 °C (Table V), these compounds may be exerting their effects through a mechanism(s) which is not directly related to dephosphorylation.

Clearly, the ability of exogenous calf intestinal alkaline phosphatase to stimulate the rate of activation of preformed glucocorticoid–receptor complexes (Figure 1, Table I) is consistent with a dephosphorylation mechanism. However, two lines of evidence suggest that endogenous activation may be the result of a phosphoprotein phosphatase enzyme rather than an alkaline phosphatase enzyme. First, although sodium molybdate and sodium tungstate block heat-induced activation, 10 mM arsenate and 10 mM levamisole, which are specific inhibitors of alkaline phosphatases (Fernley, 1971), do not prevent endogenous activation (Table II). Second, relatively high nonphysiological concentrations of the exogenous calf intestinal alkaline phosphatase are required to stimulate activation. (Activation by the exogenous alkaline phosphatase is not observed in the presence of 1 mM arsentate, a potent competitive inhibitor of the enzyme.) Recent studies report that a variety of purified alkaline phosphatases secondarily dephosphorylate phosphoproteins (Mellgren et al., 1977; Huang et al., 1976). Our observations with calf intestinal alkaline phosphatase are consistent with the idea that activation of the glucocorticoid–receptor complexes by the exogenous enzyme is due to associated phosphoprotein phosphatase activity.

Multiple forms of soluble phosphoprotein phosphatase activity have been described in a variety of tissues (Ullman & Perlman, 1975a,b; Li et al., 1978, 1979; Kato & Sato, 1974; Nakai & Thomas, 1974; Kato & Bishop, 1972). In some cases these activities have been shown to be inhibited by molybdate and tungstate (Japundzic et al., 1978; Roberts & Bazer, 1976). Although most of these enzymes when partially purified are inhibited to some extent by fluoride, the degree of inhibition varies depending on the substrate and on the extent of purification of the enzyme (Nakai & Thomas, 1974; Khandelwal, 1977; Roberts & Bazer, 1976). It is possible that our failure to detect significant inhibition of activation with NaF is the result of working with unfractionated cytosols. Preliminary experiments suggest that 100 mM NaF inhibits the rate of activation of glucocorticoid–receptor complexes approximately 20–30% (25 °C, 30 min).

Leach et al. (1979) have shown that molybdate reversibly inhibits heat activation of glucocorticoid–receptor complexes and heat inactivation of unbound receptor. Using both DNA–cellulose binding assays and ion-exchange chromatography, we have shown that molybdate and tungstate prevent the formation of activated glucocorticoid–receptor complexes (Table III, Figure 2) whereas vanadate prevents binding of the activated complexes to DNA–cellulose (Table III, Figure 3). Experiments not presented show that at a concentration of 10 mM both molybdate and tungstate prevent heat inactivation of the unbound receptor whereas vanadate is ineffective. This is in contrast to the report by Leach et al. (1979) that tungstate does not prevent inactivation of unbound receptor. The effects of these compounds are not limited to activation of glucocorticoid receptors. Nishigori & Toft (1979, 1980) have reported that heat activation of progesterone–receptor complexes from chick oviduct is prevented by molybdate, tungstate, and vanadate. Using sucrose density gradient centrifugation, Nishigori & Toft (1980) clearly demonstrate that molybdate blocks formation of the activated species; however, similar experiments with vanadate are not reported. Therefore, it is not clear whether vanadate prevents formation of the activated progesterone–receptor complex or whether, as observed with the glucocorticoid–receptor complex, it simply prevents binding of the activated species to DNA–cellulose.

The precise mechanism by which molybdate, tungstate, and vanadate exert their effects on steroid–receptor complexes is not known. All three compounds are potent phosphatase inhibitors (Paigen, 1958; VanEtten et al., 1974; Lopez et al., 1976; Rothstein & Meier, 1949; Nordlie & Arion, 1964; Paietta & Sands, 1978; Gibbons et al., 1978; Bond & Hudgins, 1979; Wallick et al., 1979), and all three are capable of forming complexes with inorganic phosphate at acidic pH (Cotton & Wilkinson, 1972). However, despite their physicochemical similarities vanadate does not prevent formation of activated glucocorticoid–receptor complexes. Leach et al. (1979) have presented data which suggest that molybdate may block activation by binding to sulfhydryl or phosphate moieties on the receptor protein. By binding to phosphate groups within the receptor molecule, molybdate (and presumably tungstate) could block a dephosphorylation which may be required for activation of the complex. A second possibility, suggested by Nishigori & Toft (1980), is that molybdate (and tungstate) may exert its effect through an interaction with polypeptide amino acid residues. Molybdate has been shown to interact with the thiol group of cysteine and the imidazole group of histidine (Weathers et al., 1979). Paigen (1958) suggested that the inhibition of a mouse liver phosphoprotein phosphatase activity by molybdate was due to its interaction with heavy metal ions. A satisfactory explanation of the effects of these compounds on steroid receptors will have to take into account our observation that vanadate does not prevent formation of activated glucocorticoid–receptor complexes.

Several lines of evidence suggest that inactivation of unbound receptors and activation of glucocorticoid–receptor complexes involve similar biochemical mechanisms. Both processes are stimulated by heat, by salt, and by dilution (gel filtration). In addition, both processes are stimulated by the addition of exogenous calf intestinal alkaline phosphatase (Nielsen et al., 1977a; Table I), by theophylline (Cake & Litwack, 1978; our unpublished data), by *p*-nitrophenol (and

its precursor *p*-nitrophenyl phosphate), and by α-naphthyl phosphate and β-naphthyl phosphate (this report). The effects of all these treatments are blocked by molybdate. Tungstate has not been tested in every case, but preliminary results indicate tungstate will be equally effective with respect to the various chemical treatments.

Our studies with low molecular weight phosphorylated compounds indicate that control of the various active and inactive states of the glucocorticoid receptor is more complex than originally believed. One group of compounds stimulates the rate of activation of glucocorticoid–receptor complexes and accelerates the rate of inactivation of unbound receptor. A second group of compounds stimulates the rate of activation of the hormone–receptor complex but either has no effect on inactivation of unbound receptor or has a protective effect. We have shown that *p*-nitrophenol stimulates the rate of activation of glucocorticoid–receptor complexes (Figures 5–7) and accelerates the rate of inactivation of unbound receptors (Figure 8). Similar effects are observed with its precursor *p*-nitrophenyl phosphate (Figures 5 and 8), with α-naphthyl phosphate, and with β-naphthyl phosphate (Table V and unpublished data). One explanation consistent with these results is that these compounds activate endogenous phosphatase activity. We recognize that substrates and end products usually inhibit enzymatic activity, and, in fact, both competitive inhibition of a phosphoprotein phosphatase activity by *p*-nitrophenyl phosphate (Khandelwal, 1977) and substrate inhibition of alkaline phosphatase activity (VanBelle, 1972) have been reported. However, studies by Adamich & Dennis (1978), Adamich et al. (1978, 1979), and Roberts et al. (1979) provide a precedent for the idea that enzymatic activity can be stimulated by both substrates and end products. These authors demonstrate quite convincingly that cobra venom phospholipase A_2 can be allosterically activated by both substrates and end products as well as by nonsubstrate lipids. It is also possible that the compounds employed in the present study (*p*-nitrophenyl phosphate, α-naphthyl phosphate, and β-naphthyl phosphate) are not substrates for the endogenous phosphatase involved in activation of steroid–receptor complexes. Several laboratories have reported that highly purified preparations of phosphoprotein phosphatase from various tissues do not exhibit activity toward low molecular weight phosphoesters (Li et al., 1978; Khandelwal et al., 1976; Chou et al., 1976; Detweiler et al., 1977; Titanji, 1977). Alternatively, it is possible that these compounds may exert their effects directly by interacting with the receptor or indirectly by interacting with nonreceptor components (other than a phosphatase) which regulate the various active and inactive states of the receptor.

Our results concerning the stimulation of activation by ATP, α-glycerophosphate, and β-glycerophosphate (Table V) are more difficult to reconcile with a dephosphorylation mechanism. Sando et al. (1979a) have shown that ATP protects unbound receptor against heat inactivation, and experiments in our own laboratory indicate that α-glycerophosphate and β-glycerophosphate have no effect on heat inactivation of unbound receptor (25 °C, 10 mM; data not shown). The fact that ATP stimulates activation raises the possibility that phosphorylation of some component(s) of the receptor activating system may also be involved in the transformation of the hormone–receptor complex to a DNA-binding form. The coordination of both phosphorylation and dephosphorylation reactions in the control of glycogen metabolism is well documented (Cohen, 1978), and it is conceivable that activation of glucocorticoid–receptor complexes involves both phosphorylation and dephosphorylation of different components of the activation complex. John & Moudgil (1979) have suggested that ATP-mediated stimulation is the result of an allosteric transformation which occurs when ATP combines with a specific nucleotide binding site on the receptor. Although we cannot exclude the possibility that ATP is exerting its effect in this manner, our results clearly show that stimulation of the rate of activation is not a specific nucleotide effect but rather it is a more general phenomenon which is observed with a variety of phosphorylated compounds.

The evidence presented by Pratt's group (Nielson et al., 1977a,b; Sando et al., 1979a,b; Leach et al., 1979) in conjunction with our own data is consistent with the idea that either the receptor or some component(s) involved in the regulation of the various active and inactive states of the receptor must be phosphorylated in order for steroid binding to occur and is dephosphorylated during activation. Experiments are in progress to determine whether exogenous calf intestinal alkaline phosphatase is dephosphorylating the glucocorticoid–receptor complexes directly or whether it is modifying another cytosolic component(s) which regulates the activation process. It is probable that this regulation of the various active and inactive states is not peculiar to glucocorticoid receptors. The activation of the [^{3}H]triamcinolone acetonide binding protein of kidney cortex (which may be the mineralocorticoid receptor) is blocked by molybdate (Figure 2) and is stimulated by calf intestinal alkaline phosphatase (data not shown). Molybdate has been reported to stabilize unbound androgen receptors in rat prostate (Tremblay et al., 1979) and vitamin D receptors in rat intestinal mucosa (McCain et al., 1979), in addition to its ability to prevent activation of progesterone–receptor complexes from avian oviduct (Nishigori & Toft, 1979, 1980). 1980). Preliminary data from our laboratory indicate that calf intestinal alkaline phosphatase stimulates the rate of activation of androgen receptors from rat seminal vesicle and estrogen and progesterone receptors from rat uterus. The studies reported in this paper emphasize the complexity of the control mechanisms regulating the active and inactive states of glucorticoid receptors and suggest that both dephosphorylation and phosphorylation may be involved at various steps of the process. The discovery that small molecular weight phosphoesters have differential effects on inactivation of unbound receptors provides a probe for differentiating the biochemical steps involved in the regulation of the glucocorticoid receptors.

References

Adamich, M., & Dennis, E. A. (1978) *Biochem. Biophys. Res. Commun. 80*, 424–428.

Adamich, M., Voss, H. F., & Dennis, E. A. (1978) *Arch. Biochem. Biophys. 189*, 417–423.

Adamich, M., Roberts, M. F., & Dennis, E. A. (1979) *Biochemistry 18*, 3308–3314.

Alberts, B., & Herrick, G. (1971) *Methods Enzymol. 21*, 198–217.

Bailly, A., Savouret, J.-F., Sallas, N., & Milgrom, E. (1978) *Eur. J. Biochem. 88*, 623–632.

Baxter, J. D., Rousseau, G. G., Benson, M. L., Garcea, R. L., Ito, J., & Tomkins, G. M (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 1892–1896.

Beato, M., & Feigelson, P. (1972) *J. Biol. Chem. 247*, 7890–7896.

Bond, G. H., & Hudgins, P. M. (1979) *Biochemistry 18*, 325–331.

Cake, M. H., & Litwack, G. (1978) *Eur. J. Biochem. 82*, 97–103.

Chou, C.-K., Alfano, J., & Rosen, O. M. (1976) *J. Biol. Chem. 252*, 2855–2859.
Cohen, P. (1978) *Curr. Top. Cell. Regul. 14*, 118–196.
Cotton, F. A., & Wilkinson, F. R. S. (1972) *Advanced Inorganic Chemistry*, p 950, Wiley, New York.
Dayan, J., & Wilson, I. B. (1964) *Biochim. Biophys. Acta 81*, 620–623.
Detweiler, T. C., Gratecos, D., & Fisher, E. H. (1977) *Biochemistry 16*, 4818–4823.
Fernley, H. N. (1971) *Enzymes, 3rd Ed. 4*, 417–447.
Gibbons, I. R., Cosson, M. P., Evans, J. A., Gibbons, B. H., Houck, B., Martinson, K. H., Sale, W. S., & Tang, W.-J. Y. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 2220–2224.
Goidl, J. A., Cake, M. H., Dolan, K. P., Parchman, L. G., & Litwack, G. (1977) *Biochemistry 16*, 2125–2130.
Higgins, S. J., Rousseau, G. G., Baxter, J. D., & Tomkins, G. M. (1973) *J. Biol. Chem. 248*, 5866–5872.
Huang, K.-P., Robinson, J. C., & Chou, J.-Y. (1976) *Biochem. Biophys. Res. Commun. 70*, 186–192.
Ishii, D., Pratt, W. B., & Aronow, L. (1972) *Biochemistry 11*, 3896–3904.
Japundzic, I. P., Mimic-Oka, J. I., & Japundzic, M. M. (1978) *Biochimie 60*, 489–498.
John, J. K., & Moudgil, V. K. (1979) *Biochem. Biophys. Res. Commun. 90*, 1242–1248.
Kalimi, M., Colman, P., & Feigelson, P. (1975) *J. Biol. Chem. 250*, 1080–1086.
Kato, K., & Bishop, J. S. (1972) *J. Biol. Chem. 247*, 7420–7429.
Kato, K., & Sato, S. (1974) *Biochim. Biophys. Acta 358*, 299–307.
Khandelwal, R. L. (1977) *Biochim. Biophys. Acta 485*, 379–390.
Khandelwal, R. L., Vandenheede, J. R., & Krebs, E. G. (1976) *J. Biol. Chem. 251*, 4850–4858.
Leach, K. L., Dahmer, M. K., Hammond, N. D., Sando, J. J., & Pratt, W. B. (1979) *J. Biol. Chem. 254*, 11884–11890.
Li, H.-C., Hsiao, K.-J., & Chan, W. W. S. (1978) *Eur. J. Biochem. 84*, 215–225.
Li, H.-C., Hsiao, K.-J., & Sampathkumar, S. (1979) *J. Biol. Chem. 254*, 3358–3374.
Litwack, G., & Rosenfield, S. A. (1975) *J. Biol. Chem. 250*, 6799–6805.
Litwack, G., Filler, R., Rosenfield, S. A., Lichtash, N., Wishman, C. A., & Singer, S. (1973) *J. Biol. Chem. 248*, 7481–7486.
Litwack, G., Schmidt, T. J., Markovič, R. D., Eisen, H. J., Barnett, C. A., Disorbo, D. M., & Phelps, D. S. (1980) in *Perspectives in Steroid Receptor Research* (Bresciani, F., Ed.) pp 113–131, Raven Press, New York.
Lopez, V., Stevens, T., & Lindquist, R. N. (1976) *Arch. Biochem. Biophys. 175*, 31–38.
McCain, T. A., Hirst, M. A., Chen, T. L., & Feldman, D. (1979) *Clin. Res. 27*, 86A.
Mellgren, R. L., Slaughter, G. R., & Thomas, J. A. (1977) *J. Biol. Chem. 252*, 6082–6089.
Milgrom, E., Atger, M., & Baulieu, E.-E. (1973) *Biochemistry 12*, 5198–5205.
Munck, A., & Brinck-Johnsen, T. (1968) *J. Biol. Chem. 243*, 5556–5565.
Munck, A., & Foley, R. (1979) *Nature (London) 278*, 752–754.
Munck, A., Wira, C., Young, D. A., Mosher, K. M., Hallahan, C., & Bell, P. A. (1972) *J. Steroid Biochem. 3*, 567–578.
Nakai, C., & Thomas, J. A. (1974) *J. Biol. Chem. 249*, 6459–6467.
Nielsen, C. J., Sando, J. J., & Pratt, W. B. (1977a) *Proc. Natl. Acad. Sci. U.S.A. 74*, 1398–1402.
Nielsen, C. J., Sando, J. J., Vogel, W. M., & Pratt, W. B. (1977b) *J. Biol. Chem. 252*, 7568–7578.
Nishigori, H., & Toft, D. (1979) *Proc. Annu. Meet. Endocr. Soc., 61st*, Abstr. 791.
Nishigori, H., & Toft, D. (1980) *Biochemistry 19*, 77–83.
Nordlie, R. C., & Arion, W. J. (1964) *J. Biol. Chem. 239*, 1680–1685.
Norman, M. R., & Thompson, E. B. (1977) *Cancer Res. 37*, 3785–3791.
Paietta, E., & Sands, H. (1978) *Biochim. Biophys. Acta 523*, 121–132.
Paigen, K. (1958) *J. Biol. Chem. 233*, 388–394.
Parchman, L. G., & Litwack, G. (1977) *Arch. Biochem. Biophys. 183*, 374–382.
Pasqualini, J. R., & Sumida, C. (1977) *Mod. Pharmacol.-Toxicol. 8*, 399–511.
Roberts, M. F., Adamich, M., Robson, R. J., & Dennis, E. A. (1979) *Biochemistry 18*, 3301–3308.
Roberts, R. M., & Bazer, F. W. (1976) *Biochem. Biophys. Res. Commun. 68*, 450–455.
Rothstein, A., & Meier, R. (1949) *J. Cell. Comp. Physiol. 34*, 97–114.
Sakaue, Y., & Thompson, E. B. (1977) *Biochem. Biophys. Res. Commun. 77*, 533–541.
Sando, J. J., La Forest, A. C., & Pratt, W. B. (1979a) *J. Biol. Chem. 254*, 4772–4778.
Sando, J. J., Hammond, N. D., Stratford, C. A., & Pratt, W. B. (1979b) *J. Biol. Chem. 254*, 4779–4789.
Titanji, V. P. K. (1977) *Biochim. Biophys. Acta 481*, 140–151.
Tremblay, R. R., Gaubert, C.-M., & Dubé, J. Y. (1979) *Proc. Annu. Meet. Endocr. Soc., 61st*, Abstr. 807.
Ullman, B., & Perlman, R. L. (1975a) *Biochem. Biophys. Res. Commun. 63*, 424–431.
Ullman, B., & Perlman, R. L. (1975b) *Biochim. Biophys. Acta 403*, 393–411.
VanBelle, H. (1972) *Biochim. Biophys. Acta 289*, 158–168.
VanEtten, R. L., Waymack, P. P., & Rehkop, D. M. (1974) *J. Am. Chem. Soc. 96*, 6782–6785.
Wallick, E. T., Lane, L. K., & Schwartz, A. (1979) *J. Biol. Chem. 254*, 8107–8109.
Weathers, B. J., Grate, J. H., & Schrauzer, G. N. (1979) *J. Am. Chem. Soc. 101*, 917–924.
Wilson, I. B., Dayan, J., & Cyr, K. (1964) *J. Biol. Chem. 239*, 4182–4185.
Zittle, C. A., & Della Monica, E. S. (1950) *Arch. Biochem. Biophys. 26*, 112–121.