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Effect of Pyridoxal Phosphate on the DNA Binding Site of Activated Hepatic Glucocorticoid Receptor*

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The binding of rat liver glucocorticoid·receptor complexes to DNA-cellulose and nuclei has been studied after activation of the complexes by heating. Subsequent exposure to pyridoxal 5′-phosphate or pyridoxal markedly inhibited this binding. In one system 0.75 mM pyridoxal 5′-phosphate or 6.5 mM pyridoxal gave 50% inhibition. Pyridoxamine 5′-phosphate, pyridoxamine, and pyridoxine did not inhibit significantly. The inhibition by pyridoxal 5′-phosphate is competitive with respect to DNA suggesting that its effect is directly on the DNA binding site of the activated receptor. The inhibition of DNA-cellulose binding by pyridoxal 5′-phosphate can be reversed by treatment with dithiothreitol or by gel filtration, but not if the modified receptor is first reduced using sodium borohydride.

These results suggest that pyridoxal 5′-phosphate acts by forming a Schiff base of an ϵ-NH_2 of a lysine which may be 1 residue appearing on the surface of the steroid·receptor complex upon activation. However, since pretreatment of the DNA-cellulose with the intercalating drug ethidium bromide also inhibits activated receptor binding, we conclude that the binding of the receptor involves more than electrostatic interactions between receptor positive charges and DNA phosphate groups.

In order to bind to nuclei (1–5), chromatin (6), or purified DNA (1, 3, 7) the glucocorticoid·receptor complex must undergo a physiochemical alteration called "activation." *In vitro*, this activation can be brought about by temporary exposure to heat (1–4), higher ionic strength (2, 3, 7), calcium ions (7), or theophylline (8). In addition, the steroid·receptor complex can be activated by dilution or by subjecting it to gel filtration (9–11). The exact chemical nature of this activation process is presently unknown. Activation of heat does not induce any change in the size of the glucocorticoid receptor as judged by sedimentation behavior in sucrose gradients (7) and by chromatographic behavior on Sepharose 4B (12). There is, however, a change in the isoelectric point of the receptor upon heat activation although there is disagreement as to the nature of this change (7, 12). A conformational change appears to be necessary for nuclear binding and this involves exposure of positively charged regions on the surface of the receptor, resulting in an increase in the affinity of the steroid·receptor complex for a variety of polyanions (3, 7). These include not only DNA (3, 6, 7, 13) but other polyanions such as carboxymethyl- and sulfopropyl-Sephadex (3, 12) and phosphocellulose (7, 14).

In an effort to understand further the chemical changes induced by activation of the cytoplasmic receptor protein, we have investigated the effect of various chemical reagents which can inhibit binding of the activated steroid·receptor complex to DNA. In a preliminary communication (15) we have reported that exposure to pyridoxal 5′-phosphate inhibits the binding of the activated receptor to DNA-cellulose. We have extended this study and data presented in this report suggest that pyridoxal 5′-phosphate acts by forming a Schiff base of an ϵ-NH_2 of a lysine which may be 1 residue appearing on the surface of the steroid·receptor complex upon activation. However, experiments with the intercalating drug ethidium bromide suggest that the binding of the activated glucocorticoid receptor to DNA involves more than just electrostatic interactions.

MATERIALS AND METHODS

Animals—Adrenalectomized rats of the Sprague-Dawley and Wistar strains were used 4 to 14 days following surgery. They were fed a normal diet and given 0.9% NaCl to drink.

Isotopes and Chemicals—[^{3}H]Dexamethasone[1] (26 Ci/mmol) was obtained from the Radiochemical Centre (Amersham). [1,2,4-^{3}H]-Triamcinolone acetonide (33.7 Ci/mmol) was obtained from New England Nuclear Corp. Pyridoxal, pyridoxal 5′-phosphate, pyridoxine, pyridoxamine 5′-phosphate, ethidium bromide, dexamethasone, dithiothreitol, and activated charcoal were obtained from Sigma Chemical Co. DNA-cellulose (1.0 mg of native calf thymus DNA/ml of cellulose) was purchased from P-L Biochemicals, Inc. and Dextran T-500 and Sephadex G-25 were obtained from Pharmacia. Phosphocellulose (Cellex P) was obtained from Bio-Rad Laboratories and 3,5-diaminobenzoic acid, dihydrochloride, was purchased from Eastman Kodak Co.

Specific Cytosol Binding of Dexamethasone or Triamcinolone Acetonide—Animals were killed by decapitation and the livers perfused *in situ* with cold 0.145 M NaCl through the portal vein. Livers were removed and homogenized in an equal volume of either Buffer TSM (50 mM Tris-HCl, pH 8.0, at 0°C, 0.25 M sucrose, 3 mM $MgCl_2$) or Buffer BSM (0.2 M boric acid, pH 8.0, at 0°C, 0.25 M sucrose, 3 mM $MgCl_2$). Cytosol was prepared as previously described (8) and incubated for 2 h at 0–4°C with 30 nM [^{3}H]dexamethasone or [^{3}H]triamcinolone acetonide. Specific macromolecular bound steroid was determined using the dextran-coated charcoal technique (16).

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[1] The trivial names used are: dexamethasone, 9-fluoro-11β,17,21-trihydroxy-16α-methylpregna-1,4-diene-3,20-dione; triamcinolone acetonide, 9-fluoro-11β,21-dihydroxy-16α,17-[1-methylethylidenebis-(oxy)]pregna-1,4-diene-3,20-dione.

Assay of Receptor Binding to DNA-cellulose and to Hepatocyte Nuclei—The DNA-cellulose consisted of native calf thymus DNA adsorbed onto a cellulose matrix. Nuclei were isolated by the method of Blobel and Potter (17). Binding of the glucocorticoid receptor to both DNA-cellulose and nuclei was determined as previously reported (18). DNA was determined by the method of Hinegardner (19) using highly polymerized calf thymus DNA as standard.

RESULTS

Pyridoxal 5'-Phosphate Inhibits Binding of Glucocorticoid Receptor to DNA-cellulose—When rat liver glucocorticoid·receptor complexes are subjected to elevated temperature they rapidly become activated and capable of binding to DNA-cellulose. However, if pyridoxal 5'-phosphate is added prior to the temperature activation step the enhanced binding to DNA-cellulose can be completely prevented (Fig. 1*B*). This diminished binding could be the result of an inhibition of activation or of binding to the DNA-cellulose. To answer this question pyridoxal 5'-phosphate was added after the heat activation step and incubation continued either at 0°C or 25°C. In both cases steroid·receptor complexes were rapidly inactivated suggesting that the effect of pyridoxal 5'-phosphate is to prevent the binding of the steroid·receptor complexes to DNA-cellulose.

In addition to this effect it was observed that pyridoxal 5'-phosphate caused a marked decrease in [^{3}H]dexamethasone binding to the receptor when incubated at 25°C (Fig. 1*A*). This was not evident if the incubation of pyridoxal 5'-phosphate with the receptor was carried out at 0°C. For this reason all further experiments involving pyridoxal 5'-phosphate employed an incubation at the lower temperature.

Specificity of Effect of Pyridoxal 5'-Phosphate—The effect of pyridoxal 5'-phosphate was concentration-dependent (Fig. 2). In one system 0.75 mM pyridoxal 5'-phosphate inhibited binding of the heat-activated receptor by 50%. Pyridoxal was also an effective inhibitor of receptor binding to DNA-cellulose but required higher concentrations; 6.5 mM gave 50% inhibition. Pyridoxamine 5'-phosphate and pyridoxamine did not inhibit receptor binding to DNA-cellulose significantly. In addition, it was shown that pyridoxine and phosphate ions were without effect (data not shown). The effectiveness of these agents to inhibit the binding of the heat-activated receptor to DNA correlates with their ability to form a Schiff base with an ϵ-NH_2 group of lysine. This suggests that a lysine may be 1 residue appearing on the surface of the steroid·receptor complex upon activation and that modification of this group impairs binding of the hormone·receptor complex to DNA.

From the data presented in Table I, it can be seen that pretreatment with pyridoxal 5'-phosphate not only inhibits binding of the heat-activated receptor to DNA-cellulose but also inhibits binding to isolated rat liver nuclei and phosphocellulose. The binding to phosphocellulose was low at this concentration of acceptor. When higher concentrations of phosphocellulose were used a much greater percentage of the activated receptor was bound, however, the inhibition of this binding by pyridoxal 5'-phosphate was much reduced (data not shown).

Pretreatment of DNA-cellulose with pyridoxal 5'-phosphate and pyridoxamine 5'-phosphate had no effect on binding of heat-activated receptor. This suggests that the effect of pyridoxal 5'-phosphate is on the receptor molecule and not an effect on components of the acceptor.

Competition between DNA-cellulose and Pyridoxal 5'-Phosphate—Pyridoxal 5'-phosphate could be acting directly on the receptor protein or, alternatively, could be inactivating a separate component present in rat liver cytosol which is necessary for binding of the complex to DNA. To definitively distinguish between these two possibilities experiments would

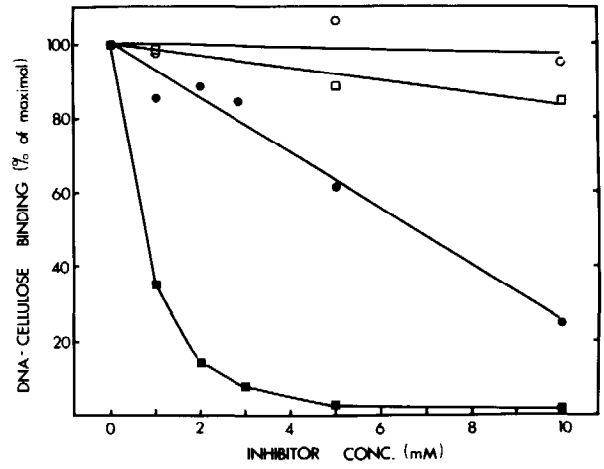

FIG. 2. Specificity of the effect of pyridoxal 5'-phosphate. A rat liver cytosol (1 g of liver + 7 ml of Buffer BSM) was incubated with 30 nM [^{3}H]dexamethasone and the steroid·receptor complexes activated by heating at 25°C for 30 min. At this point the indicated concentrations of pyridoxal 5'-phosphate (■), pyridoxal (●), pyridoxamine 5'-phosphate (□), and pyridoxamine (○) were added and incubation continued for a further 20 min. The binding of the complexes to DNA-cellulose were determined and results are expressed as the percentage of maximal binding.

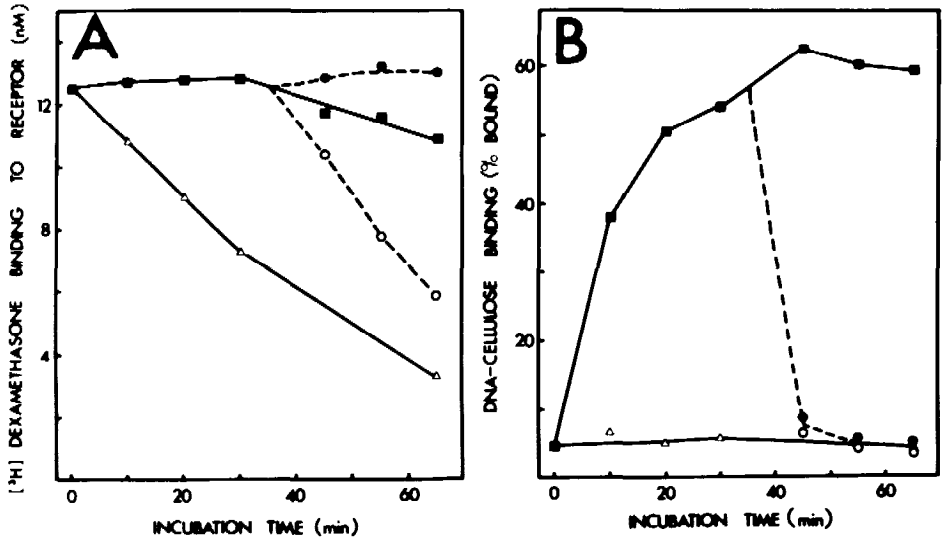

FIG. 1. Effect of pyridoxal 5'-phosphate on glucocorticoid receptor activation. Glucocorticoid·receptor complexes were formed by incubating rat liver cytosol with 30 nM [^{3}H]dexamethasone in the presence and absence of a 300-fold excess of unlabeled dexamethasone. The complexes were then heat-activated by incubation at 25°C for the times indicated in the presence (△) and absence (▲) of 10 mM pyridoxal 5'-phosphate. In addition, portions of complexes incubated for 35 min at 25°C in the absence of pyridoxal 5'-phosphate were then subjected to 10 mM pyridoxal 5'-phosphate and incubation continued at 25°C (○) or 0°C (●) for the times indicated. At each time, specific binding of [^{3}H]dexamethasone to receptor (*A*) and binding of the steroid·receptor complexes to DNA-cellulose (*B*) were determined.

need to be performed using purified receptor. However, at present there are no methods available for purification of the hepatic glucocorticoid receptor to homogeneity. Nevertheless, the data presented in Fig. 3 indicate that pyridoxal 5′-phosphate is a competitive inhibitor with respect to DNA binding and suggest that its effect is directly on the DNA binding site of the activated receptor. The effect of pyridoxal 5′-phosphate is dramatic. A DNA concentration of 0.07 g/liter is required for half-maximal binding of the glucocorticoid·receptor complex in the absence of the inhibitor. The presence of 2.5 mM pyridoxal 5′-phosphate effects the DNA binding of the complex such that 0.36 g/liter of DNA is required for half-maximal binding.

Reversibility of Inhibition by Pyridoxal 5′-Phosphate—The inhibition of DNA-cellulose binding of the activated glucocorticoid receptor by pyridoxal 5′-phosphate can be reversed by gel filtration at room temperature (Table II). This reversal can be prevented if the pyridoxal 5′-phosphate-treated sample is subjected to sodium borohydride reduction prior to gel filtration. Sodium borohydride alone has no effect on DNA-cellulose binding of the receptor. The reversal by gel filtration can also be prevented if the column is equilibrated and eluted with buffer containing 10 mM pyridoxal 5′-phosphate (data not shown). These results are in keeping with the assumption that pyridoxal 5′-phosphate inhibits DNA binding of the activated receptors by forming a Schiff base with the ϵ-NH_2 group of an essential lysine residue.

Effects of Reduction on Pyridoxal 5′-Phosphate Inhibition of Binding to DNA-cellulose under Nonactivating Conditions—In order to confirm that pyridoxal 5′-phosphate is binding to a DNA binding site which becomes unmasked as a result of the process of activation, the effect of the inhibitor was tested under nonactivating conditions (0°C). The results are shown in Table III. In these experiments, the inhibitor, control compounds, and sodium borohydride, when added, are incubated at 0°C. Subsequently, activation is carried out at 25°C for 30 min. A control experiment is also carried out in the reverse order (Experiment 9) with steroid binding and activation preceding incubation with inhibitor and then with reducing agent at 0°C. The control experiment (Experiment 1) shows that by incubating cytosol with 30 nM [^{3}H]triamcinolone acetonide for 2 h at 0°C, adding the pyridoxal-P solvent only and incubating for 20 min at 0°C and then solvent only for sodium borohydride and incubating for 10 min at 0°C, 49% of specifically bound steroid is bound to DNA-cellulose agreeing with previous results (11). When pyridoxal-P is added to the control (Experiment 4), binding of the steroid-receptor complex to DNA-cellulose is reduced from 49 to 15%. Replace-

TABLE I

Inhibition of receptor binding to DNA-cellulose, nuclei, and phosphocellulose by pyridoxal 5′-phosphate

Rat liver cytosol (1 g of liver + 1 ml of Buffer BSM) was incubated with 30 nM [^{3}H]dexamethasone for 3 h at 0°C. A portion was incubated at 25°C for 45 min to heat activate the steroid·receptor complexes. A portion of this was further incubated at 0°C for 30 min with 10 mM pyridoxal 5′-phosphate. Binding of these three fractions to DNA-cellulose, isolated rat liver nuclei, and phosphocellulose was determined. The results represent the mean ± S.E. of three separate experiments.

State of receptor	Acceptor		
	DNA-cellulose	Nuclei	Phospho-cellulose
	% bound		
Nonactivated	2.7 ± 0.4	1.0 ± 0.1	0.4 ± 0.2
Heat-activated	58.0 ± 2.8	25.9 ± 3.2	5.5 ± 0.6
Heat-activated 10 mM pyridoxal-5′-P	4.0 ± 0.8	1.4 ± 0.5	1.3 ± 0.2
Inhibition by pyridoxal-5′-P	97.6	98.4	82.4

TABLE II

Reversibility of pyridoxal 5′-phosphate inhibition by gel filtration

[^{3}]Dexamethasone-labeled cytosol was activated by heating at 25°C for 30 min and a portion further incubated at 0°C for 40 min with 10 mM pyridoxal 5′-phosphate. Half of each sample was then incubated with 50 mM sodium borohydride at 0°C for 40 min. A 400-μl aliquot of each fraction was then applied to a Sephadex G-25 minicolumn (1.5 × 6.0 cm) equilibrated and eluted at room temperature with Buffer BSM containing 30 nM [^{3}H]dexamethasone. After elution of the void volume a sample (1.6 ml) containing the macromolecules was collected. Each fraction, before and after gel filtration, was then assayed for specific cytosol binding of [^{3}H]dexamethasone and [^{3}H]-dexamethasone·receptor complex binding to a saturating amount of DNA-cellulose. The results are expressed as a percentage of receptor-bound radioactivity capable of binding to DNA-cellulose and represent the mean of duplicate experiments.

	DNA-cellulose binding	
	Before gel filtration	After gel filtration
	% bound	
Heat activated	58.9	50.9
+10 mM pyridoxal-5′-P	3.7	38.7
+10 mM pyridoxal-5′-P + 50 mM $NaBH_4$	3.4	2.8
+50 mM $NaBH_4$	52.1	58.6

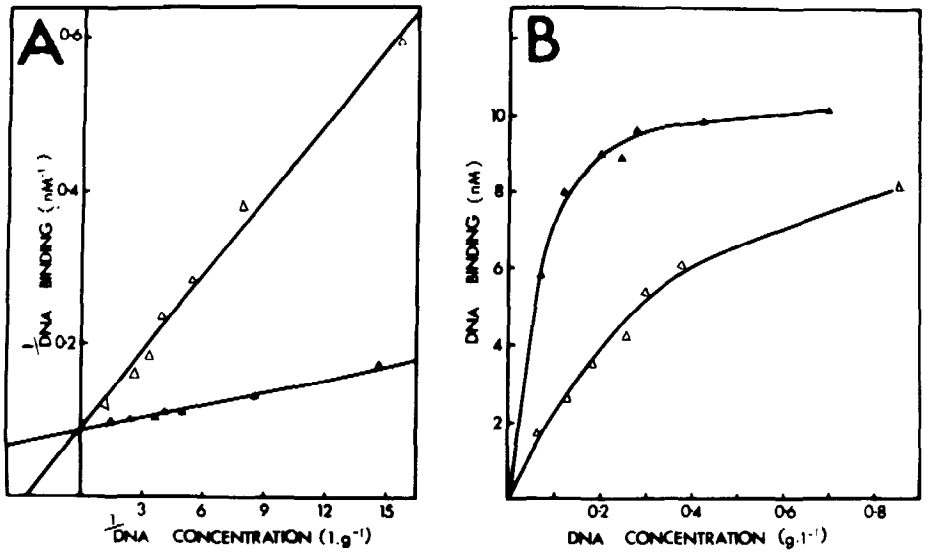

FIG. 3. Kinetics of pyridoxal 5′-phosphate inhibition. [^{3}H]Dexamethasone-labeled cytosol (1 g of liver + 1 ml of Buffer BSM) was heat-activated by incubation at 25°C for 45 min. Half was then exposed to 2.5 mM pyridoxal 5′-phosphate at 0–4°C for an additional 30 min. Aliquots of each fraction, heat-activated and then not exposed (▲) or exposed (△) to pyridoxal 5′-phosphate, were then incubated with increasing concentrations of DNA-cellulose for 4 h at 0–4°C. After thorough washing, specific binding of the steroid·receptor complexes to DNA-cellulose was determined. Results are expressed as a double reciprocal plot (*A*) or as a standard plot of DNA binding *versus* the DNA concentration (*B*). Maximal binding to DNA-cellulose of the specifically bound radioactivity is 35% in these experiments.

ment of pyridoxal-P by pyridoxamine-P produces no inhibition (Experiment 5). Addition of sodium borohydride to the steroid·receptor complex has no inhibitory effect on subsequent binding to DNA-cellulose when the entire experiment is performed at 0°C (Experiment 6). Addition of pyridoxal-P and sodium borohydride (Experiment 7) in the cold, prior to heat activation, abolishes the inhibitory effect of the coenzyme, indicating that the site to which pyridoxal-P binds is unavailable in the unactivated state of the receptor. It appears that covalent binding of excess pyridoxal-P to acceptors in the system other than the steroid·receptor complex occurs. For this reason, it is unnecessary to remove physically excess inhibitor from the system. Physical removal by the usual methods, such as gel filtration, would result in unwanted activation of the receptor (9). Other treatments, such as with alkaline phosphatase to dephosphorylate the coenzyme, could have undesirable nonspecific effects. Pyridoxamine-P in place of pyridoxal-P in the same experiment gives the expected result (Experiment 8). When a control experiment is done (Experiment 9) in which steroid is bound to receptor and activated prior to treatment with pyridoxal-P and reduction with sodium borohydride at 0°C, the inhibition of DNA binding is again evident (16% binding to DNA-cellulose equivalent to the effect of pyridoxal-P alone in Experiment 4). The best explanation of these results is that pyridoxal-P can bind only to the activated but not the unactivated form of the steroid·receptor complex. At low temperature, while the receptor remains unactivated, addition of pyridoxal-P would undoubtedly form Schiff bases with other acceptors, probably proteins, which could be stabilized by addition of sodium borohydride. Under these conditions subsequent elevation of temperature would not impede ability of activated steroid·receptor complex to bind to DNA-cellulose because previously added pyridoxal-P would be unavailable to the activated form of the receptor. Sodium borohydride is not expected to affect the aldehyde group of pyridoxal-P directly to render it inactive.

Reversibility of Pyridoxal 5'-Phosphate Inhibition by Dithiothreitol—The inhibition by pyridoxal-P was also shown to be reversed by exposure to an excess of dithiothreitol (Table IV). In like manner to the reversal by gel filtration, pretreatment with sodium borohydride prevents this reversal. This effect of dithiothreitol could be the result of an interaction of the thiol reagent with the steroid·receptor complex or of an interaction directly with pyridoxal-P. The spectrophotometric data presented in Fig. 4 imply that the latter possibility is the more likely. The specific absorption by pyridoxal-P at 390 nm in alkaline conditions is ascribed to the tautomeric

TABLE III

Effects of sodium borohydride reduction on pyridoxal 5'-phosphate inhibition of binding to DNA-cellulose under nonactivating conditions

Cytosol was incubated with [^{3}H]triamcinolone acetonide under conditions described in Table I. 5 mM pyridoxal-P was added and incubated for 20 min at 0°C. Sodium borohydride (5 mM) was added and incubated for an additional 10 min at 0°C. Activation was subsequently achieved by incubation at 25°C for 30 min. In various control experiments, the solvent was added and identical incubations performed. After activation, temperature was reduced to 0°C and binding to DNA-cellulose was for 45 min at 0°C with frequent stirring. Washings of DNA-cellulose at the end of the incubation have been described (18). The data represent the average of two experiments.

Experiment[a]	Total ligand bound	Specific ligand binding	Specific ligand binding bound to DNA-cellulose	Specifically bound ligand bound to DNA-cellulose
	dpm/ml	*dpm/ml*	*dpm/800 µl*	%
1. ^{3}H-steroid	139,700	135,800	65,900	49
2. ^{3}H-steroid + 1,000 × steroid	3,850		100	3
3. ^{3}H-steroid + 1,000 × steroid + $NaBH_4$	4,000		125	3
4. Pyridoxal-P	118,000	114,000	17,000	15
5. Pyridoxamine-P	137,000	133,000	75,000	57
6. Sodium borohydride	109,000	105,000	64,500	61
7. Pyridoxal-P + sodium borohydride	103,000	99,200	38,800	39
8. Pyridoxamine-P + sodium borohydride	107,000	103,000	65,700	63
9. ^{3}H steroid activated (25°C) to 0° incubate with pyridoxal-P, then sodium borohydride	105,200	83,200	13,600	16

[a] Experiments 2 and 3 are presented to show that sodium borohydride does not influence nonspecific binding to receptor or to DNA-cellulose.

TABLE IV

Reversibility of pyridoxal 5'-phosphate inhibition by dithiothreitol

[^{3}H]Dexamethasone-labeled cytosol was heat-activated (45 min at 25°C) and a portion treated with 10 mM pyridoxal 5'-phosphate at 0°C for 30 min. Half of each sample was then incubated with 50 mM sodium borohydride at 0°C for 30 min. A portion of each fraction was then incubated with 100 mM dithiothreitol at 0°C for 30 min. Each fraction, before and after dithiothreitol treatment, was then assayed for specific cytosol binding of [^{3}H]dexamethasone and [^{3}H]dexamethasone·receptor complex binding to a saturating amount of DNA-cellulose. The results are expressed as a percentage of receptor-bound radioactivity capable of binding to DNA-cellulose and represent the mean of duplicate experiments. The concentrations referred to in the table represent the final concentration of each reagent.

	DNA-cellulose binding	
	−Dithiothreitol	+Dithiothreitol
	% bound	
Heat activated	46.6	52.3
+7 mM pyridoxal-5'-P	12.8	50.5
+7 mM pyridoxal-5'-P + 42 mM $NaBH_4$	8.6	13.3
+42 mM $NaBH_4$	45.0	54.6

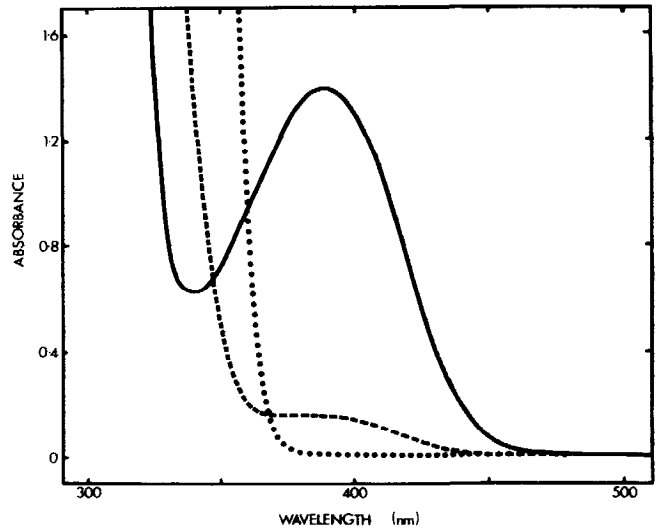

FIG. 4. Effect of dithiothreitol on the spectrum of pyridoxal 5'-phosphate. The absorption spectrum of a 10 mM solution of pyridoxal 5'-phosphate in Buffer BSM is shown (——) over the range in wavelength of 300 to 500 nm. The specific absorption peak at 390 nm was abolished by the addition of 100 mM dithiothreitol (– – –). For comparison the spectra of a 10 mM solution of pyridoxamine 5'-phosphate in Buffer BSM is shown (· · · ·).

TABLE V

Effect of ethidium bromide on receptor binding to DNA-cellulose and phosphocellulose

[^{3}H]Dexamethasone-labeled cytosol was maintained in the inactivated state or activated by heating (25°C for 45 min). Binding of each of these fractions to DNA-cellulose, ethidium bromide-pretreated DNA-cellulose, phosphocellulose, and ethidium bromide-pretreated phosphocellulose was determined. The ethidium bromide pretreatment involved incubation of the acceptors with a solution containing 50 mg of ethidium bromide/ml of 20 mM borate buffer, pH 8.0. This resulted in the binding of 0.9 μg of ethidium bromide/μg of DNA-cellulose determined spectrophotometrically at $A_{480\ nm}$ by the amount of ethidium bromide recovered in washes. Likewise, 1.9 μg of ethidium bromide were bound/μg of phosphocellulose.

Acceptor	Acceptor pretreatment	State of receptor	Receptor binding to acceptor
			dpm bound
DNA-cellulose	None	Unactivated	4,010
		Activated	34,550
DNA-cellulose	Ethidium bromide	Unactivated	250
		Activated	1,060
Phosphocellulose	None	Unactivated	570
		Activated	2,350
Phosphocellulose	Ethidium bromide	Unactivated	470
		Activated	3,980

orthoquinonoid form (20). That this absorption is abolished by the presence of dithiothreitol suggests that the thiol reagent is interacting with the aldehyde group to form a thiohemiacetal derivative. This would be expected to interfere with the formation of a Schiff base.

Effect of Ethidium Bromide on DNA-cellulose and Phosphocellulose Binding—Pretreatment of DNA-cellulose with the intercalating drug, ethidium bromide, also markedly inhibits glucocorticoid receptor binding (Table V). This was shown not to be the result of dissociation of the DNA from the cellulose. Pretreatment of phosphocellulose with ethidium bromide had no significant effect on receptor binding. Another intercalating drug, actinomycin D, caused only a 22% inhibition of receptor binding. These results suggest that there might be a hydrophobic binding site on the activated receptor which has some specificity toward the nucleotide sequence.

DISCUSSION

Very little is known about the chemical nature of the DNA binding site of the activated glucocorticoid receptor. The fact that the activated receptor not only binds to DNA but also to other polyanions such as carboxymethyl- and sulfopropyl-Sephadex (3, 12) and phosphocellulose (7, 14) indicates that activation involves exposure of positively charged regions on the surface of the receptor. Young *et al.* (21) have shown, in studies with the glucocorticoid receptor from mouse mammary tumors and from baby hamster kidney, that although the hormone·receptor complex remains intact after iodoacetamide treatment, the iodoacetamide-treated complex is no longer able to bind to DNA. Because they were using a crude receptor preparation it was not possible to distinguish between a direct effect of iodoacetamide on the receptor and an effect on some other cytosol component required for the activation or binding of the complex to DNA.

In this report we have shown that pyridoxal 5′-phosphate, when added prior to activation by exposure to increased temperature, completely prevents the enhanced binding to DNA-cellulose that occurs in its absence. The fact that pyridoxal 5′-phosphate is also inhibitory when added after heat activation indicates that its effect is by inhibiting DNA-cellulose binding rather than receptor activation. Inability of pyridoxal-P to inhibit subsequent binding to DNA-cellulose when nonactivating conditions are maintained confirms that the pyridoxal-P-sensitive site is exposed as a result of the activation process. We have shown that pyridoxal is much less effective an inhibitor and that pyridoxamine 5′-phosphate, pyridoxamine, and pyridoxine are ineffective in this system. The specificity of the effect, together with the finding that the inhibition by pyridoxal 5′-phosphate is reversable unless the modified receptor is first reduced with sodium borohydride, suggests the involvement of a Schiff base. We conclude that pyridoxal-P acts by forming a Schiff base with an ϵ-NH_2 group of a lysine which is possibly one of the residues appearing on the surface of the steroid·receptor complex upon activation. The possible interaction of the receptor directly with pyridoxal-P follows the observations made with other proteins (22). For example, the amino acid sequences of proteins which bind pyridoxal-P have been determined by interaction of the protein with pyridoxal-P, subsequent reduction with sodium borohydride, and isolation of the substituted pyridoxal amine-containing fragment after partial enzymatic proteolysis. In each case, the sequence determination has shown the pyridoxal-P acceptor to be the ϵ-NH_2 group of a lysine residue (23–26). Our assumption, therefore, at the beginning of these experiments, was that pyridoxal-P could serve as a probe for the ϵ-NH_2 group of lysine residues.

Certain experiments suggest the nature of the endogenous inhibitor named "modulator" (9, 11). We discovered that Tris buffer increased the number of steroid-receptor molecules binding to DNA-cellulose compared to other buffers. In fact, certain amino acids, especially basic amino acids, also had a stimulatory effect on this process (27). Both Tris and amino acids form Schiff bases with pyridoxal-P (28, 29). Subsequently we learned that pyridoxal-P inhibition of steroid·receptor complexes binding to DNA-cellulose occurred only in buffers which do not form a Schiff base with pyridoxal-P, such as with borate buffer. Finally, recent experiments with pyridoxine-deficient and adrenalectomized animal tissues clearly show enhanced binding of the steroid·receptor complex to DNA-cellulose compared to non-deficient, adrenalectomized controls.[2] This information suggests that, in addition to its use in *in vitro* experiments as a probe for the ϵ-NH_2 group of lysine residues, pyridoxal-P may indeed prove to be the endogenous modulator. The fact that pyridoxal is also an inhibitor infers that pyridoxal-P is not merely competing for an inorganic phosphate binding site on the receptor molecule.

If the exposure to pyridoxal-P was carried out at elevated temperature it was found that there was also a marked decrease in the amount of [^{3}H]dexamethasone bound to the receptor. This would imply that pyridoxal-P also modifies a group essential for the steroid binding site. We have not examined this effect in any detail but rather have chosen conditions under which this effect is minimized, namely lower temperature.

The effect of pyridoxal-P could be the result of a direct interaction with the receptor or, alternatively, could be the result of an interaction with a separate component of cytosol required for receptor binding to DNA. In addition, it is not possible from the data presented here to determine if multiple residues are being modified. However, the finding that pyridoxal-P is a competitive inhibitor with respect to DNA is suggestive that its inhibitory effect is the result of a direct interaction with the DNA binding site of the activated receptor. Obviously definitive information on the structure of the DNA binding site of the receptor will only be obtained using a purified receptor protein.

[2] D. M. DiSorbo and G. Litwack, unpublished experiments.

Higher levels of pyridoxal-P have been used in these experiments than those found in liver cells (30). Although experiments utilizing pyridoxine-deficient animals give anticipated results, assuming that pyridoxal-P is modulator, ultimately we need to know the binding constant of the interaction of pyridoxal-P with purified activated receptor. It is anticipated this will be much greater than binding constants with amino acids (28) or with pyridoxal-P reversibly bound to nonspecific proteins.

We conclude that the binding of the activated glucocorticoid·receptor complex to DNA involves, at least in part, electrostatic interactions between receptor positive charges and DNA phosphate groups. Furthermore, it is quite likely that a lysine residue(s) appearing on the surface upon activation contributes to this positively charged region on the activated receptor. However, the fact that the intercalating drug, ethidium bromide, also markedly inhibits activated receptor binding to DNA indicates that the DNA-receptor interaction involves more than simple electrostatic interactions, perhaps an hydrophobic binding. On the other hand, the binding of the receptor to phosphocellulose, which is much less efficient than DNA, is not impaired by ethidium bromide even though the drug binds to phosphocellulose. This implies that the binding of activated glucocorticoid receptor to phosphocellulose is simply an electrostatic interaction. Andre *et al.* (31) have similarly shown that ethidium bromide inhibits the binding of the uterine estrogen receptor to DNA but neither the binding of phosphocellulose nor poly(A)-cellulose.

Simons (32) has presented preliminary evidence suggesting that nucleotide complexity and/or sequence influences the affinity of HTC cell receptor·glucocorticoid complexes for DNA. Whether or not this hydrophobic interaction leads to a sequence specific binding remains to be elucidated.

REFERENCES

1. Baxter, J. D., Rousseau, G. G., Benson, M. C., Garcea, R. L., Ito, J., and Tomkins, G. M. (1972) *Proc. Natl. Acad. Sci. U. S. A.* **69,** 1892–1896
2. Higgins, S. J., Rousseau, G. G., Baxter, J. D., and Tomkins, G. M. (1973) *J. Biol. Chem.* **248,** 5866–5872
3. Milgrom, E., Atger, M., and Baulieu, E. E. (1973) *Biochemistry* **12,** 5198–5205
4. Kalimi, M., Beato, M., and Feigelson, P. (1973) *Biochemistry* **12,** 3365–3371
5. Wira, C. R., and Munck, A. (1974) *J. Biol. Chem.* **249,** 5328–5336
6. Simons, S. S., Jr., Martinez, H. M., Garcea, R. L., Baxter, J. D., and Tomkins, G. M. (1976) *J. Biol. Chem.* **251,** 334–343
7. Kalimi, M., Colman, P., and Feigelson, P. (1975) *J. Biol. Chem.* **250,** 1080–1086
8. Cake, M. H., and Litwack, G. (1975) *Biochem. Biophys. Res. Commun.* **66,** 828–835
9. Cake, M. H., Goidl, J. A., Parchman, L. G., and Litwack, G. (1976) *Biochem. Biophys. Res. Commun.* **71,** 45–52
10. Bailly, A., Sallas, N., and Milgrom, E. (1977) *J. Biol. Chem.* **252,** 858–863
11. Goidl, J. A., Cake, M. H., Dolan, K. P., Parchman, L. G., and Litwack, G. (1977) *Biochemistry* **16,** 2125–2130
12. Parchman, L. G., and Litwack, G. (1977) *Arch. Biochem. Biophys.* **183,** 374–382
13. Rousseau, G. G., Higgins, S. J., Baxter, J. D., Gelfand, D., and Tomkins, G. M. (1975) *J. Biol. Chem.* **250,** 6015–6021
14. Colman, P. D., and Feigelson, P. (1976) *Mol. Cell Endocrinol.* **5,** 33–40
15. Litwack, G., and Cake, M. H. (1977) *Fed. Proc.* **36,** 911
16. Beato, M., and Feigelson, P. (1972) *J. Biol. Chem.* **247,** 7890–7896
17. Blobel, G., and Potter, V. R. (1966) *Science* **154,** 1662–1665
18. Cake, M. H., and Litwack, G. (1978) *Eur. J. Biochem.* **82,** 97–103
19. Hinegardner, R. T. (1971) *Anal. Biochem.* **39,** 197–201
20. Heyl, D., Luz, E., Harris, S. A., and Folkers, K. (1951) *J. Am. Chem. Soc.* **73,** 3430–3433
21. Young, H. A., Parks, W. P., and Scolnick, E. M. (1975) *Proc. Natl. Acad. Sci. U. S. A.* **72,** 3060–3064
22. Means, G. E., and Feeney, R. E. (1971) *Chemical Modification of Proteins,* p. 132–134, Holden-Day, Inc., San Francisco
23. Hughes, R. C., Jenkins, W. T., and Fischer, E. H. (1962) *Proc. Natl. Acad. Sci. U. S. A.* **48,** 1615–1618
24. Anderson, J. A., Chang, H. W., and Grandjean, C. J. (1971) *Biochemistry* **10,** 2408–2415
25. Lerch, K., and Fischer, E. H. (1975) *Biochemistry* **14,** 2009–2014
26. Talbot, J.-C., Gros, C., Cosson, M.-P., and Pantaloni, D. (1977) *Biochim. Biophys. Acta* **494,** 19–32
27. Parchman, L. G., Goidl, J. A., and Litwack, G. (1977) *FEBS Lett.* **79,** 25–28
28. Matsuo, Y. (1957) *J. Am. Chem. Soc.* **79,** 2011–2015
29. Turano, C., Fasella, P., Vecchini, P., and Giartosio, A. (1964) *J. Chromatog.* **14,** 201–204
30. Li, T.-K., Lumeng, L., and Veitch, R. L. (1974) *Biochem. Biophys. Res. Commun.* **61,** 677–684
31. Andre, J., Pfeiffer, A., and Rochefort, H. (1976) *Biochemistry* **15,** 2964–2969
32. Simons, S. S., Jr. (1977) *Biochim. Biophys. Acta* **496,** 349–358

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