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Evidence that dephosphorylation inactivates glucocorticoid receptors

(corticosteroids/steroids/phosphatases)

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ABSTRACT **Highly purified alkaline phosphatase [orthophosphoric-monoester phosphohydrolase (alkaline optimum), EC 3.1.3.1] from calf intestine inactivates the glucocorticoid-binding capacity of soluble preparations from mouse fibroblasts (L cells) and rat liver. The unbound receptor is sensitive to inactivation whereas the steroid-bound receptor is unaffected. The ability of the enzyme preparation to inactivate the receptor, like its ability to dephosphorylate *p*-nitrophenyl phosphate, is dependent on zinc and inhibited by arsenate. Both the dephosphorylating and receptor inactivating activities coelute during DEAE-cellulose purification of the enzyme. There is no detectable proteolytic activity in the purified alkaline phosphatase preparation. In a mixed system containing both glucocorticoid and estrogen receptors, the glucocorticoid receptor is selectively inactivated. Although these observations do not prove that the receptor molecule itself is the substrate, they are consistent with the proposal that the glucocorticoid receptor can be inactivated by dephosphorylation and that only the phosphorylated form of the molecule is capable of binding steroid. A phosphorylation-dephosphorylation mechanism may be responsible for determining the level of active receptor in the cell.**

The ability of intact cells to bind glucocorticoids in a specific manner is energy-dependent (1–3). Exposure of mouse fibroblasts (3), thymic lymphocytes (4), or chick embryo retina (5) to dinitrophenol results in a loss of binding capacity. When the metabolic inhibitor is removed from the medium, the ability to bind glucocorticoids returns and the return is unaffected by the presence of inhibitors of protein synthesis (3, 5, 6). Munck *et al.* (2) have proposed that the receptor exists in two forms and that ATP may be required to generate the steroid-binding form from a nonbinding precursor. There is, as yet, no evidence for such a phosphorylation–dephosphorylation process (7). In this communication we demonstrate that the glucocorticoid-binding capacity of cell-free preparations from fibroblasts and liver is inactivated by incubation with extensively purified alkaline phosphatase [orthophosphoric-monoester phosphohydrolase (alkaline optimum), EC 3.1.3.1]. The effect is specific for the unbound form of glucocorticoid receptors.

MATERIALS AND METHODS

Materials. [*1,2,3*-^{3}H]Triamcinolone acetonide (21.6 Ci/mmol) and [*6,7*-^{3}H]estradiol-17β (47.9 Ci/mmol) were purchased from New England Nuclear, Boston, MA. The triamcinolone acetonide* was diluted to a specific activity of 5.3 Ci/mmol prior to use. ^{14}C-Labeled protein from algal cells came from Amersham/Searle Corp., Arlington Heights, IL. Alkaline phosphatase from calf intestinal mucosa (950–1100 units/mg at 37°, pH 10), *Streptomyces griseus* protease (4 units/mg of solid), dexamethasone, 1,10-phenanthroline, *p*-nitrophenyl phosphate, *p*-nitrophenol, phenylmethyl sulfonyl fluoride (PMSF), *N*-α-*p*-tosyl-L-lysine chloromethyl ketone (Tos-LysCH$_2$Cl), and L-1-tosylamido-2-phenylethyl chloromethyl ketone (Tos-PheCH$_2$Cl) were obtained from Sigma Chemical Co., St. Louis, MO. Soybean trypsin inhibitor was purchased from Worthington Biochemical Corp., Freehold, NJ.

Cell Culture and Fractionation. L 929 mouse fibroblasts were grown in monolayer culture in Joklik medium as previously described (8). Cells in the logarithmic phase of growth were harvested from Roux bottles by scraping into cold Earle's saline. They were washed once by resuspension in several volumes of the cold saline solution and centrifuged at 600 × *g* for 10 min. The washed cells were suspended in 1.5 volumes of an iced hypotonic solution of 10 mM Tris buffer at pH 7.35 and 2 μM EDTA and were homogenized with 15 strokes of a tight-fitting pestle in a Dounce-type glass homogenizer. The broken cell suspension was centrifuged at 27,000 × *g* for 15 min and the supernatant was centrifuged at 100,000 × *g* for 45 min at 2°. The 100,000 × *g* supernatant (containing 10 to 15 mg of protein per ml) is the "soluble fraction of the cell" as that term is used in this paper. No thiol reagent was added because we have not found that this provides much additional stabilization of the receptor when L cell supernatants are prepared in only the hypotonic buffer.

For experiments utilizing soluble preparations from uterus, 19-day-old female Sprague-Dawley rats were adrenalectomized by the dorsal route and maintained on 0.9% saline for 5 days prior to use. Animals were killed by decapitation and the uteri were immediately removed and placed in the iced hypotonic buffer (three or four uteri per ml). The uteri were disrupted by four 10-sec bursts with a Tissuemizer (model SDT, Tekmar Co., Cincinnati, OH) and centrifuged at 27,000 × *g* and 100,000 × *g* as described above. Soluble fractions (containing 38 to 45 mg of protein per ml) were prepared from minced liver of adrenalectomized 100-g male Sprague-Dawley rats by disruption in a Sorvall homogenizer (two 30-sec bursts at 0°; setting, 10) and centrifuged as above.

Enzyme Preparation. The commercial alkaline phosphatase was dialyzed against two 1-liter volumes of 10 mM Tris, pH 7.35/2 μM EDTA for 12 hr at 4° prior to use. Stock solutions were prepared by dilution in the same buffer. For some experiments the enzyme was further purified by chromatography on DEAE-cellulose as detailed in the legend to Fig. 4.

Incubation and Steroid-Binding Assay. In most experiments the soluble fraction was incubated at 20° with alkaline phosphatase added from stock solution in 1/10th the final incubation

Abbreviations: PMSF, phenylmethyl sulfonyl fluoride; Tos-LysCH$_2$Cl, *N*-α-*p*-tosyl-L-lysine chloromethyl ketone; Tos-PheCH$_2$Cl, L-1-tosylamide-2-phenylethyl chloromethyl ketone.

* The trivial names for steroids are used as follows: triamcinolone acetonide, 9α-fluoro-11β,16α,17α,21-tetrahydroxypregna-1,4-diene-3,20-dione 16,17-acetonide; dexamethasone, 9α-fluoro-16α-methyl-11β,17α,21-trihydroxypregna-1,4-diene-3,20-dione; estradiol-17β, 1,3,5(10)-estratriene-3,17β-diol; diethylstilbestrol, 3,4-bis(*p*-hydroxyphenyl)-3-hexene.

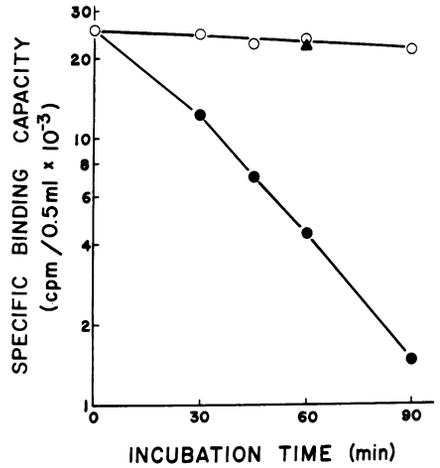

FIG. 1. Effect of alkaline phosphatase on the specific glucocorticoid binding capacity of the soluble preparation from L cells. A 100,000 × *g* supernatant from L cells was incubated at 20° with alkaline phosphatase at 80 μg/ml (●), heat-treated (90° for 10 min) enzyme (▲), or vehicle (○). At the indicated times, 0.5-ml portions were removed and the specific binding capacity was determined by incubating at 0° with [^{3}H]triamcinolone acetonide as described under *Materials and Methods.*

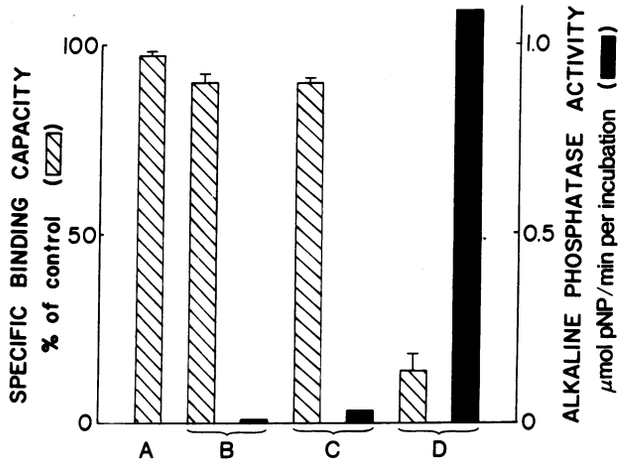

FIG. 2. Zinc dependency of the effect of alkaline phosphatase on specific glucocorticoid binding capacity (after 1 hr of incubation at 20°) of the soluble preparation from L cells. Alkaline phosphatase was dialyzed at 0° against 10 mM Tris buffer, pH 7.35, containing 1 mM EDTA and then incubated at 30° for 3 hr in this buffer with 5 mM 1,10-phenanthroline. The *o*-phenanthroline and EDTA were then removed by passing the enzyme through Sephadex G-25. A portion of this zinc-free preparation was retained as the inactivated enzyme and the remainder was incubated with 1 mM $ZnCl_2$ at 20° for 1 hr. This zinc-reactivated enzyme was again chromatographed on Sephadex G-25 to remove the unbound zinc. A portion of the zinc-reactivated enzyme was incubated at 90° for 10 min. Replicate 0.45-ml aliquots of a 100,000 × *g* supernatant from L cells were incubated for 1 hr at 20° without additions (control), with 5 μM $ZnCl_2$ (A), with heat-treated, zinc-reactivated enzyme (90 μg/ml) and $ZnCl_2$ (B), with zinc-free enzyme (150 μg/ml) (C), or with zinc-reactivated enzyme (90 μg/ml) and $ZnCl_2$ (D). The specific binding capacity for triamcinolone acetonide was assayed at the end of the incubation as described under *Materials and Methods.* The bars represent the average of two experiments with the range indicated by the line above the bar.

volume. At various times, aliquots (usually 0.5 ml) were removed and 3.0 μl of radioactive triamcinolone acetonide was added to duplicate samples from a stock solution dissolved in 10% ethanol to achieve a final concentration of 50 nM. The samples were incubated for an additional 2 hr at 0° to permit complete binding as previously demonstrated (9). Nonspecific binding was determined by incubating identical aliquots with both radioactive triamcinolone acetonide and 50 μM nonradioactive dexamethasone. The final concentration of ethanol was 1%. The difference between the binding assayed in the presence and in the absence of competing unlabeled steroid represents the specific binding capacity of the system.

In the experiment examining the effect of alkaline phosphatase on steroid-bound receptor, the 100,000 × *g* supernatant was incubated at 0° in the presence and absence of dexamethasone prior to the addition of enzyme. In this case the difference in the binding values in the presence and absence of competitor is referred to as the specific binding.

The specific binding capacity for estradiol was determined by incubating samples for 2 hr at 0° with 10 nM [^{3}H]estradiol-17β in the presence or absence of 10 μM nonradioactive diethylstilbestrol.

Bound steroid was assayed by passing each incubation mixture over a small column of Sephadex G-25 and counting the radioactivity in the macromolecular peak as described previously (10). All binding values in this paper are averages of duplicate values.

Assay for Alkaline Phosphatase Activity. Alkaline phosphatase activity was determined by a modification of the method of Cox and Griffin (11) using replicate 0.5-ml incubation mixtures containing 0.45 ml of substrate (1 mM *p*-nitrophenyl phosphate in 1 M Tris, pH 8.0) and 50 μl of enzyme preparation. Incubations were carried out at 20° for various periods, depending on the activity of the sample. Reactions were stopped by adding 4.5 ml of 0.25 M NaOH, and the yellow *p*-nitrophenol product was assayed in a spectrophotometer at 410 nm. Standard curves were prepared with *p*-nitrophenol and enzyme activity is expressed as μmol of *p*-nitrophenol produced per min in each incubation. The alkaline phosphatase preparation was shown to be free of phospholipase A activity by previously published methods (9).

RESULTS

The ability of calf intestine alkaline phosphatase to inactivate the glucocorticoid binding capacity of the 100,000 × *g* supernatant from L cells at 20° is presented in Fig. 1. At an enzyme concentration of 80 μg/ml, the effect was nearly linear on a semilogarithmic plot. There was no effect (even at much higher enzyme concentrations) at 0°, the temperature at which the binding assay is conducted.

Alkaline phosphatase from calf intestine is a zinc-dependent enzyme (12). The zinc requirement for both the action of the enzyme on the steroid-binding capacity of the L cell soluble preparation and the dephosphorylation of *p*-nitrophenyl phosphate is presented in Fig. 2. The enzyme binds Zn^{2+} quite tightly. Consequently, 5 mM *o*-phenanthroline was used to chelate the metal ion. *o*-Phenanthroline itself has a deleterious effect on the binding capacity at concentrations above 0.5 mM. Therefore, the excess chelator was removed by passage through Sephadex G-25. At concentrations above 10 μM, Zn^{2+} also adversely affects the binding capacity, so the zinc-reactivated enzyme was passed through Sephadex G-25 to remove unbound metal ion. It is clear from Fig. 2 that there was no effect of the enzyme on either *p*-nitrophenyl phosphate- or glucocorticoid-binding capacity in the absence of zinc, but both activities were present in the reactivated enzyme.

Arsenate has been shown to be a competitive inhibitor of several alkaline phosphatases, including that from calf intestine (13, 14). We found arsenate to be an effective inhibitor of the dephosphorylation of *p*-nitrophenyl phosphate, and at 1 mM it also prevented the loss of specific binding capacity caused by the enzyme preparation at 25 μg/ml (Fig. 3). As shown in Fig.

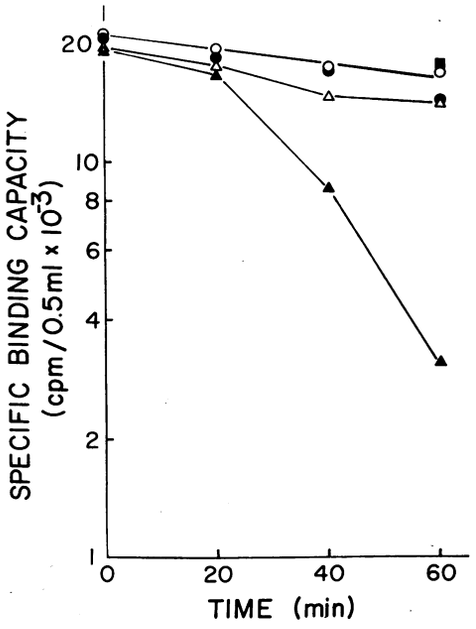

FIG. 3. Inhibition of alkaline phosphatase effect by arsenate. Replicate portions of the soluble fraction from L cells were incubated at 20° with vehicle (○), alkaline phosphatase at 25 μg/ml (▲), 1 mM arsenate (●), both arsenate and alkaline phosphatase (△), or alkaline phosphatase that was heated at 90° for 10 min (■). At various times, 0.5 ml-aliquots were removed and incubated at 0° with triamcinolone acetonide, and the specific binding capacity was determined as described in *Materials and Methods*.

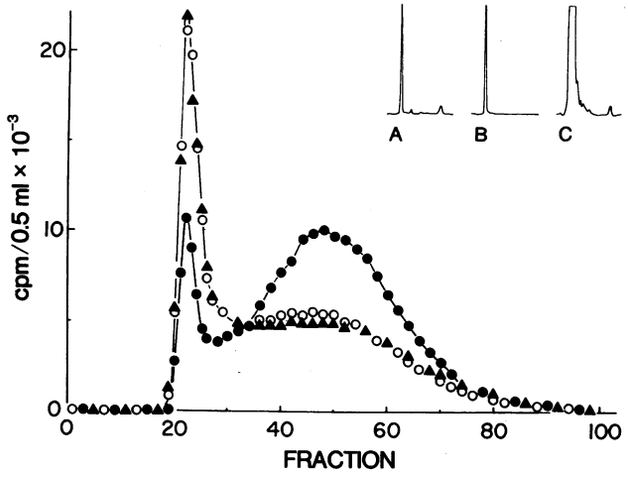

FIG. 5. Assay for proteolytic activity in alkaline phosphatase peak recovered from DEAE-cellulose. Fractions 43 through 53 from the DEAE-column of Fig. 4 were combined, concentrated against crystalline sucrose to a volume of 2 ml, and dialyzed against 15 mM Tris, pH 7.5. ^{14}C-Labeled algal protein (final concentration, 2.5 μg/ml) was incubated for 1 hr at 20° with 0.25 ml (final concentration, 600 μg/ml) of the concentrated alkaline phosphatase preparation (▲), of protease at 80 μg/ml (●), or of vehicle (○) under the usual enzyme digestion conditions. The incubation mixes were chromatographed on columns of Sephadex G-50. *Inset* Scans at 550 nm of Coomassie blue-stained sodium dodecyl sulfate/10% polyacrylamide gels of the alkaline phosphatase preparation prepared as previously described (9). *A*. Alkaline phosphatase, 8 μg, prior to chromatography on DEAE-cellulose. *B*. Concentrated peak, 8 μg. *C*. Concentrated peak, 80 μg.

4, the receptor-inactivating and dephosphorylating activities coeluted from DEAE-cellulose.

In order to detect possible proteolytic activity in the alkaline phosphatase preparation, the major peak eluted from a DEAE-cellulose column was concentrated by dialysis against crystalline sucrose and incubated under the usual assay conditions with a heterogeneous ^{14}C-labeled protein mixture from algal cells. This incubation, as well as control mixtures incubated with vehicle or protease, were passed through a column of Sephadex G-50 (Fig. 5). The alkaline phosphatase did not have detectable proteolytic activity even though it was present at a concentration of 600 μg/ml. This is 10 times the amount of enzyme that produces at least 90% inactivation of the binding capacity in the soluble fraction of L cells. The inset in Fig. 5 presents profiles of Coomassie blue stained sodium dodecyl sulfate/polyacrylamide gels of the alkaline phosphatase preparation before and after DEAE-cellulose chromatography. There still was some contamination in the DEAE-cellulose peak, as can be seen as four visible bands in the heavily loaded gel (*C*) but the preparation was highly purified. Several protease inhibitors were found to have no effect on the ability of alkaline phosphatase (25 μg/ml) to inactivate the binding capacity—they included PMSF at 0.1 mM, Tos-LysCH_2Cl at 0.1 mM, Tos-PheCH_2Cl at 10 μM, and soybean trypsin inhibitor at 0.5 mg/ml.

When the soluble fraction of L cells was mixed with a similar preparation from rat uterus, the glucocorticoid-binding ca-

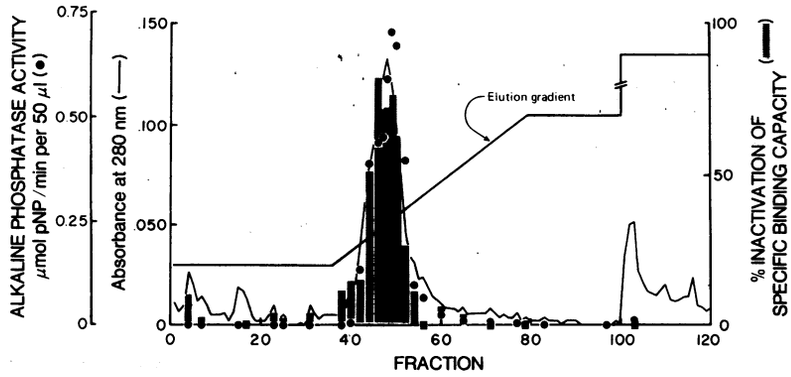

FIG. 4. Chromatography of alkaline phosphatase on DEAE-cellulose. A 6-mg sample of alkaline phosphatase was dialyzed against 15 mM Tris, pH 7.5 and adsorbed to a column of DEAE-cellulose. The column was eluted with 120 ml of the initial buffer, followed by a linear gradient to 15 mM Tris/100 mM KCl and a terminal elution with a step to 15 mM Tris/500 mM KCl. An aliquot (50 μl) of each 4-ml fraction was incubated at 20° for 1 hr with 0.45 ml of 100,000 × *g* supernatant prepared from L cells. Aliquots (10 μl) were used to determine alkaline phosphatase activity at 20° as described under *Materials and Methods*, and each activity value was multiplied by 5 to give the activity for 50 μl of sample as used in the incubation with L cell supernatant. The symbols on the abscissa represent samples without activity on either the receptor or on *p*-nitrophenyl phosphate. pNP = *p*-nitrophenol.

Table 1. Effect of alkaline phosphatase on specific binding of triamcinolone acetonide and estradiol-17β by receptors from L cells and rat uterus

		Triamcinolone acetonide		Estradiol	
	Time	Specific binding capacity, cpm/incubation	% of 0 time control	Specific binding capacity, cpm/incubation	% of 0 time control
No additions	0	12,340	100	24,870	100
No additions	1	11,700	95	18,070	73
Plus alkaline phosphatase	1	1,570	13	15,130	61
Plus heated alkaline phosphatase	1	12,640	102	17,580	71

High-speed supernatants were prepared from L cells and rat uteri and mixed in equal proportions. The mixture was incubated for 1 hr at 20° in the presence of buffer, alkaline phosphatase (100 μg/ml), or alkaline phosphatase that had been heated at 90° for 15 min. Aliquots (0.54 ml) were removed at 0 time and at 1 hr and assayed for their ability to specifically bind [^{3}H]triamcinolone acetonide or [^{3}H]estradiol-17β. The values in the table represent the average of duplicate assays.

pacity was inactivated by the enzyme whereas the estradiol-binding capacity in the same mixture was almost unaffected (Table 1). In order to determine if the inactivation observed with the soluble fibroblast preparation was common to other glucocorticoid receptor systems, we examined the effect on the binding component in liver. It has been reported that, as with L cells, the specific binding capacity in cell-free preparations from this source is normally lost at a relatively slow rate (15). As shown in Fig. 6 the binding capacity of the liver supernatant was inactivated, although higher concentrations of enzyme were required. Because the binding capacity of the soluble preparation from rat thymic lymphocytes was lost in a few minutes of incubation at 20° in the absence of alkaline phosphatase, we were not able to determine the effect of the enzyme in that well-studied system. There was a delay before extensive inactivation of specific binding capacity was observed in the liver preparation. This resembles the kinetics observed in the L cell supernatant at a low concentration of alkaline phosphatase (Fig. 3).

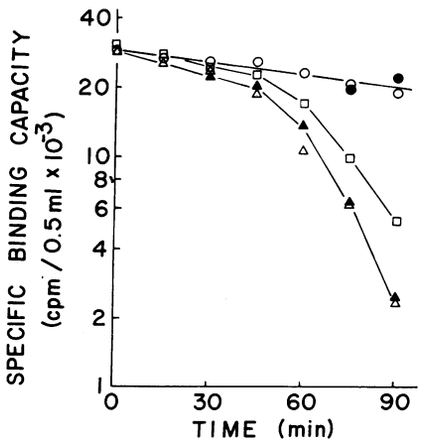

FIG. 6. Effect of alkaline phosphatase on the specific glucocorticoid-binding capacity of a 100,000 × *g* supernatant from rat liver. Replicate portions of the soluble fraction from rat liver were incubated at 20° with vehicle (○), with alkaline phosphatase at 75 μg/ml (□), 150 μg/ml (△), or 200 μg/ml (▲), or with heat-treated (90° for 10 min) alkaline phosphatase at 200 μg/ml (●). The specific binding capacity for triamcinolone acetonide was determined at various times on 0.5-ml samples as described under *Materials and Methods*.

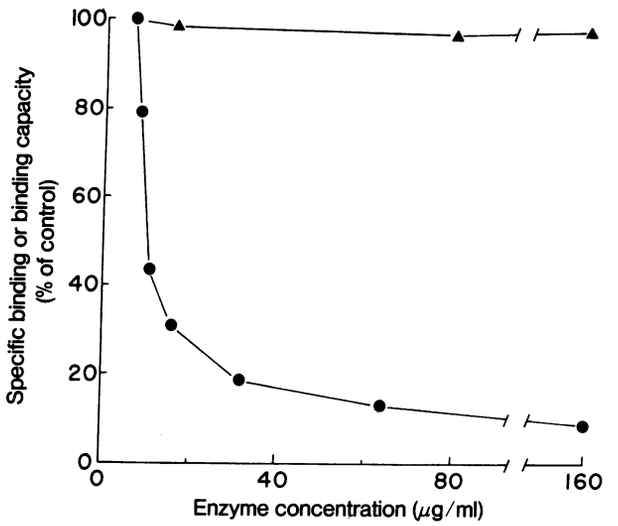

FIG. 7. Effect of alkaline phosphatase on the unbound receptor and on the triamcinolone acetonide-bound receptor. A 100,000 × *g* supernatant from L cells was divided into two portions; one portion was incubated for 2 hr at 0° with 50 nM [^{3}H]triamcinolone acetonide. Aliquots of the steroid-bound and unbound preparations were incubated at 20° for 1 hr with the indicated concentrations of alkaline phosphatase that had been chromatographed on DEAE-cellulose as in Fig. 4. At the end of the incubation, the specific binding (steroid-bound receptor, ▲) and the specific binding capacity (unbound receptor, ●) were determined on duplicate samples as described under *Materials and Methods*.

The triamcinolone acetonide-bound receptor was not inactivated by alkaline phosphatase (Fig. 7). We have tested concentrations as high as 370 μg/ml without producing any release of bound steroid during the 1-hr incubation.

DISCUSSION

The conclusion that we are observing a phosphatase effect is supported by the facts that the dephosphorylating and receptor-inactivating activities were coeluted from a DEAE-cellulose column (Fig. 4), both effects were zinc-dependent (Fig. 2), and both actions are inhibited by arsenate (Fig. 3), a competitive inhibitor of alkaline phosphatase. No proteolytic activity was observed on a ^{14}C-labeled protein mix at high concentrations of enzyme under our assay conditions. The observations that the triamcinolone acetonide-bound receptor was not affected and that glucocorticoid-binding capacity was extensively inactivated while that of estradiol-17β in the same incubation mixture was not are evidence against an effect due to proteolytic contamination of the enzyme.

It is important to consider how the observations presented in this paper may fit into the glucocorticoid receptor cycle proposed for mouse fibroblasts (Fig. 8) and, with one difference, for thymic lymphocytes. According to the model, the glucocorticoid binds to a receptor that is normally located in the cytoplasm (16, 17). As has been shown for other glucocorticoid-responsive systems and for other classes of steroid hormones, the steroid–receptor complex undergoes a temperature-dependent change to a form (RS^n) that can bind to nuclear components (3, 18). It has been suggested from studies in hep-

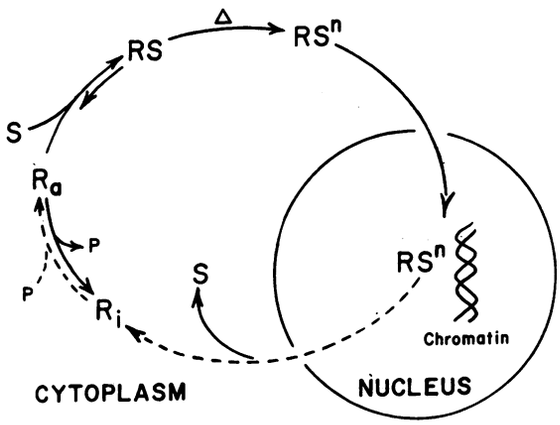

FIG. 8. Proposed cycle of events controlling the binding state and cellular location of the glucocorticoid receptor. R_i dephosphorylated form of the receptor that is inactive and cannot bind steroid; R_a, phosphorylated form of the receptor that binds steroids in the cytoplasm of the cell; *RS*, steroid–receptor complex; RS^n, form of the steroid–receptor complex that can bind to nuclear components; S, steroid. Broken lines refer to processes that appear to require energy in L cells.

atoma cells that the ultimate nuclear acceptor site for the glucocorticoid–receptor complex may be DNA, presumably as it exists in the chromatin complex (19, 20). Direct demonstration of binding of the steroid–receptor complex from L cells to either the DNA or protein components of chromatin has not been carried out. Middlebrook *et al.* (18) suggested that the steroid–receptor complex of L cells bind to the chromatin fraction because at least a portion of the complex recovered in a low-speed pellet was released into soluble form by DNase.

Munck *et al.* (2), from studies with intact rat thymocytes, and Ishii *et al.* (3), on the basis of observations made in mouse L cells, suggested that the steroid receptor is released from the nucleus in a form that cannot bind glucocorticoids (R_i). It is proposed (3, 18) that release of the receptor from its association with L cell nuclei (low-speed particulate fraction) requires energy. From a study of steroid binding in the particulate and soluble fractions of rat thymocytes, Sloman and Bell (7) did not conclude that energy is required for release of receptor from the nucleus, and this stands as the principal difference in the observations made with respect to the cell cycle in the two systems.

Observations in intact thymocytes and fibroblasts also suggest that the inactive glucocorticoid receptor (R_i) is converted to a form that binds steroid (R_a) by a process that requires energy and is independent of protein synthesis (2, 3, 6). Two observations are consistent with the suggestion that this activation mechanism may involve phosphorylation. First, the capacity of thymocytes to bind steroids varies according to their ATP content (2, 7). Second, the ability of cell-free preparations from fibroblasts and liver to bind glucocorticoids specifically has been shown in this paper to be inactivated by alkaline phosphatase. Direct phosphorylation of the receptor has not yet been demonstrated because it has not been possible to achieve any degree of purification of the unbound form. It is possible that alkaline phosphatase is dephosphorylating ATP or some other compound required for activation of the receptor by other means. It is also possible that alkaline phosphatase is activating a receptor-degrading enzyme by dephosphorylation. However, a simple and direct interpretation of the data is that the receptor itself can be inactivated by dephosphorylation. An attractive feature of the activation–inactivation model is that it may provide a site for controlling the cell's capacity to respond to the hormone.

There are some observations that indicate that the effect of alkaline phosphatase may be similar to the rapid inactivation of glucocorticoid-binding capacity observed in many cell-free preparations. As Bell and Munck (6) have noted, it is primarily (or perhaps solely) the unbound form of the receptor that is inactivated. This is also true for the phosphatase effect (Fig. 7). Ishii and Aronow (21) reported that glucose 1-phosphate, glucose 6-phosphate, fructose 1,6-diphosphate, and ribose 5-phosphate have a stabilizing effect on the unbound receptor in soluble preparations from L cells but have no effect on the bound form. This stabilization could be explained by competition by these phosphorylated substrates for an endogenous inactivating system that dephosphorylates the receptor.

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