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Progesterone-binding Sites of the Chick Oviduct Receptor

PRESENCE OF A WEAKER LIGAND SITE WHICH IS DESTROYED BY PHOSPHATASE TREATMENT*

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Titration of chick progesterone receptor over a wide range of [^{3}H]progesterone concentration (0.15 to 90 nM) shows two distinct types of binding sites in cytosol and in partially purified receptor samples prepared from oviducts of estrogenized chicks. The difference in affinity between the two sites (K_d = 1 nM; K_d = 25 nM) is sufficient to allow analysis by Scatchard plot methods. Ligand competition studies show that both sites have the same relative specificity for progesterone compared to other steroids. Both sites seem to be on the same receptor molecule as shown by their copurification and chromatographic properties. No cooperativity between the two sites has been detected in analysis using either rate kinetics or equilibrium methods. Thus, the function of the low affinity sites is not apparent at this time; it does not appear to function as a "helper" site which influences binding to the high affinity site previously described. The binding constant of the low affinity site is sufficiently strong to allow potential occupancy of these sites *in vivo*, at least at certain stages of the female reproductive cycle. The hormone-binding activity of the low affinity site can be destroyed after *in vitro* treatment with alkaline phosphatase, but the high affinity site remains functional under these conditions. Inhibitors of the enzyme block the inactivation. Furthermore, preliminary data *in vivo* suggest that estrogen administration to the animal can influence the relative titer of the low affinity sites.

The progesterone receptor of chick oviduct is a protein complex of two hormone-binding subunits, A (M_r = 79,000) and B (M_r = 108,000) (Schrader and O'Malley, 1972; Coty *et al.*, 1979). Studies on the ligand-binding properties of crude receptors revealed a single, high-affinity steroid-binding site on both A and B; the sites had indistinguishable binding kinetics and ligand specificity (Schrader *et al.*, 1977; Hansen *et al.*, 1976). Those studies however, demonstrated discrepancies between hormone binding kinetics, as determined by equilibrium and rate methods, which led us to predict the presence of progesterone binding site heterogeneity (Schrader *et al.*, 1977). Other laboratories have reported complex kinetics of cytosolic receptor dissociation for glucocorticoids (Rousseau *et al.*, 1972) or estrogens (Weichman and Notides, 1977, 1979; Notides *et al.*, 1981) and suggested that the hormone could modulate receptor activation by shifting the equilibrium from a lower affinity state (the nonactivated receptor) toward a higher affinity state characterized by a slower dissociation phase.

Recently we achieved partial purification (500 ×) of the progesterone receptor as an apoprotein (*i.e.* lacking hormone) (Maggi *et al.*, 1980). The purified aporeceptor had binding kinetics and ligand specificity very close to the crude starting material and, therefore, represented a good tool to reconsider the characteristics of receptor-hormone interactions.

In this paper we report the existence of a second low affinity binding site for progesterone on the receptor subunits. The relative amount of this second site fluctuates over a wide range in the oviduct of estrogenized immature chicks. Therefore, we were interested in examining the mechanisms of control of this second site. Recently, a number of authors (Nielsen *et al.*, 1977; Auricchio *et al.*, 1981; Yuh and Keyes, 1981) have suggested that phosphorylation is involved in the regulation of the binding activity of various steroid receptors. Furthermore, we demonstrated recently the existence of charge heterogeneity for the progesterone receptor which is correlated with its state of phosphorylation (Weigel *et al.*, 1981). We, therefore, tested for the effect of receptor phosphorylation on the two sites for progesterone. In this communication we report that exposure of the receptor to alkaline phosphatase preferentially inactivates the binding activity of the weaker site.

EXPERIMENTAL PROCEDURES

Animals—White Leghorn chicks, 5 to 7 weeks old, were obtained from Texas Animal Specialities and used as the source of tissue for all experiments. The animals were implanted weekly with 20-mg pellets of diethylstilbestrol until the stimulated oviduct tissue reached about 2 g per 5-week-old bird. Laying hen oviducts were obtained from Tex-Hens, Nixon, TX.

Chemicals—All chemicals were reagent grade, unless otherwise stated in the text, and were purchased from J. T. Baker Chemical Co. Ion exchange resins were from Whatman; nonionic cellulose (Cellex N-1) used to prepare DNA-cellulose resin was obtained from Bio-Rad. [1,2-^{3}H]Progesterone (43 Ci/mmol) was from Amersham Corp. Nonradioactive steroids, bovine intestine alkaline phosphatase, and glucose 1-phosphate were purchased from Sigma. Hydroxylapatite was purchased from Bio-Rad. Scintillation counting fluid (Type ACS) was from Amersham/Searle.

Preparation of Resins—DNA-cellulose was prepared by the method of Alberts and Herrick (1971) as described in Coty *et al.* (1979). Phosphocellulose and DEAE-cellulose were precycled and equilibrated with Buffer A (10 mM Tris-HCl, 1 mM Na_2EDTA, 12 mM 1-thioglycerol, pH 7.4) as previously described (Coty *et al.*, 1979). Hydroxylapatite was washed with several volumes of 0.08 M potassium phosphate buffer, pH 7.4, prior to use. The capacity and ion exchange characteristics of these resins for receptor chromatography have been described previously (Schrader, 1975).

Preparation of Cytosol—The crude cytoplasmic soluble fraction was obtained by homogenization and centrifugation exactly as de-

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scribed previously (Schrader and O'Malley, 1972).

Hormone-binding Assay—Saturation analysis of cytosol and partially purified progesterone receptors was performed in the presence of [^{3}H]progesterone at final concentrations ranging from 0.15 to 90 nM. Specific binding was calculated by determining total binding and subtracting the value obtained in the presence of a 100-fold excess of nonradioactive progesterone. Single point analyses were made with two different [^{3}H]progesterone concentrations (±100-fold excess nonradioactive progesterone) to detect only higher affinity sites (0.3 nM) or total sites (50 nM). Tubes (in duplicate) were incubated at 4 °C for 12 h, and the specific binding was determined by the hydroxylapatite assay as described below. Data plotted according to Scatchard (1949) were corrected by the method of Rosenthal (1967) and Feldman (1972). The hydroxylapatite assay was based on the method of Pavlik and Coulson (1976). Twenty microliters of cytosol or 1 pmol of purified receptor were incubated with ^{3}H ligand in Buffer B (10 mM Tris-HCl, 1 mM Na_2EDTA, 1% ethanol, pH 7.8) to a final volume of 0.5 ml. At equilibrium (12 h at 4 °C) samples were combined with 0.5 ml of 60% v/v hydroxylapatite in phosphate buffer (10 mM, pH 7.8). The mixture was then incubated at 4 °C for 30 min. The samples were stirred a few times during this period and then centrifuged for 5 min at 500 × *g*. The supernatant was decanted, and the precipitate was washed three times with phosphate buffer (0.08 M, pH 7.8). The [^{3}H]progesterone was then extracted with petroleum ether, and the radioactivity was determined by scintillation counting.

Prelabeled Sucrose Density Gradient Analysis—Linear 5 to 20% sucrose gradients were prepared using a Beckman density gradient apparatus. Sucrose solutions were prepared in Buffer C (10 mM Tris, 1 mM Na_2EDTA, pH 7.8).

Cytosolic receptor preparations were incubated at 4 °C for 12 h with a final concentration of either 0.3 or 40 nM [^{3}H]progesterone or the same concentration of [^{3}H]progesterone plus a 100-fold molar excess of cold progesterone. The progesterone receptor preparations (200 μl) were layered on the gradients and centrifuged in a Beckman VTi65 rotor at 4 °C for 90 min at 65,000 rpm.

After centrifugation, the gradients were fractionated from the bottom into forty 0.12-ml fractions. Water (0.3 ml) was added for counting in 4 ml of ACS. The fractions were counted by liquid scintillation spectrometry in a Beckman LS8000 spectrometer at 33% efficiency. A linear standard curve of sedimentation coefficient *versus* migration was obtained using the following proteins as standards: (*a*) ovalbumin, 3.7 S; (*b*) IgG 7.2 S; (*c*) *Escherichia coli* aspartate transcarbamylase, 11.7 S.

Gel Filtration Chromatography—Gel filtration analysis of the high affinity and the high plus low affinity progesterone-binding sites was performed on a Sephadex G100 (Pharmacia) column (2.4 × 30 cm) which was equilibrated in Buffer A containing 0.3 M KCl. Protein standards used for the calibration of the G100 column were from a protein calibration kit (Size II) (Boehringer Mannheim). Typically, 1-ml samples were applied, and 1.5-ml fractions were collected at a flow rate of 7.5 ml/h.

Progesterone Receptor Purification Procedures—Purification of progesterone receptor 8 S aggregate was carried out as previously described (Maggi *et al.*, 1980). Subunits A and B of progesterone receptor were partially purified following the purification procedure shown in Fig. 1.

Enzyme Preparation—The commercial alkaline phosphatase was desalted on Sephadex G-25 columns and stored overnight at 4 °C in 10 mM Tris, pH 7.8, 0.1 mM $ZnCl_2$, 1 mM $MgCl_2$. Alkaline phosphatase activity was determined by the method of Cox and Griffin (1967).

RESULTS

Titration analysis of cytosol receptor over a wide range of [^{3}H]progesterone concentrations yielded the curves shown in Fig. 2*A*. This part shows the uncorrected total ligand binding, the values obtained in the presence of a large excess of nonradioactive hormone, and the specific binding curve derived from the difference between the two experimental curves. The nonspecific binding data are a straight line, indicating that the method adequately corrects for this parameter over this concentration range. Therefore, the specific saturable binding data of Fig. 2*A* were suitable for analysis by a linear transformation method. The Scatchard plot of the corrected receptor binding curve is shown in Fig. 2*B*. The data were fitted initially to a one-component binding model by least square regression, yielding a correlation coefficient of 0.89. Fitting to a two-component model yielded a correlation coefficient of 0.99. Thus, the data shown in Fig. 2*B* is better approximated by the binding expected for at least two independent binding sites of different affinities.

This curvature of the Scatchard plot also could be due to negative cooperativity among ligand sites. The possibility of negative cooperativity was excluded by the fact that the data yielded a Hill coefficient of nearly 1.0 (Hill, 1910) for both components as shown in Fig. 2*C*.

The kinetics of hormone dissociation from the apparent higher affinity sites was investigated for evidence of site-to-site interaction as shown in Fig. 2*D*. Receptor B subunit-hormone complexes were prepared by adding 1.5 nM [^{3}H] progesterone and incubating overnight at 4 °C. Then nonradioactive progesterone was added in various amounts to block reassociation of ligand with receptor and thus to promote first order dissociation of the existing complexes. We have previously shown (Schrader *et al.*, 1974) that receptor-hormone complexes show an infinite half-life in the absence of added cold progesterone. The half-life dropped to 10 h at 0 °C in the presence of 100 nM cold hormone. In Fig. 2*D*, the apparent half-life fell from infinity to 10 h as the cold hormone concentration approached 5 nM. This concentration is far below the K_{diss} for occupancy of the lower affinity component (Fig. 2*B*). Thus, Fig. 2*D* shows that the alterations in kinetics of dissociation from the higher affinity sites do not require occupancy of the lower affinity class; furthermore, no additional alternation in rate was seen even at cold progesterone concentrations five times higher than the K_{diss} for the lower affinity class.

Thus, the kinetics of hormone dissociation in the presence of differing amounts of cold progesterone and hormone-binding titrations at nonequilibrium (data not shown) did not indicate the presence of cooperativity among cytosolic progesterone-binding sites. Rather, the results were consistent with the existence of binding site heterogeneity.

The mixture of steroid hormone-binding sites could be resolved by using the methods of Rosenthal (1967) for correction of nonlinear Scatchard plots. This correction was applied to the data of Fig. 2*B* to yield the *dashed lines*, calculated on the basis of two classes of sites.

Following this correction, values for K_{diss} were obtained from the reciprocal slope of the *dashed lines*, and values for N, the site molarity, were determined from the respective x intercepts. The higher affinity class in Fig. 2*B* contained the characteristics of the strong progesterone site described previously with K_{diss} = 1.2 nM. The remaining class of sites had a lower affinity for progesterone (K_{diss} = 26 nM) but a somewhat higher concentration relative to the stronger binding sites.

Since progesterone has a finite affinity for other steroid receptors, especially glucocorticoids, we tested to see if the weaker class could represent progesterone interaction with receptors for other hormones. Fig. 3 contains results obtained when [^{3}H]progesterone titration was performed in the absence and presence of 10 nM testosterone and 10 nM triamcinolone acetonide, added together to block progesterone binding to the androgen and glucocorticoid receptors, respectively. Fig. 3 shows that both the ratio of the two classes of sites and their binding constants were unaffected. Some reduction in the N values occurred due to occupancy by the nonradioactive androgen and glucocorticoid of [^{3}H]progesterone-binding sites on the progesterone receptor. Parallel reduction of both classes of site in this experiment provided circumstantial

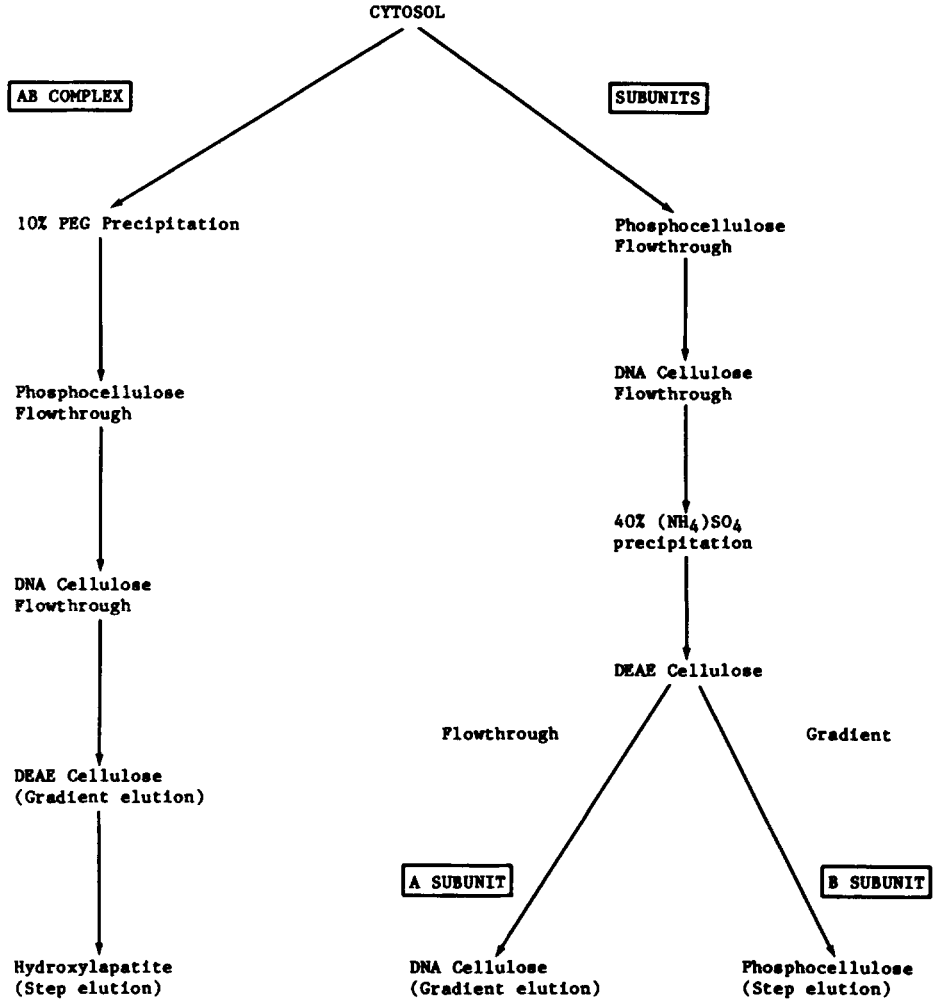

FIG. 1. **Preparation of progesterone aporeceptor 8 S aggregate (AB) and subunits (A and B).** For the preparation of AB aggregate, 150 ml of cytosol were precipitated by addition of 30% (w/v) polyethylene glycol (*PEG*) to a final concentration of 10%. After centrifugation at 38,000 rpm in a Beckman Ti45 rotor for 30 min, the receptor was resuspended in about one-eighth of the original volume of Buffer A and passed through a 20-ml phosphocellulose column and a 20-ml DNA-cellulose column. Then the receptor was applied to a 10-ml DEAE-cellulose column. Elution of the receptor was obtained with a 0.05 to 0.5 M KCl gradient in Buffer A. Fractions eluting at 0.25 KCl molarity were pooled, diluted 1:3 with 0.05 M sodium phosphate buffer, pH 7.8, and applied to 3-ml hydroxylapatite column. The AB aggregate was then step eluted with 0.3 M sodium phosphate buffer. For the A and B subunit preparations, 150 ml of cytosol were passed through a 50-ml phosphocellulose column and then through a 40-ml DNA-cellulose column. The DNA-cellulose flowthrough was precipitated by addition of saturated $(NH_4)_2SO_4$ in Buffer A to a final concentration of 40% saturation. The receptor subunits were collected by 30-min centrifugation at 1000 × *g* and then resuspended in Buffer A, and the solution was diluted with Buffer A until the conductivity was equivalent to 0.1 M KCl. The receptor solution was then applied to a 10-ml DEAE-column. The A subunit was collected from the DEAE-cellulose flowthrough and applied to a 3-ml DNA-cellulose column. Elution of the A subunit was obtained with a 0.1 to 0.5 M KCl gradient in Buffer A. The A subunit eluted at 0.25 M KCl. B receptor subunit was eluted from the DEAE-cellulose column with a 0.1 to 0.5 M KCl gradient in Buffer A. Fractions eluting at 0.28 M KCl were pooled, diluted 1:3 with Buffer A and applied to a 2-ml phosphocellulose column. The B subunits were step eluted with 0.4 M KCl in Buffer A. Periodically the elution profile of the aporeceptor from the columns was checked by single point analysis to ascertain that the fractions routinely pooled were actually those containing the highest amount of receptor.

evidence that the ligand specificities of the two site classes were similar.

The cytosol receptor preparations tested in Figs. 2 and 3 were prepared in low ionic strength; under these conditions subunits A and B were present in a larger complex. It was possible that steric interaction between the subunits of the complex had altered the ligand site of one subunit but not the other. To test this, receptor titrations with [^{3}H]progesterone were performed using cytosol receptors in the presence of 0.3 M KCl. Under these conditions, subunits A and B were completely dissociated from each other and behaved during gel filtration and ultracentrifugation as individual proteins not associated with other macromolecules (Schrader and O'Malley, 1972). The titrations done in 0.3 M KCl gave two-component Scatchard plots indistinguishable from those of Figs. 2 and 3 (data not shown).

A second explanation for the presence of the lower affinity sites was that some of the receptor's hormone-binding sites had been altered or partially denatured during isolation. To examine this possibility, both receptor subunits and the intact complex were isolated by the purification protocol shown in Fig. 1. Purification of each apoprotein (*i.e.* lacking bound

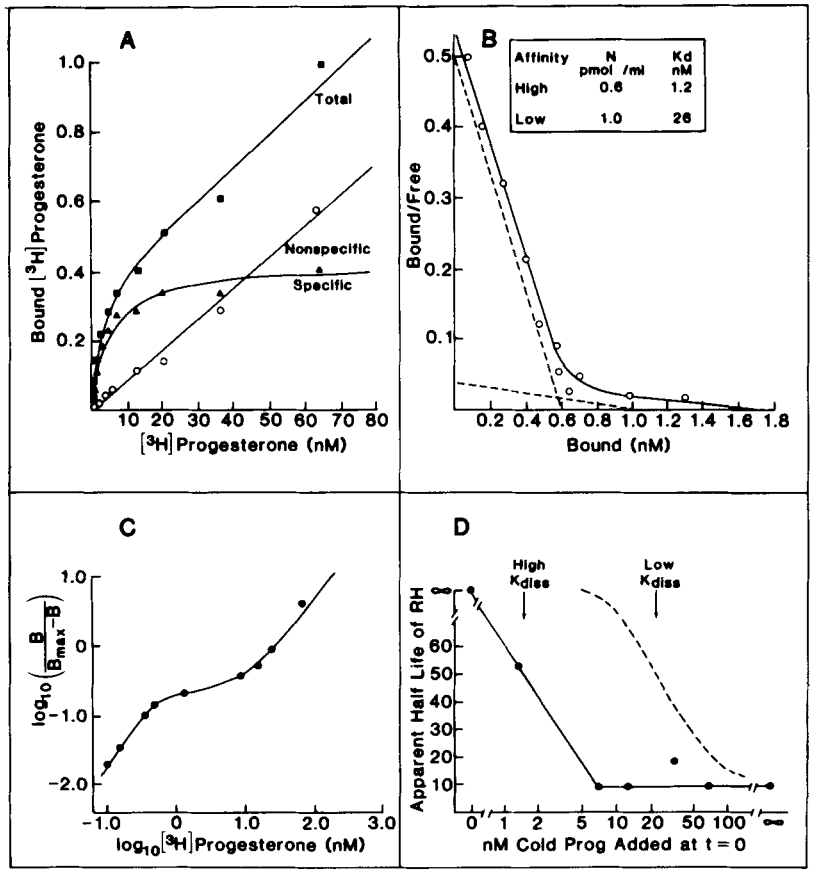

FIG. 2. **Hormone-binding titration of cytosol receptors using [^{3}H]progesterone.** *A*, aliquots (25 μl) of cytosol were incubated with increasing concentrations of [^{3}H]progesterone in the absence (total binding) or presence (nonspecific binding) of 100-fold excess of cold hormone at 4° C for 12 h. The quantity of [^{3}H]progesterone binding was determined by hydroxylapatite adsorption assay (see under "Experimental Procedures"). Specific binding (▲——▲) was determined by subtracting nonspecific binding (○——○) from total binding (■——■). *B*, Scatchard analysis of the data in *A*. The quantity of [^{3}H]progesterone specifically bound was plotted according to Scatchard (1949). The *dashed lines* were derived by the method of Rosenthal (1967). The *inset* indicates the values for K_{diss}, obtained from the reciprocal slope of the *dashed lines*, and for the site molarities, *N*, calculated from their *x* intercepts. *C*, Hill plot of the binding data. *D*, apparent half-life of stronger class of site as a function of cold progesterone concentration added to preformed B receptor complexes. Receptor B prepared as in Fig. 1 was labeled 18 h at 0 °C with 1.5 nM [^{3}H]progesterone. Dissociation was begun at $T = 0$ in 500-μl aliquots by additions of cold progesterone (0 to 200 nM) as indicated on the abscissa. Aliquots (75 μl) were removed at intervals from 0 to 30 h, and bound [^{3}H]progesterone was determined by hydroxylapatite assays. Semilogarithmic plots of complexes remaining *versus* time gave values for apparent dissociation rate constants, from which half-lives were determined. Apparent half-life is shown as a function of final progesterone concentration (●——●). The *dashed line* shows expected perturbation of half-life if occupancy of a weak class of site were involved. *Arrows* show apparent equilibrium constants for the two sites as determined in *B*.

hormone) was measured by ligand-binding assays. The degree of purification was about 1000-fold for A, 800-fold for B, and 500-fold for AB complexes. These preparations were devoid of other receptors for steroids tested, as shown by the example of Table I.

The three receptor preparations were then assayed for progesterone binding as shown in Fig. 4. This figure shows that both high and low affinity ligand sites were present in both the A and B fractions, as well as in the fraction containing intact AB complexes. When the ratio between the two sites of the three receptor preparations was compared to each starting material, it was clear that, despite considerable purification, the three receptor preparations had not been substantially enriched or depleted in one binding site class or the other. Furthermore, the binding constants were highly reproducible. Since the two classes co-purified, the data suggested that both binding sites could be on the same receptor proteins.

Although the two binding activities appeared to co-purify through a wide range of steps, the purification procedure did not include any steps involving separation on the basis of molecular size. Thus we used gel filtration to assay for coincident elution of the two-site classes. Since endogenous proteases can selectively cleave the progesterone receptor (Sherman *et al.*, 1976, 1977; Vedeckis *et al.*, 1980), we also used this gel filtration step to examine the possibility that the weaker site was a consequence of such proteolytic action. The behavior of the two sites was examined by Sephadex G-100 gel filtration chromatography, as shown in Fig. 5. The column did not resolve subunits A and B, which coeluted in fraction 25.

To perform this experiment, aliquots of purified B subunits were incubated overnight in the presence of [^{3}H]progesterone (0.3 nM) to label exclusively the high affinity site or 30 nM label both sites. Volumes of 1 ml were then chromotographed on G-100 as described under "Experimental Procedures." The elution profiles of the receptor sample were the same in both

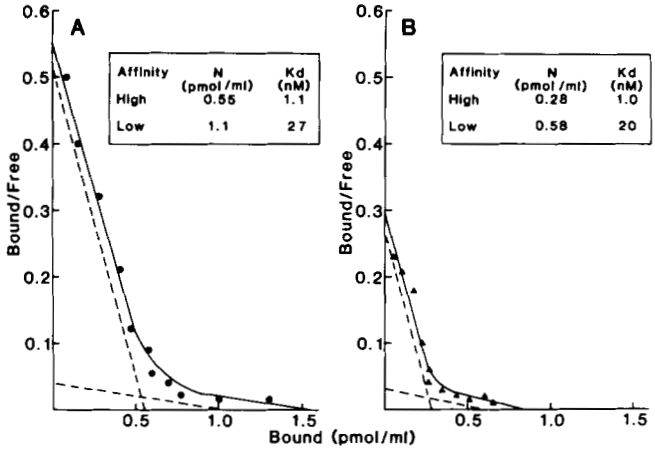

FIG. 3. **Glucocorticoid and androgen competition of [^{3}H] progesterone binding in cytosol of chicken oviduct.** Aliquots (25 μl) of cytosol preincubated (8 h at 4 °C) in the absence (*A*) or presence (*B*) of 10 nM triamcinolone acetonide and 10 nM testosterone were added to tubes containing increasing amounts of [^{3}H]progesterone for hormone-binding titration as described under "Experimental Procedures." The data obtained were plotted according to Scatchard. Site molarities and K_{diss} were calculated as described in Fig. 2.

TABLE I

Assays for various steroid receptor activities in preparations of partially purified receptor B subunits

Ligand	Concentration	Specific binding[a]
	nM	*pmol/ml*
[^{3}H]Estradiol	20	0.003
[^{3}H]Triamacinolone acetonide	20	ND[b]
[^{3}H]Testosterone	32	0.002
[^{3}H]Progesterone	30	1.9

[a] Portions (100 μl) of purified receptor B subunits were incubated with the concentrations of [^{3}H]steroid indicated in the table for 12 h at 4 °C in the presence or absence of a 100-fold excess of cold steroid. Binding was determined by use of dextran-coated charcoal to separate unbound [^{3}H]steroid from protein-bound radioactivity (Hansen *et al.*, 1976). The values represent means of duplicate determinations.

[b] ND, not detectable.

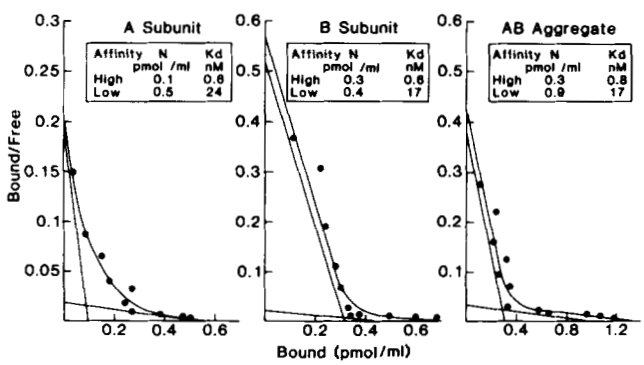

FIG. 4. **Scatchard plots of [^{3}H]progesterone binding to partially purified receptors.** Subunits A and B or AB receptor complex were prepared on different days from cytosol obtained from different animals. The scheme of purification is presented in Fig. 1. The extent of purification typically achieved was about 1000-fold for A, 800-fold for B, and 500-fold for AB. The extent of cross-contamination of A in B preparations and vice versa was less than 10%. Binding assays were carried out exactly as described in Fig. 2.

labeling conditions; a peak of receptor was seen in fraction 25. No binding was observed in fractions 32 or 51, where the predominant proteolytic fragments of receptor elute (Vedeckis

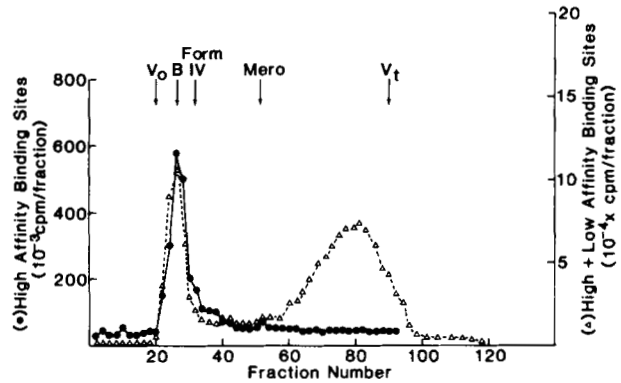

FIG. 5. **Sephadex G-100 gel filtration chromatography of two classes of progesterone-binding sites in subunit B preparation.** 1-ml aliquots of progesterone receptor B subunits (purified as shown in Fig. 1) were incubated with [^{3}H]progesterone at a concentration of 0.3 nM (●——●) or 30 nM (▵---▵) for 12 h at 4 °C prior to chromatography on Sephadex G-100. Experimental details are given under "Experimental Procedures." V_0, blue dextran; V_t, KCl; *B*, elution position of B subunit; *Form IV* and *Mero*, elution position of the proteolytic fragments described by Sherman and Diaz (1977) and Vedeckis *et al.* (1980). Protein standards eluted in the following fractions: bovine serum albumin (M_r = 68,000) fraction 32; ovalbumin (M_r = 42,000) fraction 44; cytochrome *c* (M_r = 12,500) fraction 57.

et al., 1980). We concluded that significant proteolysis of receptor to yield smaller fragments had not occurred and thus could not explain the presence of the lower affinity class of binding sites. Rather, the data of Fig. 5 were again consistent with the existence of both sites on the same protein.

A second method based upon protein size was chosen to determine if both sites were contained on the same proteins. For this purpose, sucrose gradient ultracentrifugation was used. Since both subunits are highly asymmetric, analysis by both gel filtration and sedimentation velocity are excellent criteria for identity. Furthermore, the receptor complex could be reversibly dissociated from the intact 8 S state to a mixture of 4 S A and B monomers. Thus, if the weaker sites were indeed associated with the receptor itself, the two types of sites should shift their sedimentation coordinately as a function of ionic strength. The results of the sedimentation studies are shown in Fig. 6. For these experiments, receptor samples were either treated with 0.3 or 40 nM [^{3}H]progesterone to label either the high affinity sites alone or both classes together. The figure shows coincident sedimentation of the two classes of binding sites in both high and low ionic strength. The data shown are not corrected for nonspecific adsorption of [^{3}H]progesterone to abundant proteins sedimenting at about 3 to 4 S. Label bound to these other proteins sedimented as a broad peak in fractions 2–10 (Fig. 6, *left*). In the presence of a large excess of nonradioactive progesterone, the two types of sites were both undetectable due to occupancy by the unlabeled ligand (data not shown). The lower affinity receptor sites were detected coincident with the strong sites in both parts of Fig. 6. Thus, by this criterion as well, both sites behaved as if they were present on the same receptor proteins.

Studies of Receptor Binding by Photoaffinity Labeling—We have shown previously that the progesterone receptor can be covalently labeled with [^{3}H]R5020 (17α-, 21-dimethyl-19-norpregn-4,9-diene-3,20-dione), a synthetic progestin (Dure *et al.*, 1980). The high affinity binding site has been mapped to a single receptor peptide on both A and B using this method (Birnbaumer *et al.*, 1983). Thus, it was possible to undertake a more stringent test for the presence of the lower affinity site on the authentic receptors by receptor photoaffinity la-

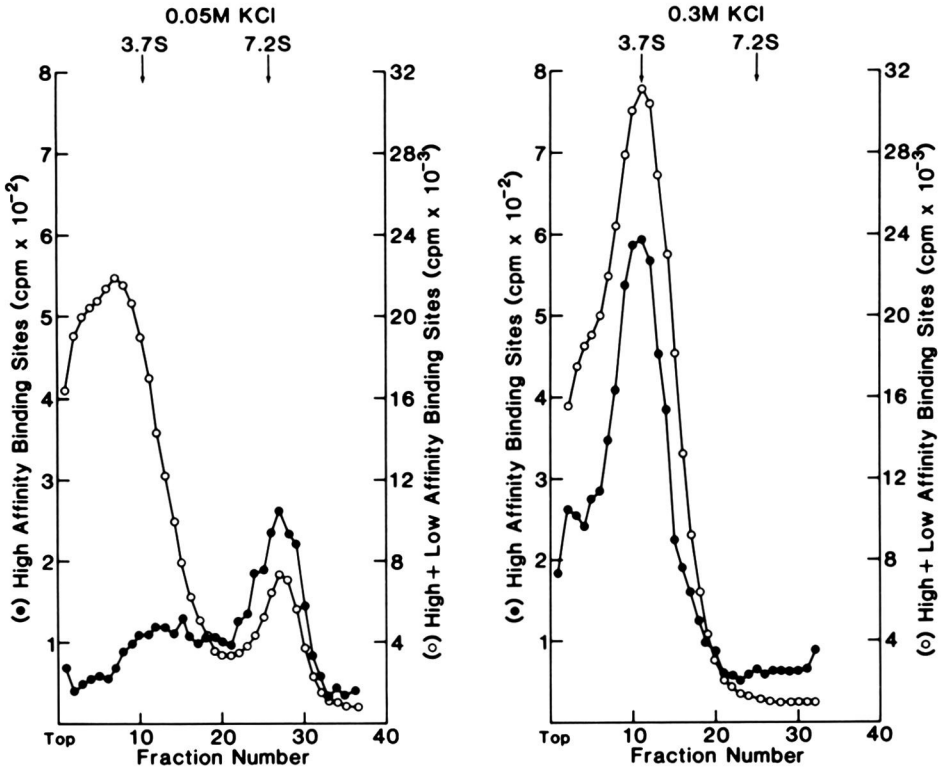

FIG. 6. **Sucrose gradient analysis of high and low affinity progesterone-binding sites.** Cytosol progesterone receptor was incubated overnight in the presence of 0.3 (●) or 40 nM (○) [^{3}H] progesterone. Dissociation of the 8 S complex to 4 S subunits was obtained by incubating the samples for 2 h in a final KCl concentration of 0.3 M. The sucrose gradients were run as described under "Experimental Procedures." *Left*, gradient containing 0.05 M KCl; *right*, gradient containing 0.3 M KCl.

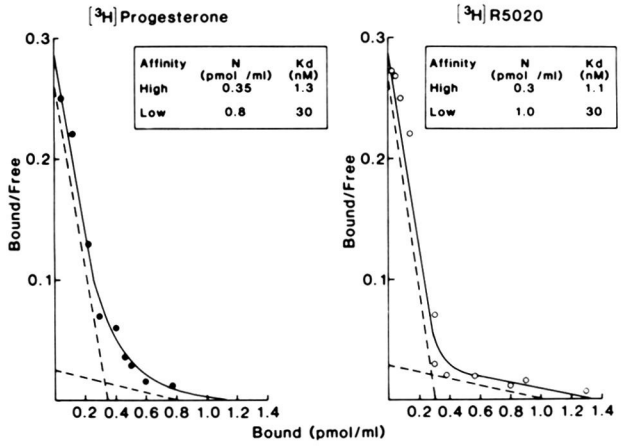

FIG. 7. **Hormone binding titration in cytosol of chicken with [^{3}H]progesterone and [^{3}H]R5020.** The titration of cytosolic receptor and the analysis of the data were performed as described in Fig. 2. *Left*, progesterone data. *Right*, R5020 data. ---, resolved plots for two binding sites.

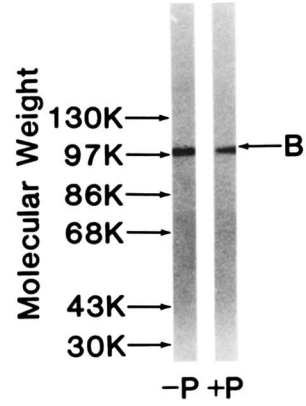

FIG. 8. **Analysis by gel electrophoresis of covalent [^{3}H] R5020-subunit B complex under denaturing conditions.** After partial purification (as described in Fig. 1) A and B subunits were incubated in the presence or absence of 1 nM progesterone for 12 h at 4 °C. Then 40 nM [^{3}H]R5020 was added for 2 h at 4 °C prior to irradiation at 320 nm. Aliquots were then run on a sodium dodecyl sulfate-polyacrylamide gel electrophoresis slab gel (7.5% total acrylamide) at 33 mA for 2 h. The gel was stained with Coomassie Blue, impregnated with EN^{3}HANCE (New England Nuclear) and dried. The figure shows the fluorography of the slab gel using Kodak XRP film (21 days, −70 °C). *Left lane*, no cold progesterone added; *right lane*, with addition of 1 nM progesterone.

beling followed by polyacrylamide gel electrophoresis under denaturing conditions in sodium dodecyl sulfate followed by fluorography of the gel.

The binding of [^{3}H]R5020 to both sites was tested first as shown in Fig. 7. The same receptor preparation was titrated with either [^{3}H]progesterone or [^{3}H]R5020. The figure shows Scatchard plots of the binding data after correction for nonspecific adsorption by the method of Fig. 2. Both classes of sites were detected by [^{3}H]R5020; furthermore, the ratio of sites and the K_{diss} values for the two compounds were similar. The titrations with R5020 further substantiated the similarity of hormone preference for the two site types.

Next, we covalently coupled [^{3}H]R5020 to the receptor at concentrations of ligand which would label preferentially the lower affinity sites (Fig. 8). In this experiment, receptors were incubated first with nonradioactive progesterone at a concentration of 1.0 nM to bind to (and thereby block) about half of the strong binding sites (as estimated from the K_{diss} data of Fig. 7). Then 40 nM [^{3}H]R5020 was added for 2 h at 4 °C. Since the half-life of the strong sites is 12 h at this temperature (Hansen *et al.*, 1976), negligible exchange of [^{3}H]R5020 onto occupied strong sites could occur. Since Fig. 7 showed three times as many lower affinity sites as higher affinity ones, the progesterone blockage should have resulted in a 6-fold difference in the apparent number of labeled low affinity sites relative to the high affinity site. The receptor B preparations were then irradiated with UV light as described elsewhere (Dure *et al.*, 1980), a procedure which photocouples

about 10% of the bound [^{3}H]R5020 to its receptor site.

The analysis of this photolabeling study is shown in Fig. 8. Fluorography of the slab gel showed ^{3}H bands at 108,000 for the B subunit, as we reported previously. Thus, at 40 nM [^{3}H] R5020 only the receptor bands were labeled covalently. This study suggested that the weak sites have thereby been labeled and that they exist on the authentic receptor molecules. Confirmation of this concept awaits peptide mapping of the photoadducts. Although less likely, we cannot presently exclude the possibility that *no* labeling of the low affinity sites occurred and that the signals seen in Fig. 8 were simply the labeling by [^{3}H]R5020 of the residual high affinity sites which were not occupied by the prior addition of 1 nM unlabeled progesterone.

Analysis of Receptor-binding Sites by Ion Exchange and Adsorption Chromatography—The foregoing experiments revealed by four separate criteria that a weaker class of progesterone-binding sites exists. There still remained the possibility that the lower affinity sites were created during receptor isolation, perhaps due to partial receptor denaturation. To test this possibility, we attempted first to resolve the two classes of sites by DNA-cellulose chromatography of the receptor A subunits as shown in Fig. 9. We reasoned that the two classes of sites might have been resolved on DNA-cellulose if denaturation were involved. The results in Fig. 9 showed that the application of this chromatographic step did not lead to the resolution of the two site classes. Receptor A apoprotein was chromatographed on DNA-cellulose. Aliquots of each fraction were then analyzed by single point hormone-binding assays using either 0.5 or 50 nM [^{3}H]progesterone to detect either only the high affinity sites or both sites, respectively. Fig. 9, *top*, demonstrates the coincidence of the specific hormone-binding curves obtained by these two assays. Since the single concentration assays are not sufficiently quantitative, we determined the general shape of the elution profile from the *top part* and then performed Scatchard plot saturation curves on three fractions indicated by the *hatched bars*. These plots are shown in the *lower portion* of the figure. Significantly, all three fractions gave the same ratio of the two sites, about 1:2. Thus the two classes of sites on receptor A co-eluted from DNA-cellulose. By this criterion, it was unlikely that the weak sites arose from partial denaturation.

A similar analysis of receptor B was carried out using DEAE-cellulose chromatography as shown in Fig. 10. The receptor was labeled with 0.5 nM [^{3}H]progesterone prior to chromatography on DEAE-cellulose. The DEAE-cellulose elution profile is shown in the *top part*. Subsequent saturation curves on the three fractions (*hatched bars*) gave the results shown in the *lower part*. In this experiment, the relative concentration of lower affinity sites was increased to 4-fold in the fractions eluting at the higher ionic strength. This result indicated that the weak sites were preferentially localized in the more acidic fractions of receptor B and somewhat under-represented in the less acidic fraction eluting at lower salt. Since we knew from earier studies that a charge heterogeneity existed in the receptor population, we were encouraged

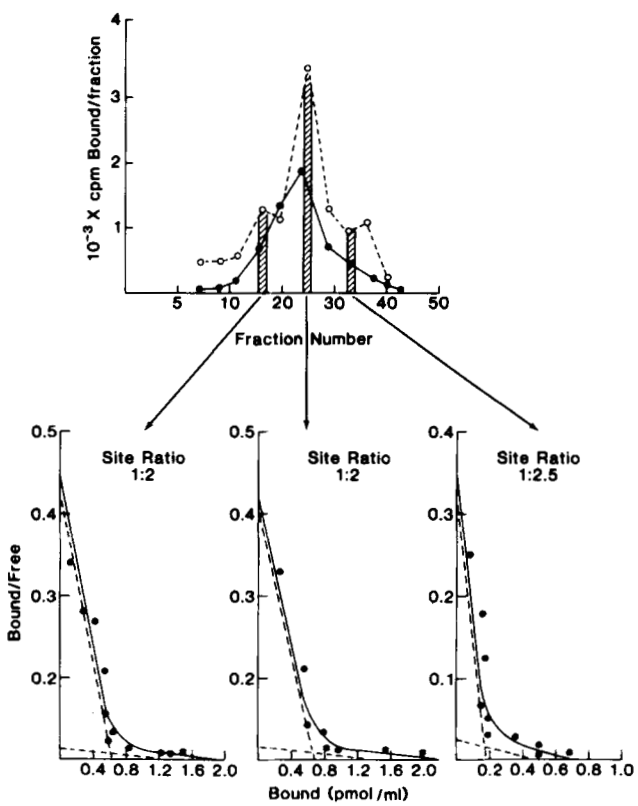

FIG. 9. **DNA-cellulose elution profile of high and low affinity hormone-binding sites in subunit A preparation.** Progesterone aporeceptor A from a DEAE-cellulose column (see Fig. 1) was diluted to 0.01 M KCl with Buffer A and applied to a 3-ml DNA-cellulose column. After washing with 15 ml of Buffer A containing 0.01 M KCl, the adsorbed receptors were eluted with a linear 0.01 to 0.4 M KCl gradient in Buffer A, and 1-ml fractions were collected. *Top*, elution profile of the high affinity sites only (●——●), the sum of both sites (O---O), obtained by single point analysis of each fraction at [^{3}H]progesterone concentrations of 0.5 and 50 nM, respectively. *Hatched bars* indicate the fractions collected to perform the hormone titrations. *Bottom*, Scatchard plots from the titrations as described in Fig. 2.

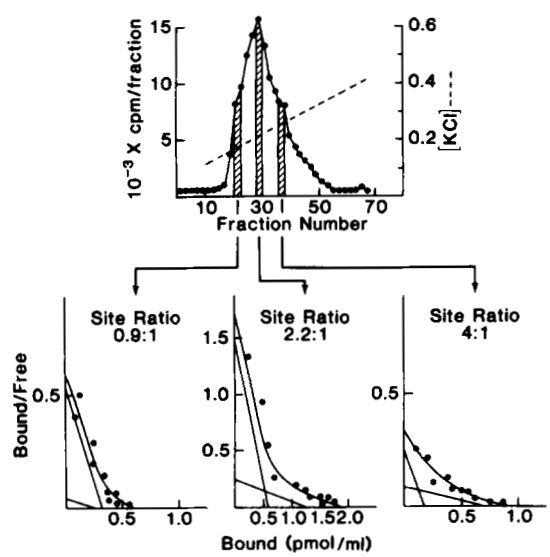

FIG. 10. **DEAE-cellulose elution profile of high and low affinity progesterone subunit B-binding sites.** Progesterone receptor A and B subunits precipitated with $(NH_4)_2SO_4$ were resuspended in Buffer A, incubated with 0.5 nM [^{3}H]progesterone for 2 h at 4 °C (in order to label the higher affinity sites), and applied to a 5-ml DEAE-cellulose column. Subunit A was washed off the column by extensive washing at 0.1 M KCl in Buffer A. Then subunit B was eluted using a 0.1 to 0.5 M KCl gradient, and 1-ml fractions were collected. *Top*, elution profile of the receptor from the column. *Hatched bars* indicate the fractions which were pooled to quantify the two site classes. A small aliquot of each fraction was also pooled and applied to a DNA column at a KCl concentration of 0.1 M to assay for the presence of A subunit. More than 95% of the total counts were observed in the DNA-cellulose flowthrough indicating the absence of subunit A in the DEAE peak. The *lower part* of the figure shows Scatchard analysis from the titration of the receptor eluting at different KCl concentrations.

to perform the experiments listed below.

Effect of Receptor Phosphorylation on the Low Affinity Progesterone Sites—Recent experiments conducted in our laboratory have demonstrated that progesterone receptors are excellent substrates for cAMP-dependent protein kinase (Weigel *et al.*, 1981). Furthermore, A and B subunit analysis on two-dimensional gels demonstrated the presence of charge heterogeneity which could be correlated with the state of phosphorylation of the receptor.

On the basis of these data and those already published in the literature (Nielsen *et al.*, 1977; Auricchio *et al.*, 1981), we speculated that receptor phosphorylation could have altered the activity of the lower affinity site and simultaneously altered the receptor elution behavior during DEAE chromatography. We tested this hypothesis by treating partially purified B subunit preparations (containing both types of sites) with highly purified bovine intestine alkaline phosphatase.

We first tested the effect of different concentrations of alkaline phosphatase on the two sites. As shown in Fig. 11, the enzyme preferentially inactivated the lower affinity site; at high concentrations (36 units) the high affinity site was also affected. To test whether the effect observed was due to phosphatase activity or to a contaminating protease, we performed a time course using low amounts of enzyme (3.6 units) in the presence and absence of a competitive inhibitor, glucose 1-phosphate. The results are shown in Fig. 12. Using this concentration of enzyme for up to 2 h at 20 °C, only the lower affinity site was inactivated. Furthermore, glucose 1-phosphate inhibited the phosphatase effect by over 50%. Since glucose 1-phosphate did not completely prevent the inactivation of the lower affinity site and high concentrations of the enzyme also affected the high affinity site, we postulated that the commercial phosphatase had also some proteolytic activity. To test for this possibility we incubated progesterone receptor covalently labeled [^{3}H]R5020 together with 36 units of phosphatase for 2 h at 20 °C. The samples were then electrophoresed on sodium dodecyl sulfate-polyacrylamide gel electrophoresis slab gel and fluorographed. As shown in Fig. 13, phosphatase substantially decreased the total amount of receptor protein. Thus, protease contamination also existed in these experiments. Similar experiments were carried out testing alkaline phosphatase from other sources, with similar results on the destruction of the lower affinity site class.

Additional control experiments using phosphatase are presented in Table II. The data show a requirement for native

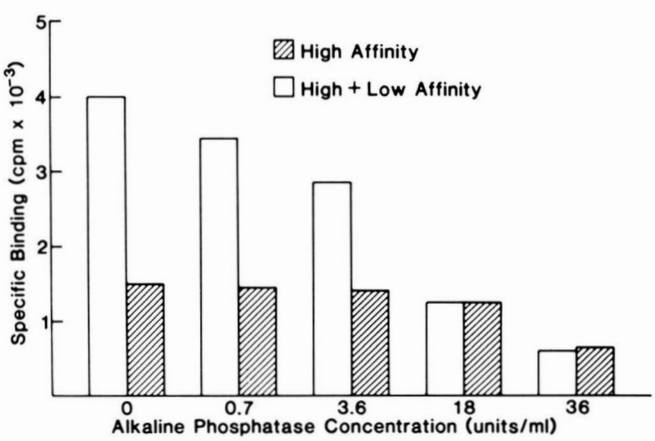

FIG. 11. **Effect of alkaline phosphatase on hormone-binding capacity of progesterone receptor.** Partially purified aporeceptor B subunits (in 500 μl of Buffer A, pH = 8 with 0.3 M KCl, 0.1 mM $ZnCl_2$, and 1 mM $MgCl_2$) were incubated with increasing amounts of alkaline phosphatase (0.7 to 36 units) to 20 °C for 30 min. Specific binding was calculated (as described under "Experimental Procedures") at 0.3 nM [^{3}H]progesterone (*hatched bars*) and at 30 nM [^{3}H] progesterone (*open bars*).

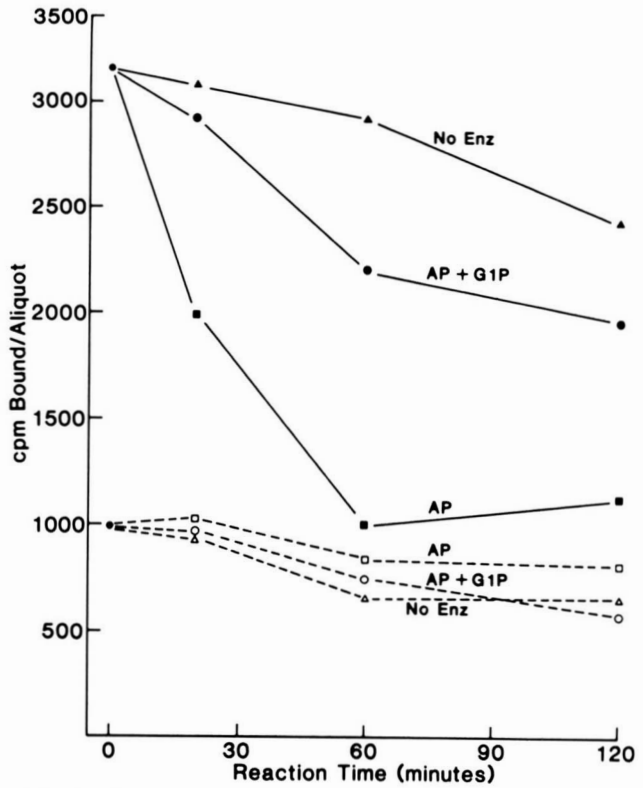

FIG. 12. **Time course of bovine intestine alkaline phosphatase effect on high and low affinity progesterone binding sites.** Aliquots (1500 μl) of purified B aporeceptor were incubated at 20 °C (△---△, ▲——▲); at 20 °C in the presence of 3.6 units of alkaline phosphatase (*AP*; □---□, ■——■); or at 20 °C in the presence of 3.6 units of alkaline phosphatase and 10 nM glucose 1-phosphate (*G1P*, ○---○, ●——●). At the indicated times, 500-μl portions were removed, and the enzymatic reaction was stopped by addition of 10 nM EDTA. The specific binding was determined at 4 °C with [^{3}H]progesterone 0.5 nM (△, ○, □) and 50 nM (▲, ●, ■) as described under "Experimental Procedures."

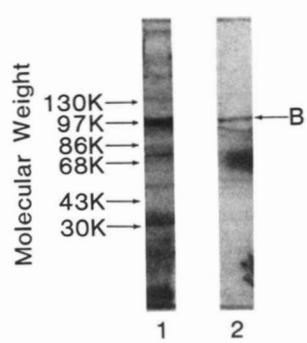

FIG. 13. **Assay for proteolytic activity in bovine intestine alkaline phosphatase.** Receptor B apoprotein was purified as shown in Fig. 1 and labeled with 20 nM [^{3}H]R5020 for 12 h at 4 °C. After irradiation at 320 nm to photolabel the progesterone receptor, 100-μl aliquots were incubated at 20 °C for 2 h either alone or in the presence of 36 units of alkaline phosphatase. The incubation was stopped by the addition of 3% sodium dodecyl sulfate and by boiling. 30-μl aliquots of each sample was then run on a 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel electrophoresis system. The gel was then dried and processed as described in the legend of Fig. 7. *Lane 1*, control; *lane 2*, [^{3}H]R5020-receptor complex incubated with alkaline phosphatase.

TABLE II

Effect of alkaline phosphatase and inhibitors on receptor progesterone-binding sites

Treatment	Specific binding[a]	
	Using 0.3 nM [^{3}H]progesterone	Using 30 nM [^{3}H]progesterone
	pmol/ml	
Control at 20 °C[b]	0.014	0.19
Phosphatase[c]	0.013	0.04
Phosphatase + Na_2MoO_4 (10 mM)	0.015	0.18
Phosphatase + $NaVO_3$ (1 mM)	0.013	0.18
Heat-inactivated phosphatase[d]	0.015	0.18
Na_2MoO_4 (10 mM)	0.013	0.17
$NaVO_3$ (1 mM)	0.015	0.17

[a] Determined by single point analysis as described under "Experimental Procedures" at two ligand concentrations.

[b] Samples of receptor B from hen oviduct partially purified by the protocol of Fig. 1, incubated under the conditions described in Fig. 13.

[c] Bovine intestinal phosphatase, prepared as described under "Experimental Procedures," was added at a concentration of 4 units/tube.

[d] Enzyme was inactivated by boiling for 10 min.

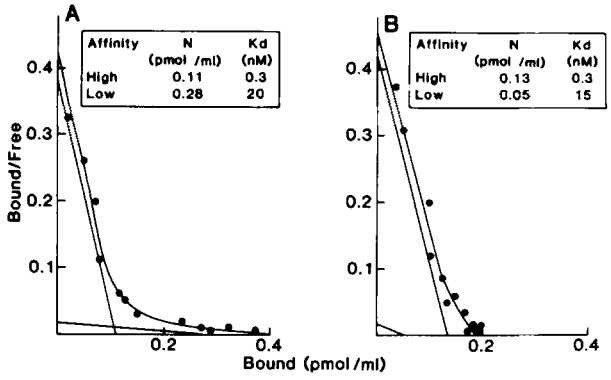

FIG. 14. **Scatchard plot of alkaline phosphatase effect on receptor B apoprotein.** Portions of partially purified B subunit were incubated at 20 °C for 30 min with alkaline phosphatase buffer (*A*) or with 4 units of alkaline phosphatase (*B*). Hormone titration analysis was then carried out at 4 °C as described in Fig. 2.

enzyme, since boiling blocked its activity. Similarly, inhibitors such as $NaMoO_4$ and $NaVO_3$ were effective, again affecting only the lower affinity sites. In order to quantify more accurately the phosphatase effect, progesterone titration studies were performed on the B subunit apoprotein which had been incubated with alkaline phosphatase. As shown in Fig. 14, exposure to 4 units of alkaline phosphatase for 30 min at 20 °C destroyed over 80% of the lower affinity sites with no loss of the high affinity sites. Furthermore, the value for K_{diss} obtained from the slopes of Fig. 14 were unchanged, as shown in the *inset boxes.*

Alterations in the Concentration of Low Affinity Binding Sites as Affected by the Hormonal Status in Vivo—We observed frequent variation in the concentration of weak sites relative to strong sites when receptors were assayed in oviducts from estrogenized immature chicks. However, the concentration ratio was less variable when laying hen oviducts were used. Thus, we evaluated this parameter as a function of the developmental status of the oviduct and as influenced by estrogen. These results are presented in Table III. Scatchard plots were performed on [^{3}H]progesterone titration data obtained for crude cytosol preparations at each stage tested. The table shows that there was a low but finite amount of progesterone receptor in unstimulated oviducts, a number

TABLE III

Effect of estrogen treatment on receptor-binding sites

Hormonal state	Average weight of single oviduct	High affinity sites	Low affinity sites	Number of observations
	g	*pmol/g fresh tissue (±S.E.)*	*pmol/g fresh tissue (±S.E.)*	
Unstimulated	0.025	10.7	31.3	1[a]
Acute stimulation[b]	0.035	28.0	16	1[a]
Chronic stimulation[c]	3.0 ± 0.2	203 ± 24	227 ± 80	7
Withdrawal[d]	1.2 ± 0.1	265 ± 15	ND[e]	6
Restimulation[b]	2.3 ± 0.1	280 ± 20	ND	3
Mature animal	25 ± 3	19 ± 6	43 ± 4	6

[a] One single saturation experiment was performed in cytosol obtained from 20 oviducts (unstimulated chicks) or 10 oviducts (stimulated chicks).

[b] 24-h acute stimulation was obtained with a single intramuscular injection of 1 mg of estradiol benzoate (Sigma).

[c] Two-week-old female chicks were implanted weekly with 20-mg pellets containing 20 mg of diethylstilbestrol (Rhodia, Inc.) and were sacrificed within 7 days from the last implant.

[d] Chicks were withdrawn for periods of 2 weeks before being sacrificed.

[e] ND, not detectable.

which rose about 20-fold by chronic estrogen (diethylstilbestrol) treatment. This change is similar to that reported earlier (Toft and O'Malley, 1972). The binding site ratio (high to low affinity) varied in this series but was generally between 1:3 and 1:1. Significantly, upon acute withdrawal of estrogen for 10 days, the weak sites became undetectable, whereas the strong sites were unaffected. Readministration of estrogen for 48 h did not alter this ratio nor did it restore the weak sites to a measurable titer. Finally, when laying hen oviducts were examined, the site ratio was again 1:2. The concentrations of receptor sites in laying hens were only one-tenth of those seen in chronically estrogenized chicks, due to the presence of endogenous progesterone bound to the receptors in the former, but not in the latter (Schrader *et al.*, 1977). Thus, we conclude that the lower affinity sites for progesterone were altered somehow in their abundance by the hormonal status of the animal.

DISCUSSION

In this paper we have demonstrated the presence of two distinct classes of binding sites for progesterone in chicken oviduct. These sites can be distinguished because of their different affinities for the hormone. Many authors, working in different systems, have reported multiplicity of binding sites for glucocorticoids (Barlow *et al.*, 1979; Do *et al.*, 1979) and estrogens (Clark *et al.*, 1978; Smith *et al.*, 1979; Capony and Rochefort, 1978; Markaverich and Clark, 1979). Not all of them are thought to be true receptors (Rousseau and Baxter, 1979). For this reason it was necessary to establish the physicochemical characteristics of a newly identified binding site before assuming its status as a putative receptor. The only system in which this multiplicity of binding sites has been well characterized is the estrogen receptor in rat uterus. Clark's laboratory has demonstrated the existence of two sets of estrogen-binding sites (Clark *et al.*, 1978; Markaverich and Clark, 1979) which were named Type I (the classical estrogen receptor) and Type II (binders of lower affinity for estradiol). Such binding sites seem to be located in different molecules as demonstrated by their different sedimentation on sucrose

gradients and varying capacities to be translocated to the nuclear compartment.

In the case of the progesterone receptor, our study suggests that both sites are located on the same receptor molecule. In fact, they (*a*) comigrate on sucrose gradients as an 8 S complex in hypotonic buffer and also as a 4 S complex in high salt buffer (Fig. 6), (*b*) coelute from gel filtration columns (Fig. 5), and (*c*) copurify through a wide variety of chromatographic steps (Fig. 4). In addition, they are both translocated into the nuclear compartment (data not shown). However, it is interesting to note that a careful study of the elution of the two sites from DEAE-cellulose clearly demonstrates that the lower affinity sites are preferentially associated with the more acidic molecules (Fig. 10). Since we have recently demonstrated that the progesterone receptor can be phosphorylated, we hypothesized that this type of site is found in association with the phosphorylated form of the receptor. Further support for this hypothesis is provided by the observation that alkaline phosphatase dramatically reduced the number of low affinity sites (Fig. 14). Unfortunately, the presence of proteolytic activity in the commercial alkaline phosphatase (Fig. 11) complicates the interpretation of the data. However, since we could demonstrate that phosphatase inhibitors prevented the effect observed on the lower affinity site (Table II, Fig. 12), but not on the high affinity site, we postulate that the effect observed on the latter at high doses of phosphatases (Fig. 11) was mainly due to proteolysis of the entire receptor protein. A role of phosphorylation in activation of the binding sites of steroid hormone has been proposed by many authors (Nielsen *et al.*, 1977; Yuh and Keyes, 1981; Auricchio *et al.*, 1981). However, nobody yet has reported whether phosphorylation affects all the populations of steroid receptor or specific subsets of them. In this regard, it is of interest to note that the study conducted by Auricchio *et al.* (1981) on deactivation of estrogen receptors in calf uterus by endogenous phosphatases showed that only 40% of the total population of the estrogen receptor could be inactivated by the enzyme. Further investigation is needed to understand whether this lack of sensitivity of 60% of the receptor population is associated with any specific type or subclass of the estrogen receptor.

We have demonstrated that the two avian progesterone-binding activities (*a*) are located on the same molecule; (*b*) do not act cooperatively; (*c*) have similar ligand specificity and the same relative affinities for R5020 (Fig. 7), testosterone, and triamcinolone acetonide (Fig. 3). The question can be raised whether they actually are distinct sites or one single site at which affinity for the hormone is modulated, for example, via phosphorylation. The demonstration that alkaline phosphatase dramatically affects the number of low affinity sites without altering (increasing) the number of high affinity sites argues against the possibility of interconversion between the two forms. Yet, since proteases are active in the commercial enzyme, the definitive proof of a lack of interconversion would be obtained only by studying the effect of phosphorylation on the two classes of binding sites.

Initial attempts to phosphorylate *in vitro* crude preparations of aporeceptor were unsuccessful, probably due to the relatively low concentration of receptor protein (with regard to the K_m of the enzymes used) and due to the presence of contaminating proteins which acted as competitive substrates.

With regard to the exact relative abundance of the two sites, only rough approximations can be made at present. In fact, all our studies were conducted in the presence of a population of molecules containing various degrees of phosphorylation. The results shown in Fig. 10 suggest that fully phosphorylated progesterone receptors contain lower and higher affinity sites at a ratio of at least 2:1. From the data shown, even a higher ratio was possible, but in such experiments we had no way to determine how many of the molecules eluting in the late peak had functional high affinity sites.

The physiological role and importance of this newly described site in the mechanism of action of progestrone may be considerable. Many authors have suggested that phosphorylation could be a way to transform steroid hormone receptors. Barnett *et al.* (1980) suggested that dephosphorylation of glucocorticoid receptors is associated with an increase in their DNA-binding activity. Nishigori and Toft (1980) demonstrated that molybdate inhibits transformation of the avian progesterone receptor, as determined by increased sedimentation rate and augmented affinity for nuclei. Our results on elution from a DNA column (Fig. 9) suggest that the two classes of binding sites have similar DNA-binding activity.

We still do not know whether "activation" of the lower affinity site has any effect on the translocation of the cytoplasmic receptor to the nuclear compartment. At the present time, we are attempting to purify the cellular phosphorylated form of the receptor to determine whether it preferentially binds to nuclei or to specific sequences of the ovalbumin gene.

Finally, in light of the phosphorylation results included in Figs. 10 and 11, it is tempting to speculate that the reason for the estrogen effect on the concentration of the weaker sites is that estrogen alters the activity of cellular phosphatases and kinases which act on the receptor substrate. A candidate phosphatase in cell nuclei has been identified for the uterine estrogen receptor (Auricchio *et al.*, 1981). We reported earlier the characterization of at least three distinct protein kinases of chick oviduct (Keller *et al.*, 1976). The enzymes are suitable candidates for tests on the receptor proteins.

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