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Glucocorticoid Receptors in Mouse Mammary Tumors: Specific Binding to Nuclear Components†

G. Shyamala

ABSTRACT: The specific interaction of glucocorticoids with nuclei of mouse mammary tumor was studied *in vitro* by incubation of the tissue with [^{3}H]dexamethasone at 25°. It was demonstrated that the mammary tumors contain a limited number of specific nuclear binding sites which were saturated with low hormone concentrations (10^{-8} M). The concentrations of specific binding sites in the nuclei were related to the concentration of cytoplasmic binding sites of unincubated tissues and varied between individual tumors. The binding component in the nuclei appeared to be a protein and was easily solubilized with 0.4 M KCl containing buffers. The ability of various corticoids to block the nuclear localization of the steroid correlated well with their glucocorticoid potency. Estradiol and progesterone at concentrations of 10^{-6} M were also effective in competing for the glucocorticoid receptor binding sites. However, while the glucocorticoids such as hydrocortisone and corticosterone translocated to nuclear sites also specific for dexamethasone, estradiol and progesterone competed for the cytoplasmic binding sites and did not translocate to the nucleus. The possible significance of the interaction of various steroids with the glucocorticoid receptors in mammary tumors is discussed.

It is currently believed that an initial step in the mechanism of steroid hormone action involves the specific binding of the hormones to the receptor proteins in target cells (Raspe, 1971). In the case of glucocorticoid hormones, such receptors have been demonstrated in liver (Beato *et al.*, 1971), thymus (Munck and Wira, 1971), kidney (Funder *et al.*, 1973), fetal lung (Giannopoulous, 1973a,b; Ballard and Ballard, 1972; Toft and Chytil, 1973), hepatoma cells (Baxter and Tomkins, 1971), steroid sensitive lymphoma cells (Baxter *et al.*, 1971), normal mammary glands (Shyamala, 1973), and pituitary tumor cells (Watanabe *et al.*, 1973, 1974).

Corticosteroid hormones are directly involved in milk secretion in mice (Lyons *et al.*, 1958) and it has been suggested that the hormonal influence, while not inductive, is a permissive one which is essential for mammary tumor ap-

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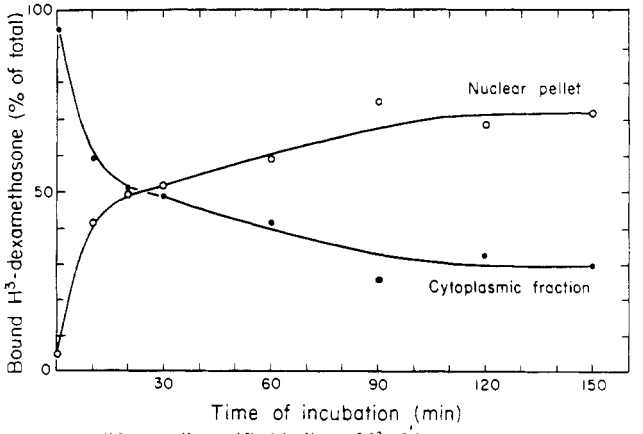

FIGURE 1: "*In vitro*" specific binding of [^{3}H]dexamethasone by mammary tumor slices. Mammary tumor slices were incubated with 3×10^{-8} M [^{3}H]dexamethasone for varying time intervals at 25°. Parallel sets of incubations were done in the presence of 100-fold excess of nonradioactive dexamethasone to determine nonspecific binding. The control samples (0 min) were incubated for 2 hr at 4°. At end of incubations, cell fractions were prepared and assayed for binding as described under Materials and Methods.

pearance (Bern and Nandi, 1961). Studies from our laboratory (Nandi, 1959) on the mammary tumor virus (MTV)[1] infected mouse strains indicate that corticosteroids are one of the principal classes of hormones required for maintenance of precancerous lesions and for development of tumors. Mammary tumor virus production increases during lactation (Huseby *et al.*, 1950) and cortisol treatment of tumor bearing mice results in the appearance of cytoplasmic inclusion bodies composed of large aggregations of virus like particles (Smoller *et al.*, 1961). Cortisol is effective in inducing MTV replication in cell cultures of mammary tumors (McGrath, 1971). Cortisol can also cause atrophy of mammary adenocarcinomas (Sparks *et al.*, 1955). Thus the MTV induced mammary tumors are clearly responsive to glucocorticoid hormones.

Recently we have shown that MTV induced mammary tumors contain a specific cytoplasmic glucocorticoid hormone receptor (Shyamala, 1974). Glucocorticoid receptors are also present in mammary tumors of rats (Gardner and Witliff, 1973). It has been proposed that the effects of glucocorticoids in target tissues result from steroid-mediated interaction of the receptor with the genome (Gemmeltoft and Schaumburg, 1972). In all steroid–receptor systems studied so far the nuclear localization of the hormone seems to involve a two-step mechanism whereby the hormone is translocated from the cytoplasm to the nucleus (Raspe, 1971). Such interactions with glucocorticoids have not been reported for mouse mammary tumors. In this report specific interactions of glucocorticoids in mammary tumors and the isolation and characterization of the binding components are described.

Materials and Methods

The GR strain mice were from our own colony; these mice carry the MTV and show about 82% incidence of mammary tumors in breeding females.

All radioactive steroids were purchased from New England Nuclear Corporation and were chromatographically pure. The specific activities of the steroids were as follows: [^{3}H]dexamethasone (35.2 Ci/mmol); [^{3}H]hydrocortisone (51.7 Ci/mmol); [^{3}H]corticosterone (40 Ci/mmol); [^{3}H]progesterone (44 Ci/mmol); [^{3}H]estradiol (48 Ci/mmol); [^{3}H]deoxycorticosterone (50 Ci/mmol).

Nonradioactive steroids were obtained from either Calbiochem (A grade) or from Steraloids. Radioactive steroids were prepared in ethanol after evaporation of the benzene in which they had been dissolved originally. Nonradioactive steroid solutions were prepared in ethanol. The ethanol content in the final reaction mixture did not generally exceed 2%.

The buffers used were: buffer A, 0.01 M Tris–0.0015 M EDTA (pH 7.4) containing 0.012 M thioglycerol; buffer B, 0.01 M Tris–0.0015 M EDTA–0.4 M KCl (pH 7.4) with 0.012 M thioglycerol.

The mice were killed by cervical dislocation, and the tissues were chilled immediately after their removal. The tissues were rinsed in buffer A and weighed. Slices of tumor of known weight (300–500 mg) were incubated at 25° for 120 min in 4.0 ml of Eagle's minimum essential medium containing known concentrations of radioactive and nonradioactive steroids in an atmosphere of 95% O_2–5% CO_2. At the end of the incubation period, tissues were homogenized in buffer A (gram per milliliter) at 4°. The homogenate was centrifuged at 800*g* in a refrigerated Sorvall centrifuge. The nuclear pellet was resuspended and centrifuged once in phosphate-buffered saline, once in phosphate-buffered saline containing 0.25 M sucrose, and finally in phosphate-buffered saline containing 0.25 M sucrose and 0.05% Triton X-100. The pellet was then extracted with buffer B and centrifuged and the supernatant extract called nuclear extract was analyzed for the binding protein. To obtain purified nuclei, the nuclear pellet was gently homogenized in a Triton-containing buffer (0.25 M sucrose, 3 mM $CaCl_2$, 0.01 M Tris, 0.02 M thioglycerol, and 0.25% Triton X-100), resuspended, and centrifuged twice in the buffer. The nuclei were then centrifuged at 24.000 rpm in a Spinco SW-50L through 2.2 M sucrose in 0.01 M Tris buffer containing 3 mM $CaCl_2$. Supernatants incubated under cell free conditions with [^{3}H]dexamethasone at 4° were also assayed in some experiments.

The binding in the cytoplasmic extract was studied by an assay using a Sephadex G-25 filtration technique previously described (Shyamala, 1973) or by the DEAE-filter assay (Santi *et al.*, 1973). The binding capacity determined by the two procedures did not differ significantly. The total radioactivity present in washed nuclear pellets was extracted with 3 ml of ethanol and counted with 10 ml of scintillation fluid. Separation of bound from unbound labeled steroid in nuclear extracts was achieved by gel filtration on Sephadex G-25 using buffer B.

Sucrose gradient techniques used for the characterization of the binding component have been described previously (Shyamala, 1973).

Protein concentration in the cytoplasmic extracts was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as the standard. DNA content of the tissue homogenate was measured by the method of Webb and Levy (1955) using calf thymus DNA as the standard.

Radioactivity was measured in a liquid scintillation spectrometer (Beckman, LS 250) with 55% counting efficiency for tritium.

Results

In Vitro Uptake of [^{3}H]Dexamethasone by Mammary Tumor Slices. When [^{3}H]dexamethasone is added to mam-

[1] Abbreviation used is: MTV, mammary tumor virus.

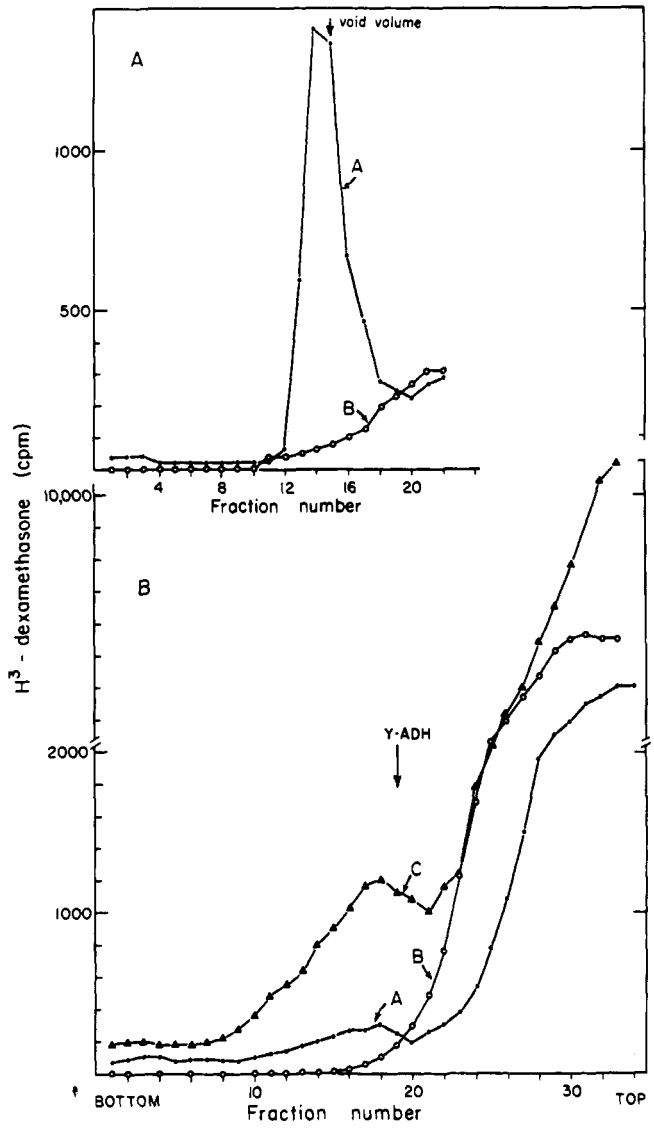

FIGURE 2: (A) Gel filtration on Sephadex G-25 columns of nuclear extracts of mammary tumors. The tumor slices were incubated at 25° for 2 hr with (A) 10^{-8} M [^{3}H]dexamethasone only or (B) 10^{-8} M [^{3}H]dexamethasone + 10^{-5} M unlabeled dexamethasone. Techniques for preparation of nuclear extracts and assay are as described under Materials and Methods. (B) Sucrose density gradient patterns of cytoplasmic fractions of mammary tumors. The tissues were incubated at 25° for 2 hr in a medium containing (A) 10^{-8} M [^{3}H]dexamethasone only; (B) 10^{-8} M [^{3}H]dexamethasone + 10^{-5} M unlabeled dexamethasone; (C) cytoplasmic fraction of unincubated tissue was treated with 10^{-8} M [^{3}H]dexamethasone for 90 min at 4°. Techniques for the preparation and assay of the cytoplasmic fraction are described under Materials and Methods. Centrifugations were performed at 48K at 4° for 16 hr. Yeast alcohol dehydrogenase (Y-ADH) was used as standard to calculate sedimentation coefficient. The migration of standard was determined by enzymatic assays on the effluent fractions.

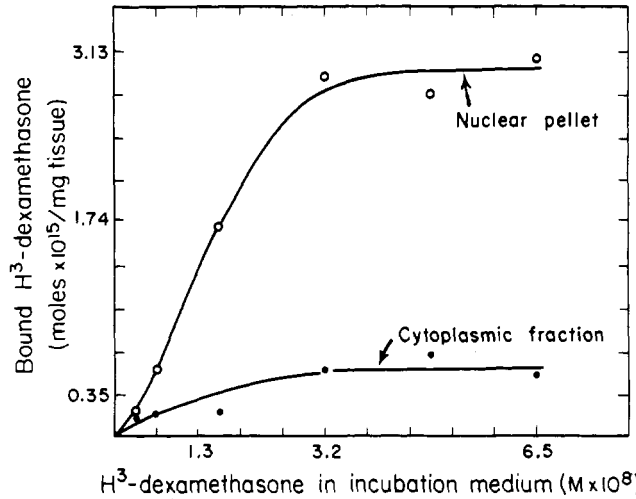

FIGURE 3: Specific binding of [^{3}H]dexamethasone by mammary tumor slices *in vitro*. Mammary tumor slices of known weights were incubated with increasing concentrations of [^{3}H]dexamethasone in 4.0 ml of Eagle's minimum essential medium for 2 hr at 25°. The cell fractions were prepared and assayed as described under Materials and Methods. Nonspecific binding was determined by carrying out parallel sets of incubations in the presence of 100-fold excess of nonradioactive dexamethasone and has been subtracted from the total binding.

mary tumor slices and incubated at 25°, the steroid binds specifically, to both cytoplasmic and nuclear fractions, the equilibrium being almost complete by 60 min (Figure 1). The amount of radioactivity remaining bound to the cell fractions in incubations containing an excess of competing nonradioactive steroid is referred to as the nonspecific binding. At equilibrium, the specific binding in the nuclear fraction represented roughly 80% of the total specific binding in the tissue (range: 60–90%). There is little nuclear localization of [^{3}H]dexamethasone under 4° incubations (see Figure 1, control sample), a phenomenon known to occur in most steroid–receptor systems (Raspe, 1971). Analyses of cytoplasmic fraction on sucrose density gradients revealed that the [^{3}H]dexamethasone was bound to a macromolecule sedimenting in the region of 6 S (Figure 2B) and the binding of the steroid in this region was absent in the cytoplasm from tumors incubated also with an excess of nonradioactive steroid. The cytoplasmic glucocorticoid receptor of unincubated tumors also sediments in the region of 6 S on sucrose density gradients (Shyamala, 1974) and is shown in Figure 2B. About 70–80% of the bound radioactivity in the nuclear pellet could be solubilized with buffer B and only about 50–60% of the labeled steroid in the soluble nuclear extract was bound to a macromolecule (Figure 2A). Figure 2A also shows that the amount of bound steroid in the solubilized nuclear extract is diminished when the tumor slices have been incubated in a medium also containing excess nonradioactive steriod indicating that the macromolecular bound dexamethasone in the extract is specific. In this connection it is important to mention that approximately 50–60% of the total labeled dexamethasone associated with the nuclear pellet is nonspecific and this nonspecifically bound steroid is also easily solubilized with buffer B. However, as shown in Figure 2A, only the specifically bound labeled steroid is stable during column chromatography.

The specific binding of [^{3}H]dexamethasone in both cytoplasmic and nuclear fractions reached saturation with 3×10^{-8} M dexamethasone in the incubation medium (Figure 3). The binding data obtained at saturation indicate that there are roughly 0.7 pmol of [^{3}H]dexamethasone bound/mg of DNA. Assuming 6.6×10^{-16} μg of DNA/cell nucleus (Davidson, 1960) and based on the average DNA content of the tumors used (4.0 μg of DNA/mg of tissue), the binding at saturation roughly corresponded to 2700 binding sites per cell assuming a homogeneous cell population and one molecule of steroid per binding site. As reported earlier (Shyamala, 1974), there are individual variations in the total number of cytoplasmic binding sites between tumors and this is also reflected in the nuclear localization.

Nature of Bound Radioactive Dexamethasone. After incubation of mammary tumor slices with [^{3}H]dexametha-

Table I: Effect of Various Unlabeled Steroids on the Uptake of [^{3}H]Dexamethasone by Mammary Tumor Slices *in Vitro*.[a]

Competing Steroid	Specific Binding[b] of [^{3}H]Dexamethasone (% of Control)	
	Cytoplasmic Fraction	Total Nuclear Pellet
None	100	100
Testosterone	93.1	87.0
Estradiol	96.0	43.9
Progesterone	86.0	22.3
Deoxycorticosterone	43.7	27.1
Hydrocortisone	39.0	20.2
Corticosterone	15.2	0
Dexamethasone	0	0

[a] Mammary tumor slices of known weight were incubated in 4.0 ml of Eagle's minimum essential medium containing 10^{-8} M [^{3}H]dexamethasone with or without 10^{-7} M of unlabeled steroids. All incubations were done at 25° for 2 hr. Tissue fractions were prepared and assayed as described under Materials and Methods. Each value is an average of six determinations. [b] Specific binding is the difference in the bound radioactivity in samples with and without the competing steroid.

sone, the cytoplasm and nuclear extracts were assayed on Sephadex columns; the bound steroid was extracted from the eluates of the column and chromatographed as described earlier (Shyamala, 1973). Virtually all of the bound radioactivity migrated with authentic dexamethasone. This indicated that the cell fractions primarily bind unaltered steroid.

Effect of Various Steroids on Uptake of [^{3}H]Dexamethasone by Mammary Tumor Slices in Vitro. The ability of various steroids to compete for the [^{3}H]dexamethasone binding sites of mammary tumor cell fractions under *in vitro* incubations of whole tissue is shown in Table I. There was a positive correlation between the glucocorticoid potency of various corticoids and their ability to inhibit the uptake of [^{3}H]dexamethasone by tumor slices; their order of relative affinity to the binding sites was similar in the cytoplasm and the nuclear fraction. Testosterone did not have any significant effect on [^{3}H]dexamethasone binding in either cell fraction. Estradiol and progesterone did not compete significantly with the cytoplasmic binding sites of [^{3}H]dexamethasone but inhibited the total nuclear localization of the labeled steroid. Further experiments were carried out to see if the inhibition of [^{3}H]dexamethasone in the total nuclear pellet with the various unlabeled steroids was also apparent in the solubilized nuclear extracts. In those cases where the addition of an unlabeled steroid to the incubation medium resulted in a decreased nuclear localization of labeled dexamethasone, the decrease was also apparent in the solubilized nuclear extracts. However, chromatography of the soluble extracts on Sephadex columns revealed some important differences. The macromolecular bound [^{3}H]dexamethasone in the nuclear extract was greatly diminished only when the initial incubation medium contained unlabeled hydrocortisone, corticosterone, or deoxycorticosterone (Figure 4A) and not when it contained unlabeled estradiol or progesterone (Figure 4B). As mentioned earlier a substantial amount of labeled dexamethasone associated with the nuclear pellet is nonspecific which can be solubilized but appear as free steroid during column chromatography. Thus the data presented in Table I and Figure 4 can be explained if estradiol and progesterone at 10^{-7} M concentration compete only for these nonspecific binding sites in the nuclear pellet and not for the receptor sites.

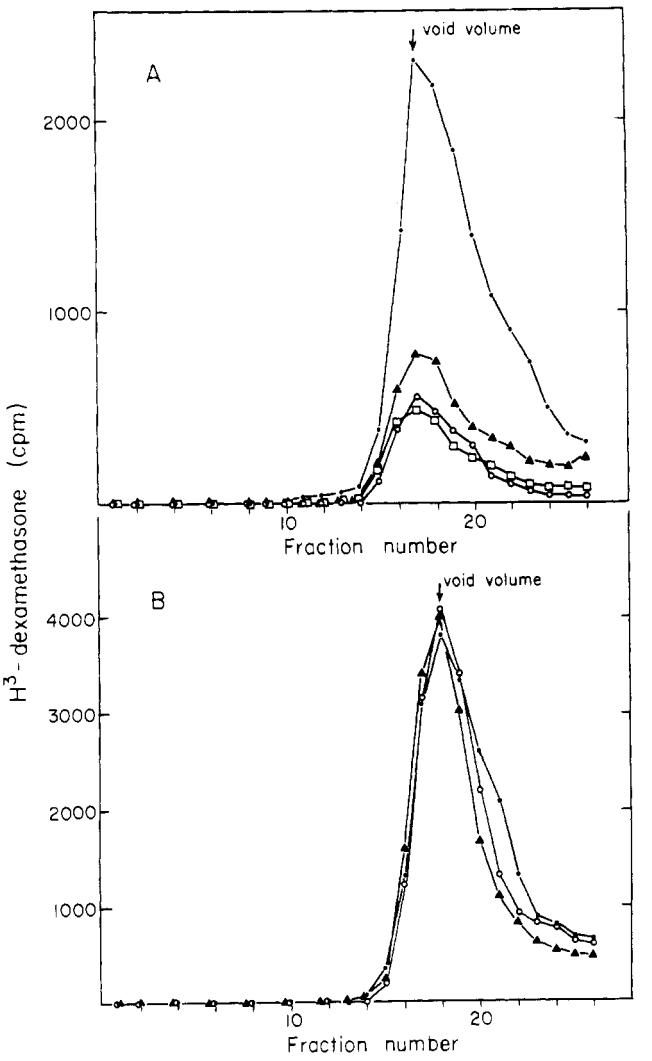

FIGURE 4: Gel filtration on Sephadex G-25 of mammary tumor nuclear extracts. In all cases, mammary tumor slices were incubated in a medium containing 3×10^{-8} M [^{3}H]dexamethasone while nonradioactive steroids, when added, were present at 3×10^{-7} M. All incubations were done at 25° for 2 hr. Techniques of tissue incubation, preparation, and assay of nuclear extracts are described under Materials and Methods. The data represent binding equivalent to 1 g of tissue. A and B are two separate experiments. (A) (●) [^{3}H]Dexamethasone; (○) [^{3}H]dexamethasone + unlabeled hydrocortisone; (□)][[^{3}H dexamethasone + unlabeled corticosterone; (▲) [^{3}H]dexamethasone + unlabeled deoxycorticosterone. (B) (●) [^{3}H]Dexamethasone; (○) [^{3}H]dexamethasone + unlabeled estradiol, (▲) [^{3}H]dexamethasone + unlabeled progesterone.

Earlier studies from our laboratory have shown that progesterone can compete with [^{3}H]dexamethasone for the cytoplasmic glucocorticoid receptor binding sites of normal mammary glands (Shyamala, 1973) and mammary tumors (Shyamala, 1974). Because of its relative lower affinity to the binding sites, progesterone exhibits its inhibitory effect significantly on dexamethasone binding only when used at concentrations of or greater than 10^{-6} M. In the whole tissue incubation studies presented in Table I, only 10^{-7} M of progesterone was used with [^{3}H]dexamethasone; this can account for the lack of any significant inhibition of specific

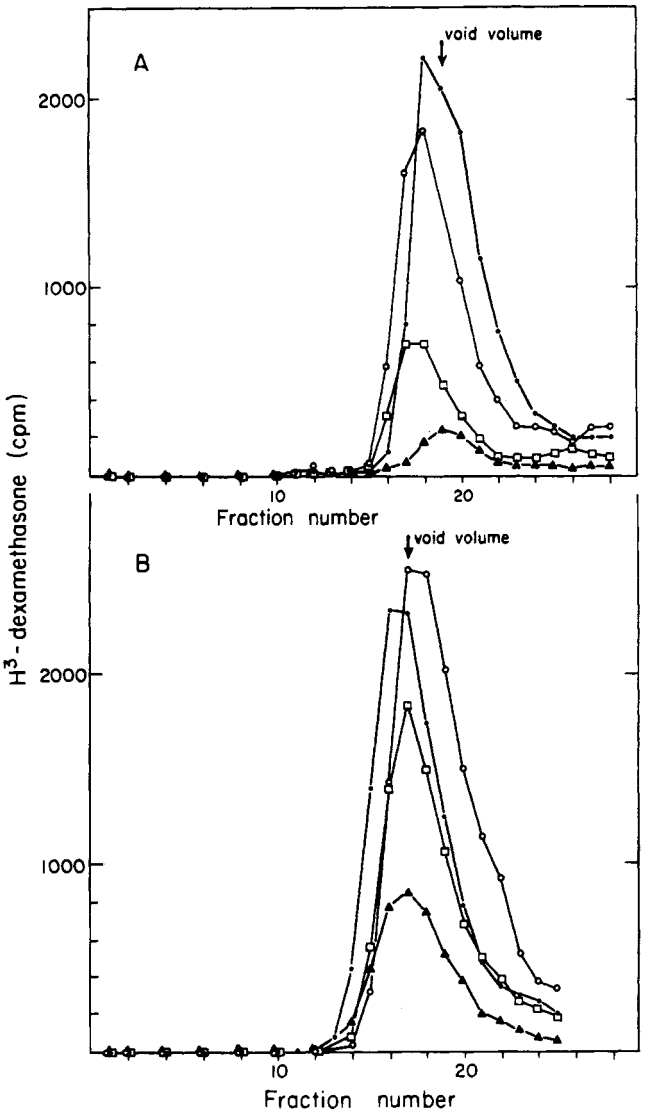

FIGURE 5: Gel filtration on Sephadex G-25 of nuclear extracts from mammary tumor slices. Tissues were incubated for 2 hr at 25° in a medium containing 10^{-8} M [^{3}H]dexamethasone either alone or with competing progesterone or estradiol. (A) Unlabeled progesterone was added as follows: (●) none; (○) 10^{-7} M, (□) 10^{-6} M; (▲) 10^{-5} M. (B) Unlabeled estradiol was added at: (●) none; (○) 10^{-7} M, (□) 10^{-6} M; (▲) 10^{-5} M. The data represent binding equivalent to 1 g of tissue. Techniques of tissue incubation, preparation, and assay of nuclear extracts are as described under Materials and Methods.

[^{3}H]dexamethasone binding either in the cytoplasm or in the solubilized nuclear extracts. However, with higher concentrations of unlabeled estradiol or progesterone in the incubation medium, there was a significant inhibition of [^{3}H]dexamethasone associated with the macromolecules in the solubilized nuclear extract. Progesterone (Figure 5A) was more effective than estradiol (Figure 5B) in abolishing the localization of nuclear bound [^{3}H]dexamethasone. Similar results were also obtained with estradiol and progesterone for the specific binding of [^{3}H]dexamethasone in the cytoplasmic fractions obtained from incubated tumor slices and this is compared with the binding data obtained with cytoplasmic fractions of unincubated tumors (Table II). Although, basically there were no differences, with all steroids, incubation of the tissue seemed to increase the affinity of the steroid to the cytoplasmic receptor as evidenced by a greater competition.

Table II: Effect of Unlabeled Estradiol or Progesterone on the Binding of [^{3}H]Dexamethasone to the Cytoplasmic Fraction of Mammary Tumors.

Nonradioactive Steroid		Cytoplasmic Binding[a] of [^{3}H]Dexamethasone	
Name	Concn(M)	Unincubated Tissue[b]	Incubated Tissue[c]
None		100	100
Dexamethasone	3×10^{-7}	72.9	29.3
	1.5×10^{-6}	58.3	N.D.[d]
	3×10^{-6}	48.7	16.6
	3×10^{-5}	34.7	11.7
Progesterone	3×10^{-7}	100	77.1
	1.5×10^{-6}	95.3	N.D.
	3×10^{-6}	84.7	37.0
	3×10^{-5}	63.8	39.4
Estradiol	3×10^{-7}	100	100
	1.5×10^{-6}	100	96.5
	3×10^{-6}	100	87.6
	3×10^{-5}	100	66.5

[a] Binding data represent total binding of [^{3}H]dexamethasone and are averages of at least four separate determinations. [b] Aliquots of cytoplasmic extracts of mammary tumors were incubated in a total volume of 0.4 ml with 3×10^{-8} M [^{3}H]dexamethasone alone or with competing nonradioactive steroids at 4° for 2 hr. [c] Cytoplasmic fractions were prepared from mammary tumor slices which had been incubated with 3×10^{-8} M [^{3}H]dexamethasone alone or with competing nonradioactive steroids at 25° for 2 hr. Binding was measured by DEAE-filter assays as described under Materials and Methods. [d] N.D. = Not done.

Effect of Various Steroids on the Subcellular Distribution of Receptors. It has been suggested that the cytoplasmic glucocorticoid receptor can exist in two forms, one active and one inactive in promoting the nuclear binding of the receptor (Rousseau *et al.*, 1973). Thus according to this model, various glucocorticoids will bind to the active form of the receptor and depending upon their relative affinity to the receptor will promote nuclear binding while antagonists will bind to the inactive form of the receptors and prevent the translocation of the receptor to the nucleus. Thus all unlabeled steroids that prevented the nuclear localization of [^{3}H]dexamethasone could have interacted with either the active or the inactive form of the receptor. In order to see if such a phenomenon was operable in mammary tumors the steroids that competed for dexamethasone binding sites were used in their radioactive form to study their interaction with the receptor. In order to ensure that the sites were specific for glucocorticoids, unlabeled dexamethasone was used as the competing steroid in these experiments. Both the cytoplasmic fractions and the solubilized nuclear extracts were assayed for binding in each case. The binding of various steroids in nuclear extracts of mammary tumors is shown in Figure 6. As may be seen, hydrocortisone and corticosterone were bound to macromolecules in nuclear extracts and this binding was abolished when the initial incubation medium also contained an excess of unlabeled dexamethasone. In the case of deoxycorticosterone, progesterone, and estradiol, although the labeled steroid was associated with a macromolecule in the nuclear extract, this binding was not diminished when unlabeled dexamethasone was

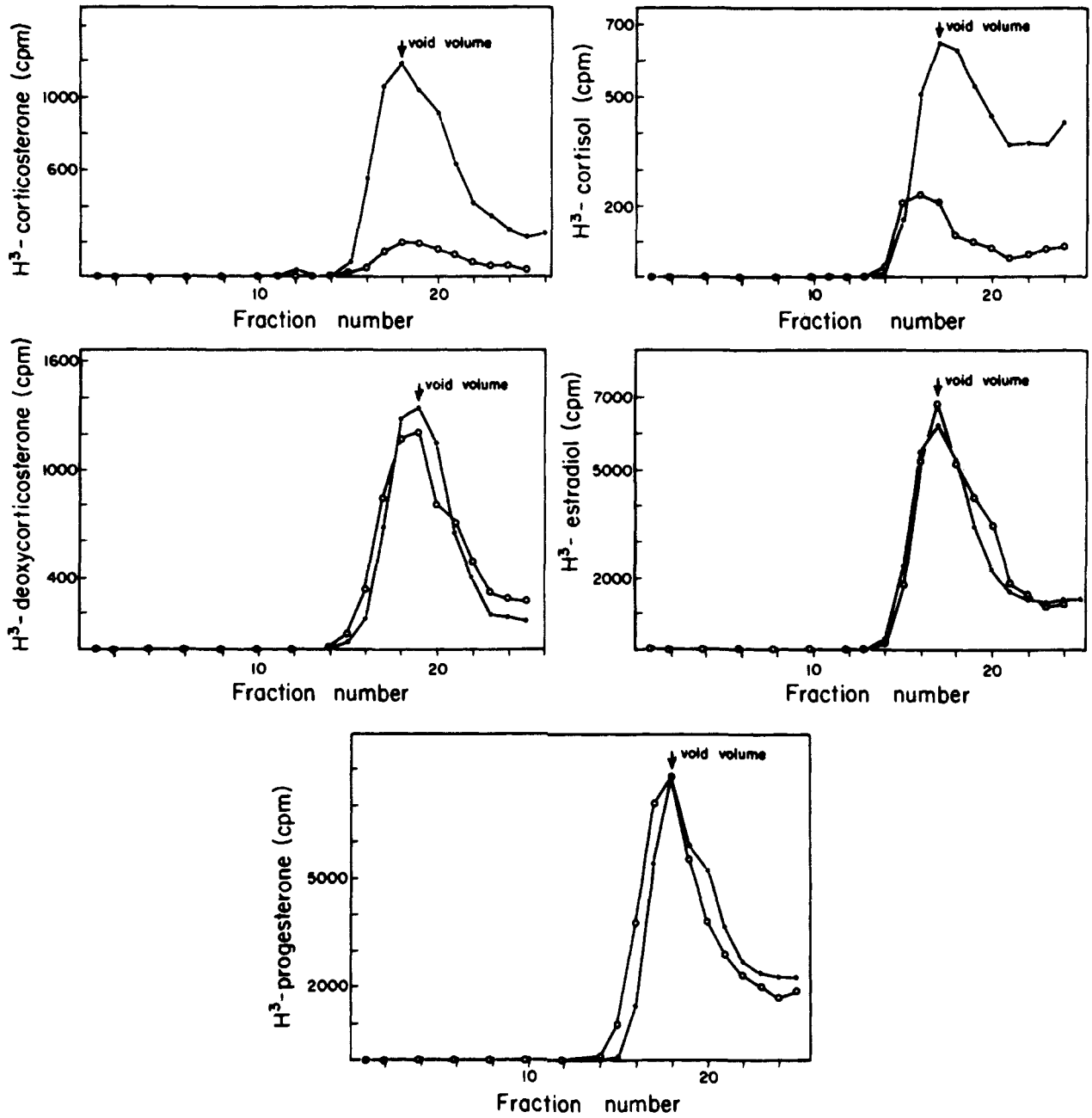

FIGURE 6: Gel filtration of Sephadex G-25 of nuclear extracts prepared from mammary tumor slices incubated with various ^{3}H-labeled steroids. Mammary tumor slices were incubated for 2 hr at 25° in a medium containing labeled steroids (●——●) at 10^{-8} M concentration in all cases. Parallel sets of incubations were also carried out where the medium in addition to labeled steroid contained 100-fold excess of unlabeled dexamethasone (○). The data represent binding equivalent to 1 g of tissue. Techniques of tissue incubation, preparation, and assay of nuclear extracts are as described under Materials and Methods.

used as the competing steroid thus indicating that the binding was not specific for glucocorticoids. Similar "nonspecific" binding with labeled deoxycorticosterone in the nuclear fraction has been observed with the glucocorticoid receptor system of hepatoma cells (Rousseau *et al.*, 1973). The subcellular distribution of the binding sites with various steroids is shown in Table III. As may be seen, the amount of steroid localized in the nuclear fraction correlates well with the glucocorticoid potency of the tested steroid. At 10^{-8} M concentration of steroids, the total number of steroid molecules bound in both the cytoplasmic and nuclear extracts reflect once again the relative affinity for various steroids to the receptor sites. At higher concentrations of steroid ($>10^{-8}$ M) the total number of steroid molecules bound to the receptor is approximately the same in all cases (data not shown). The subcellular distribution data on deoxycorticosterone and progesterone are not presented since a meaningful estimate on the nuclear binding of these steroids could not be made due to the large amount of "nonspecific" nuclear binding.

Discussion

The role of adrenal steroid hormones on the mammary tumors have been well documented and if steroid hormones exert their effect directly on the tissue, they are most likely mediated through the specific steroid receptors. Recently it was shown that specific cytoplasmic glucocorticoid receptors are present in mouse mammary tumors (Shyamala, 1974). The present report provides evidence that under *in*

vitro incubation of tumor slices the nuclei are also capable of binding unaltered [^{3}H]dexamethasone in a specific manner similar to its cytoplasm. The binding protein in the nuclei is easily solubilized with a buffer of high ionic strength (0.4 M KCl) and satisfies all the general criteria by which hormone receptors are characterized. As with other glucocorticoid receptor systems (Beato *et al.*, 1971; Munck and Wira, 1971; Funder *et al.*, 1973; Giannopoulous, 1973b), the nuclear localization of the steroid is accelerated by elevated temperatures (25 or 37°).

Again, as with other glucocorticoid receptor systems, the ability of an unlabeled steroid to compete with [^{3}H]dexamethasone binding corelated well with its known biological potency as a glucocorticoid. These steroids include corticosterone, hydrocortisone, and deoxycorticosterone, and the order of affinity to the receptor was corticosterone > hydrocortisone > deoxycorticosterone. These studies also show that dexamethasone, corticosterone, and hydrocortisone have similar subcellular distribution of glucocorticoid receptors. Thus it is interesting to note that while corticosteroids have been shown to be required for the maintenance of precancerous lesions and development of mammary tumors (Nandi, 1959; Elias and Rivera, 1959), deoxycorticosterone in organ culture systems is less effective than hydrocortisone in maintaining the precancerous lesions (Elias and Rivera, 1959). In mouse cell lines derived from mammary adenocarcinoma, dexamethasone stimulates production of MTV (Parks *et al.*, 1974). In this respect it is interesting to note that dexamethasone, hydrocortisone, and corticosterone are all optimally effective in inducing the replication of MTV in cultured tumor cells while deoxycorticosterone is submaximally effective at the same concentrations (C. Dickson, unpublished observations). It is worthwhile to mention in this respect that corticosterone which is the major glucocorticoid in mouse has as high an affinity to the receptor as the synthetic glucocorticoid, dexamethasone, used in these studies. Thus in all likelihood the characteristics of the glucocorticoid receptor described in this report are very similar to those operative *in vivo*.

The interaction of ovarian steroids, namely, estradiol and progesterone, with the glucocorticoid receptors in mammary tumors requires comment. In the case of glucocorticoid receptors of kidney (Funder *et al.*, 1973) and hepatoma cells (Baxter and Tomkins, 1971), progesterone can compete with specific binding sites but has a lower affinity than dexamethasone. Estradiol does not compete with the specific binding sites in kidney (Funder *et al.*, 1973) but has been shown to inhibit [^{3}H]dexamethasone binding in the hepatoma cells (Baxter and Tomkins, 1971). Our studies on mammary tumors show that both progesterone and estradiol when used at high concentrations can compete for the specific glucocorticoid binding sites both in the cytoplasmic and soluble nuclear extracts. It is unlikely that estradiol at higher concentration binds to the estrogen receptors of tumors, resulting in the translocation of the steroid to the same nuclear binding sites as those for the glucocorticoid receptors, since our earlier studies show that the mouse mammary tumors used in this study contain cytoplasmic estradiol receptors but do not have the ability to translocate the bound steroid to the nucleus (Shyamala, 1972). Studies with labeled progesterone or estradiol designed to detect specific binding to glucocorticoid binding sites in the nuclear fraction were unsuccessful. Thus the behavior of these steroids in mammary tumors is analogous to that of progesterone with the glucocorticoid receptor of hepatoma cells (Rousseau *et al.*, 1973) where progesterone is believed to act as an antagonist of glucocorticoids.

Table III: Effect of Various Steroids on the Subcellular Distribution of Glucocorticoid Receptors.

^{3}H-Labeled Steroid	Specific Binding[a] of ^{3}H-Labeled Steroid (mol/mg of Tissue)	
	Cytoplasmic Fraction	Nuclear Extract
Dexamethasone	6.0×10^{-16}	23.2×10^{-16}
Hydrocortisone	3.0×10^{-16}	5.1×10^{-16}
Corticosterone	3.1×10^{-16}	4.3×10^{-16}

[a] Specific binding is the difference in the bound radioactivity in samples with and without an excess of unlabeled dexamethasone. Mammary tumor slices were incubated at 25° for 2 hr with the labeled steroids (10^{-8} M) alone or with 10^{-6} M unlabeled dexamethasone. The preparation and assay for bound steroid in the cell fraction were carried out as described under Materials and Methods.

At present one can only speculate on the biological significance of high levels of progesterone and estradiol causing inhibition of binding of glucocorticoids to their receptor sites in mammary tumors. In the case of DMBA-induced mammary tumors in rats, it has been observed that large doses (10–20 μg) of estradiol and progesterone when administered *in vivo* can reduce the tumor incidence and prolong the latent period of tumor induction in rats while physiological doses of estradiol are without an effect on the growth rate of mammary cancer (Huggins *et al.*, 1962). Large doses of estrogen can also inhibit the growth rate of established mammary tumors in rats (Huggins and Yang, 1972). But this inhibition is probably exerted by a direct effect on the mammary tumors and not due to alterations in prolactin secretion through the pituitary since it appears that large doses of estrogen do not inhibit prolactin secretion (Chen and Meites, 1970). One of these direct effects on mammary tumors by high concentrations of estrogen and progesterone may be to inhibit the binding of glucocorticoids to their receptors sites thus counteracting the growth promoting effect of glucocorticoids.

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Cholesterol in Aqueous Solution: Hydrophobicity and Self-Association†

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ABSTRACT: Free energies of transfer of cholesterol monomer from water to organic solvents show that the hydrophobicity of this sterol molecule is significantly less than predicted from hydrophobic surface area considerations. It is suggested that this phenomenon may arise from unusual orientation of water molecules at the surface of the solute. From the direct measurement of the hydrophobic free energy of transfer and comparison with thermodynamic data on micelle formation reported previously we calculate specific attractive interactions between cholesterol monomers in the micelle of 2–4 kcal/mol, which suggests the possibility of self-association (phase separation) in mixed micellar systems such as sterol–lipid complexes.

Cholesterol is a biologically important amphiphile in mammalian systems as a component of cell membranes and serum lipoproteins. However, little is known about the physical properties of cholesterol in aqueous solution despite the fact that this binary system, cholesterol–water, must be well defined in order to describe the more complex systems sterol–lipid–protein–water.

We have previously shown that cholesterol forms a heterogeneous micelle in aqueous solvents at a critical micelle concentration of 20–40 $\times 10^{-9}$ M which corresponds to a unitary free energy of micellization of −12.6 kcal/mol (Haberland and Reynolds, 1973). The cholesterol micelle has a significantly lower partial specific volume than the monomer suggesting specific strong interactions between the monomers in the aggregated state. In order to verify this suggestion we need to know exactly how hydrophobic the cholesterol molecule is, *i.e.*, can the hydrophobic repulsion by water account entirely for the free energy of micellization, and, if not, how much free energy is derived from the specific interactions previously suggested by us? The studies in this paper were designed with this question in mind, but the results are of interest from another point of view as well: the hydrophobicity of the cholesterol molecule in water is significantly less than one would predict from current theory.

The unitary free energy of transfer of a nonpolar solute from water to hydrocarbon is a direct measurement of hydrophobicity (Tanford, 1973) and has been shown to be a regular function of the cavity surface area of the nonpolar

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