Comparison of Glucocorticoid-Binding Proteins in Normal and Neoplastic Mammary Tissues of the Rat†

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ABSTRACT: Kinetic and molecular properties of components binding [^{3}H]triamcinolone acetonide were studied using 105,000*g* supernatants of lactating mammary gland, R3230AC, and dimethylbenz[*a*]anthracene (DMBA) induced mammary tumors of the rat. Using a dextran-coated charcoal adsorption procedure, the relationship between specific glucocorticoid binding and protein concentration was linear in the range of 0.5–4.0 mg/reaction. These cytoplasmic macromolecules bound [^{3}H]triamcinolone acetonide with limited capacity (50–400 fmol/mg of cytosol protein) and high affinity, $K_d \sim 10^{-8}$–10^{-9} *M*. Optimal binding was obtained when homogenizations were made in Tris buffers, at pH 7.4, containing monothioglycerol. Time course of association of [^{3}H]triamcinolone acetonide and its binding sites showed maximal binding by 6–8 hr at 3° which remained unchanged up to 24 hr. The rate constant of association at 3° was in the range of 2–4 × 10^5 M^{-1} min^{-1}. The rate constant of dissociation of bound [^{3}H]triamcinolone acetonide could not be calculated accurately since the reaction was essentially irreversible for 5 hr at 3°. Estimation of the half-life of the steroid-binding protein complexes from the K_d and the rate constant for association gave a value of 11–12 hr. From ligand specificity studies, the glucocorticoids, triamcinolone acetonide, corticosterone, cortisol, and dexamethasone competed well for [^{3}H]triamcinolone acetonide binding sites. Progesterone, aldosterone, and the anti-glucocorticoid, cortexolone, were also good competitors while androgens and estrogens were weak inhibitors of binding. The binding components sedimented at 7–8 S in sucrose gradients of low ionic strength and dissociated into lower molecular weight components sedimenting at 4–5S in high ionic strength gradients. Studies in vivo using animals bearing the DMBA-induced tumor demonstrated that [^{3}H]triamcinolone acetonide binding complexes were present in cytoplasmic and nuclear compartments. Sedimentation coefficients of the cytoplasmic and nuclear forms of these receptors labeled in vivo were 7–8S and 4–5S, respectively. These studies suggest that the molecular and kinetic binding properties of glucocorticoid receptors in neoplastic mammary tissues are similar to those of the normal mammary gland.

Glucocorticoids are known to influence development and differentiation of the mammary gland. Cellular differentiation, characterized histologically by an alveolar-secretory appearance and by increased rates of synthesis of milk proteins, has been observed in explants of pregnant mouse mammary gland cultivated in vitro in the presence of cortisol, insulin, and prolactin (Juergens et al., 1965; Stockdale et al., 1966). Evidence also indicates that the adrenal corticoids are required for lactation (Lyons et al., 1958; Nandi, 1959; Thatcher and Tucker, 1970). These effects have been related to the accumulation of rough endoplasmic reticulum in the epithelial cells of mammary tissue, presumably by a membrane-stabilizing effect of the glucocorticoids (Oka and Topper, 1971).

Recently this laboratory demonstrated the presence of components in cytosol of lactating mammary gland of the rat which bound [^{3}H]triamcinolone acetonide[1] specifically and with high affinity (Gardner and Wittliff, 1973a). In another report (Gardner and Wittliff, 1973b), we presented evidence that the R3230AC mammary tumor, a well-differentiated adenocarcinoma of the rat whose growth is inhibited by the administration of hydrocortisone to the host (Hilf et al., 1965), also contained [^{3}H]triamcinolone acetonide binding components in the cytoplasm.

Mammary tumors induced by dimethylbenz[*a*]anthracene[2] represent another hormonally responsive neoplasm in that the majority regress after ovariectomy–adrenalectomy of the host animal (Dao, 1964). This observation suggested that the mechanism of regression due to the administration of glucocorticoids may involve specific glucocorticoid-binding components similar to those we reported earlier (Gardner and Wittliff, 1973a,b). These early reports were concerned largely with the sedimentation properties of glucocorticoid-binding components and provided little quantitative information.

In this paper we describe the application of a rapid, quantitative procedure for measurements of [^{3}H]triamcinolone acetonide binding using dextran-coated charcoal to adsorb unbound steroid. Using this procedure we now present a more extensive characterization of the [^{3}H]triamcinolone acetonide binding components of the mammary gland and the R3230AC mammary tumor and demonstrate for the first time their presence in the DMBA-induced mammary tumor of the rat.

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[1] 9α-Fluoro-11β,16α,17α,21-tetrahydroxypregna-1,4-diene-3,20-dione-16,17-acetonide.

[2] Abbreviations used are: DMBA, 9,10-dimethyl-1,2-benzanthracene; PCMB, *p*-chloromercuribenzoate.

Materials and Methods

Chemicals. All chemicals were reagent grade unless otherwise specified. [1,2,4-^{3}H]Triamcinolone acetonide (10.7 Ci/mmol) was purchased from Schwarz/Mann and checked periodically for purity by thin-layer chromatography. Unlabeled triamcinolone acetonide, dexamethasone,[3] and aldosterone were obtained from Sigma Chemical Co., while corticosterone, hydrocortisone, progesterone, estradiol-17β, estrone, and estriol were supplied by Calbiochem. Unlabeled cortexolone,[4] dihydrotestosterone, and testosterone were purchased from Steraloids, Inc. Tris-HCl (Trizma base) and DMBA were products of Sigma Chemical Co. Schwarz/Mann supplied the RNase-free sucrose while Norit A was obtained from Matheson Coleman and Bell. Omnifluor from New England Nuclear Corp. and Triton X-100 from Beckman were used in the preparation of scintillation cocktail.

Source and Maintenance of Animals. All animals were purchased from the Charles River Company, Wilmington, Mass., and housed in the vivarium of the University of Rochester Medical Center. Female Fischer 344 rats were used as hosts for R3230AC tumor transplants while Sprague Dawley rats lactating 10–22 days were used as a source of normal mammary tissue. Mammary tumors were induced by the carcinogen, dimethylbenz[*a*]anthracene in young Sprague Dawley female rats.

Transplantation of the R3230AC Mammary Tumor. Tissue implants (10–20 mg) which were obtained from 21-day-old tumors were transplanted by a sterile trocar technique into the ventral subcutaneous tissue of female Fischer rats weighing approximately 150 g. Implants of tumor tissue were deposited along the axillary-inguinal line of the mammary bed where neovascularization occurred readily.

Induction of Mammary Tumors by DMBA. Virgin female rats, 51–52 days of age, were each intubated intragastrically each week for 5 weeks with 1 ml of sesame oil containing 5 mg of DMBA. Approximately 4 weeks after the last intubation, animals were palpated for the development of mammary tumors. Tumors were obtained either by excision of the neoplasm from an ether-anesthetized animal or from an animal which had been sacrificed by cervical dislocation. Tissue specimens were routinely taken from the neoplasm and stained and evaluated histologically.

Preparation of Cytosols. All procedures were performed at 0–3° unless otherwise noted. Mammary glands or tumors were excised quickly and placed in 10 m*M* Tris-HCl buffer (pH 7.4) containing 1.5 m*M* EDTA, 250 m*M* sucrose, and 10 m*M* monothioglycerol. Tissues were minced with a McIlwain tissue chopper, stirred in buffer to remove blood and milk proteins, then blotted dry and weighed. Using Duall homogenizers, mammary tissues were disrupted in buffer using weight/volume ratios of 1:4, 1:1, and 1:3 for the lactating mammary gland, R3230AC, and DMBA-induced mammary tumors, respectively. These homogenates were then centrifuged for 30 min at 105,000*g* to prepare the cytosol fractions. The supernatants were drawn from beneath the lipid layer with a Pasteur pipet and stored in an ice bath until used. The pellet containing the particulate material was used to prepare the nuclear extracts.

Preparation of Nuclear Extracts. For certain experiments the particulate fractions from the above procedure were resuspended and washed twice in 4–5 volumes of 10 m*M* Tris-HCl buffer (pH 7.4) containing 1.5 m*M* EDTA and 10 m*M* monothioglycerol, then centrifuged at 4000 rpm in a J-21 centrifuge (Beckman Instruments) and the supernatants discarded. Pellets were then placed in Ten-Broeck homogenizers and extracted (1:1, w/v) with buffer (pH 7.4) which contained KCl at a final concentration of 0.4 *M*. This homogenization was continued intermittently for 1–1.5 hr at 3° to provide optimal extraction before centrifugation at 105,000*g* for 30 min at 3°.

Dextran-Coated Charcoal Procedure. This procedure is similar to the [^{3}H]estradiol-17β binding assay we reported earlier (Gardner and Wittliff, 1973c). Constant volumes, 0.2 ml, of the 105,000*g* supernatants were added to each of 0.5-dram glass shell vials (Fischer Scientific) containing [^{3}H]triamcinolone acetonide alone (total binding) or in the presence of unlabeled triamcinolone acetonide (nonspecific binding) which had been dried down previously under nitrogen. The concentration of the tritium-labeled and unlabeled steroid varied with the experiment; in routine analyses a 100-fold excess of unlabeled triamcinolone acetonide was used. Triplicate reactions were incubated for 16 hr at 3° unless noted. After the incubation period, 1 ml of a suspension of dextran-coated charcoal (10 m*M* Tris-HCl buffer (pH 7.4) containing 1.5 m*M* EDTA, 0.25 *M* sucrose, 0.05% dextran, and 0.5–1.0% Norit A) was added. Following an additional incubation of 10 min, the vials were centrifuged at 1500 rpm for 10 min at 3°. Then an aliquot of each supernatant was removed and counted in toluene-based scintillation fluor containing Triton X-100 (4 g of Omnifluor in 300 ml of Triton X-100 and 700 ml of toluene).

Sucrose Gradient Centrifugation of Steroid-Binding Proteins. The assay was similar to that previously described (Gardner and Wittliff, 1973a). In each of several 0.5-dram glass shell vials, an appropriate quantity of [^{3}H]triamcinolone acetonide (usually 40–50 n*M* final concentration) alone or in combination with an excess (usually 100–500-fold) of unlabeled triamcinolone acetonide was dried under nitrogen just prior to the assay. An aliquot of cytosol (600 μl) prepared as described previously was added to each vial and incubated for ~6 hr at 3°. Following the incubation period, cytosols were mixed with a pellet of dextran-coated charcoal, which was prepared earlier by centrifuging 3 ml of the charcoal suspension as employed by Boylan and Wittliff (1973). After an incubation of 10 min, samples were centrifuged at 500*g* for 10 min at 3° to sediment the charcoal. Aliquots (0.2 ml) of the clear supernatant were removed and layered onto linear sucrose gradients (usually 5–40% or 5–20% sucrose prepared in Tris-HCl buffer (pH 7.4) containing 1.5 m*M* EDTA and 10 m*M* monothioglycerol). For certain experiments, gradients were prepared with sucrose solutions containing 0.4 *M* KCl. Following sample addition, gradients were centrifuged at 308,000*g* for 15–16 hr at 3° in an SW56Ti rotor in a Beckman L5-65 ultracentrifuge. Gradients were collected from the bottom into scintillation vials. Scintillation fluor was added to each vial and the radioactivity was measured in a liquid scintillation counter.

Human serum albumin (4.6 S) and human γ-globulin (7.1 S) were used as marker proteins to estimate the sedimentation coefficients of binding proteins according to the method of Martin and Ames (1961). Following centrifugation, fractions (6 drops each) were collected into 10 × 75 mm round bottom test tubes, diluted with H_2O (0.3–0.5 ml), and then read at OD_{280nm} in a Gilford spectrophotometer.

[3] 9-Fluoro-16α-methyl-1,4-pregnadiene-11β,17α,21-triol-3,20-dione.
[4] 4-Pregnene-17α,21-diol-3,20-dione.

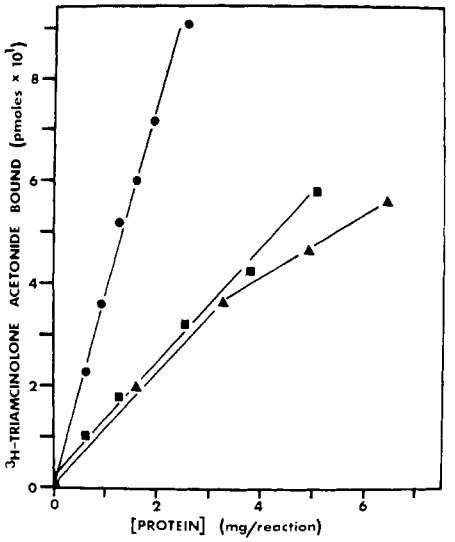

FIGURE 1: Specific glucocorticoid binding by cytosols from mammary tissues as a function of protein concentrations. Supernatants were prepared in Tris-HCl buffer (pH 7.4) by homogenization of either mammary glands from a 14-day postpartum female (●), an ovarian-dependent tumor induced by DMBA treatment (■), or an R3230AC tumor (▲). Portions of each were diluted to 0.2 ml with an appropriate volume of buffer and incubated at 3° for 16–17 hr with [^{3}H]triamcinolone acetonide in the presence and absence of unlabeled triamcinolone acetonide. Triplicate determinations were made by the dextran-coated charcoal procedure. Specific binding, as plotted, was determined as the difference between total binding and binding in the presence of unlabeled triamcinolone acetonide.

Binding in Vivo of ^{3}H-Labeled Ligands. Each animal of matched pairs of rats received a mid-ventral subcutaneous injection (0.5 ml) of 0.4 μCi of [^{3}H]triamcinolone acetonide in 0.15 *M* saline. To determine the specificity of binding, one of the paired animals received an injection of 400 μg of unlabeled triamcinolone acetonide 30 min prior to the administration of isotopically labeled ligand. The animals were sacrificed 60 min after the injection of [^{3}H]steroid and 105,000*g* supernatants were prepared as described earlier. The nuclear extracts were prepared as outlined in the preceding section.

Protein Determination. Protein concentrations were estimated by the method of Lowry et al. (1951) or by the Geiger and Bessman (1972) modification when sulfhydryl reagents were present.

Calculations of Results. Data expressed as counts/unit time were printed out on a teletypewriter (Teletype Corp.). Computer programs described earlier (Brooks and Wittliff, 1973) for the Olivetti Programma 101 were used to determine counting efficiencies and to convert radioactivity data to amounts of steroid. Estimates of specific binding by the dextran-coated charcoal assay were determined as the difference between total and nonspecific binding. In the sucrose gradient procedure, steroid binding components were identified by isotopic profiles. Specific binding was estimated as the difference in the areas under the peak sedimenting in the 7–8 S and 4–5 S regions depending upon the experiment. Specific binding capacity was expressed as fmol (10^{-15} mol)/mg of cytosol protein.

Results

Dependence of Triamcinolone Acetonide Binding on Protein Concentration. The relationship between specific glucocorticoid binding and the concentration of cytosol protein in a reaction was determined by incubating a single concentration of [^{3}H]triamcinolone acetonide with varying amounts of cytosol from each of the mammary tissues. Binding was linear with respect to protein concentration up to and including 2 mg/reaction for the lactating mammary gland, 5 mg/reaction for the DMBA-induced tumor, and 3 mg/reaction for the R3230AC tumor (Figure 1). In general, glucocorticoid-binding studies were performed using cytosol protein concentrations which were in the linear range of binding capacity.

Time Course of Specific Binding of [^{3}H]Triamcinolone Acetonide. Association of [^{3}H]triamcinolone acetonide with sites in cytosols from mammary tissues is shown in Figure 2. Maximal binding was reached within 6 hr at 3° using preparations from lactating mammary gland and the R3230AC tumor. Cytosol from the DMBA-induced tumor (Figure 2C) required approximately 10 hr to reach maximum association. Apparently the receptor–ligand complexes were stable up to 24 hr in the presence of 10 m*M* thioglycerol in the cytosol preparations from each tissue. Although the data are not presented, binding of [^{3}H]triamcinolone acetonide to specific sites in the cytosol from the lactating mammary gland also was studied at 25° as a function of time. Specific binding was rapid, reaching a maximum by 15 min then subsequently falling to undetectable levels by 2–3 hr. Since maximal binding capacity in cytosols from each of the tissues was stable at 3° for at least 16 hr, all subsequent experiments were performed under these conditions unless noted otherwise.

Effect of Sulfhydryl Reagents on [^{3}H]Triamcinolone Acetonide Binding. The participation of sulfhydryl groups in the binding reaction between [^{3}H]triamcinolone acetonide and the binding component was investigated by the addition of several sulfhydryl reducing agents to the homogenizing and incubating medium. The presence of monothioglycerol at a concentration of 10 m*M* in the Tris-HCl buffer increased specific glucocorticoid binding nearly twofold. 2-Mercaptoethanol increased binding to a lesser extent while dithiothreitol failed to elevate binding significantly. These data indicated the importance of the presence of a sulfhydryl reagent in the extraction and incubation medium. As a result, 10 m*M* thioglycerol was routinely added to the homogenizing buffer unless noted otherwise.

Influence of Buffer Type and pH on Binding of [^{3}H]Triamcinolone Acetonide. A systematic analysis of buffer type and pH was investigated to determine the optimal conditions for measuring glucocorticoid binding capacity by the charcoal adsorption procedure. Using Tris-HCl buffers, it was found that pH ~7.5 for both the homogenizing and reaction buffers resulted in maximal binding capacity at 3° (data not presented).

The effects on triamcinolone acetonide binding of other buffer systems such as phosphates and several "Good" buffers (Good et al., 1966) at various pH values were compared with those obtained using Tris-HCl buffers. In general, Tris-HCl buffer (pH 7.5) provided the best environment for maximal binding at 3° when compared to these buffers at the same molar concentrations (data not presented). Therefore, 10 m*M* Tris-HCl buffer (pH 7.4) in the mixture described earlier was chosen for tissue homogenization and binding reactions as well as for the suspension of dextran-coated charcoal.

Titration of Specific Binding Sites with [^{3}H]Triamcinolone Acetonide. Increasing concentrations of [^{3}H]triamcinolone acetonide were incubated with cytosols from the

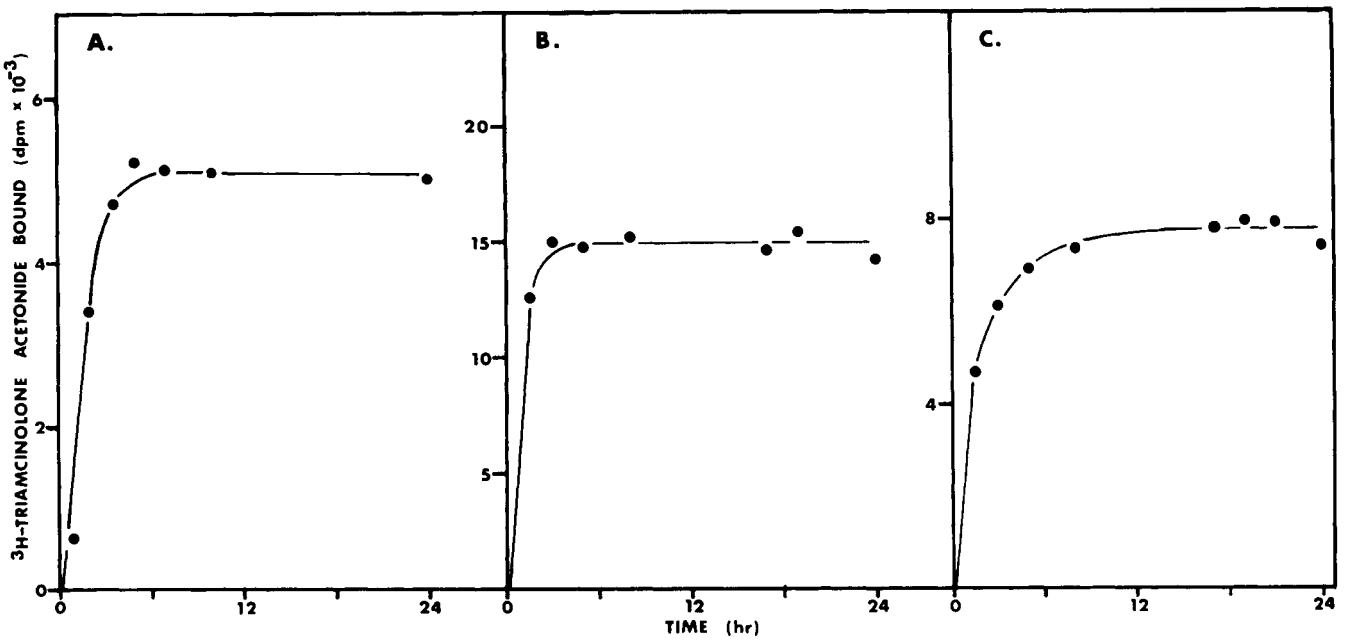

FIGURE 2: Time course of specific glucocorticoid binding by cytosol from normal and neoplastic mammary gland. Constant volumes (0.2 ml) of cytosol from either lactating mammary gland (A), R3230AC tumor (B), or DMBA-induced tumor (C) prepared in Tris-HCl buffer (pH 7.4) were incubated at 3° with 28 nM [^{3}H]triamcinolone acetonide in the presence and absence of 2.5×10^{-6} M unlabeled triamcinolone acetonide. At various times, triplicate determinations of binding capacity were made by the dextran-coated charcoal procedure. Specific binding, as plotted, was determined as outlined under Materials and Methods.

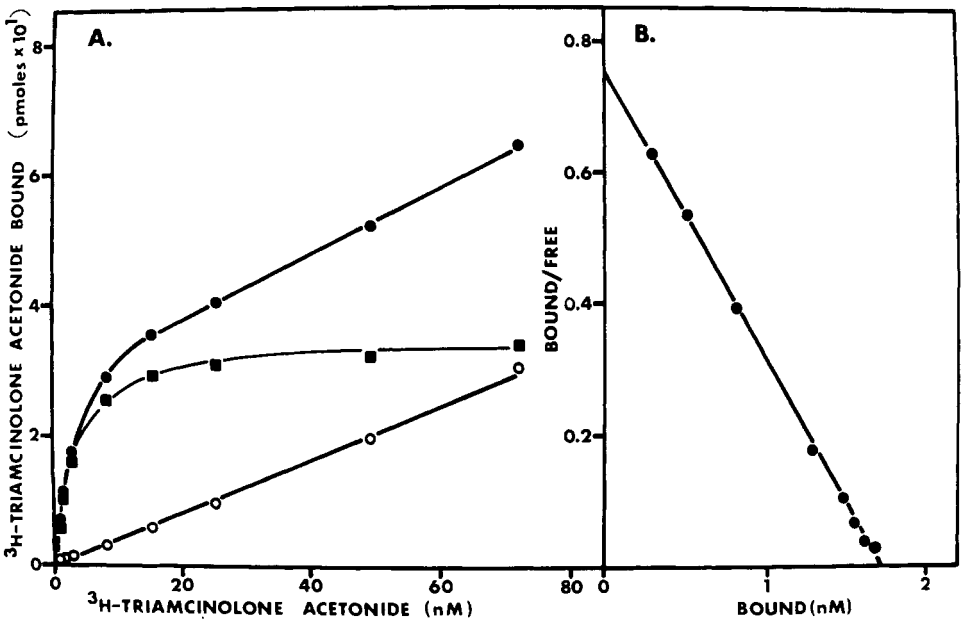

FIGURE 3: (A) Titration curve of glucocorticoid binding by cytosol from lactating mammary gland. Constant volumes (0.2 ml) of supernatant prepared from a 12-day postpartum mammary gland homogenized (1:4, w/v) in Tris-HCl buffer (pH 7.4) were incubated with increasing concentrations of [^{3}H]triamcinolone acetonide in the presence (○) and absence (●) of 10^{-5} M unlabeled triamcinolone acetonide. Each point represents the mean of triplicate determinations. Specific binding (■) was estimated as the difference between total binding and binding in the presence of unlabeled steroid. (B) Scatchard analysis of titration data for specific glucocorticoid binding. The dissociation constant of complexes formed between [^{3}H]triamcinolone acetonide and binding sites was 2.2×10^{-9} M estimated from this plot. The n was 177 fmol/mg of protein.

lactating mammary gland (Figure 3), R3230AC adenocarcinoma, and the DMBA-induced tumor in the presence and absence of unlabeled triamcinolone acetonide for 16 hr at 3°. Specific binding sites in the cytosol from the lactating mammary gland were saturated at ~25 nM [^{3}H]triamcinolone acetonide (Figure 3A). Scatchard analysis (Scatchard, 1949) of these data yielded a straight line indicating a single class of binding sites with high affinity (Figure 3B). An apparent dissociation constant (K_d) of the ligand-binding complexes of 2.2×10^{-9} M was obtained. From the intercept on the abscissa, a binding capacity of 177 fmol/mg of cytosol protein was calculated for this particular preparation. The mean of three separate determinations of the dissociation constant gave a value of $3.3 \pm 0.7 \times 10^{-9}$ M and the average number of specific binding sites (n) was 113 ± 32 fmol/mg of cytosol protein.

Saturation of specific glucocorticoid-binding sites in the cytosol from the R3230AC tumor occurred at ~40 nM [^{3}H]triamcinolone acetonide. From four separate determinations, a K_d of $\sim 1.1 \pm 0.1 \times 10^{-8}$ M was calculated; the

Table I: Ligand Specificity of [^{3}H]Triamcinolone Acetonide Binding by Cytosol from Normal and Neoplastic Mammary Tissue.[a]

	Inhibition of Specific [^{3}H]-Triamcinolone Acetonide Binding (%)					
	Lactating Mammary Gland[b]		R3230AC Tumor[c]		DMBA-Induced Tumor[d]	
	[Competitor] × 10^6 M					
Competitive Substance	2.5	12.5	4	20	2	10
None	0	0	0	0	0	0
Triamcinolone acetonide	100	100	100	100	98	100
Corticosterone	94	99	84	97	89	98
Hydrocortisone	81	96	84	99	73	88
Dexamethasone	98	100	95	100	96	97
Cortexolone	52	87	48	81	41	73
Progesterone	81	98	67	91	65	87
Aldosterone	74	91	67	86	46	71
Estradiol-17β	4	23	7	29	7	17
Estrone	0	0	20	37	4	10
Estriol	2	0	0	9	7	6
Dihydrotestosterone	0	7	10	25	5	9
Testosterone	4	28	13	42	1	11

[a] Cytosol was prepared from homogenization of mammary tissues in Tris-HCl buffer (pH 7.4). Constant volumes of supernatant were incubated at 3° for 16–17 hr with [^{3}H]triamcinolone acetonide either alone or in the presence of two concentrations of the unlabeled competitors. Triplicate determinations of binding were made by the dextran-coated charcoal procedure. [b] Cytosol (8.6 mg of protein/ml) prepared from mammary gland of a 10-day post-partum rat was incubated with 27 nM [^{3}H]triamcinolone acetonide. [c] Cytosol (11.8 mg of protein/ml) prepared from 26-day old R3230AC tumor was incubated with 42 nM [^{3}H]triamcinolone acetonide. [d] Cytosol (15.9 mg of protein/ml) prepared from a DMBA-induced tumor was incubated with 20 nM [^{3}H]triamcinolone acetonide.

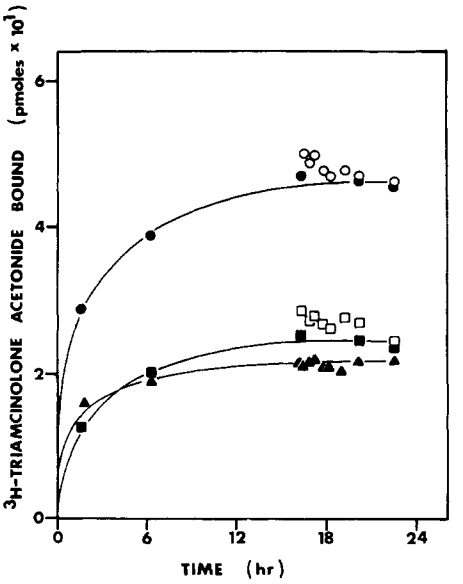

FIGURE 4: Time course of association and dissociation of [^{3}H]triamcinolone acetonide to binding sites in cytosol of mammary gland. Constant volumes of cytosol prepared from mammary gland of a 19-day postpartum rat were incubated with 23 nM [^{3}H]triamcinolone acetonide either alone (●) or in the presence of 2 × 10^{-5} M unlabeled triamcinolone acetonide (■). At various times aliquots were removed and binding was measured by the dextran-coated charcoal procedure. Specific binding (▲) was estimated as the difference in total binding and binding in the presence of unlabeled ligand. After 16 hr of incubation, 2 × 10^{-5} M unlabeled triamcinolone acetonide was added to each reaction. Exchange of "free" unlabeled triamcinolone acetonide for [^{3}H]triamcinolone acetonide previously bound to sites was studied as a function of time by the dextran-coated charcoal procedure. Measurements of total (○), nonspecific (□), and specific binding (▲) made after the addition of excess unlabeled triamcinolone acetonide to the reaction for determining total binding are indicated.

mean of the n was 377 ± 52 fmol/mg of cytosol protein. Similarly, titration curves for cytosol from the DMBA-induced tumor indicated saturation of specific binding sites at ~20 nM [^{3}H]triamcinolone acetonide. The average of five separate determinations of the apparent K_d was 8.8 ± 2.3 × 10^{-9} M and the mean of the number of binding sites was 181 ± 60 fmol/mg of cytosol protein.

Ligand Specificity of [^{3}H]Triamcinolone Acetonide Binding Sites. Various unlabeled steroid hormones and synthetic steroids were incubated with [^{3}H]triamcinolone acetonide to determine which were competitive for specific binding sites in cytosols from the lactating mammary gland, R3230AC tumor, and the DMBA-induced mammary tumor (Table I). In these mammary tissues, all of the natural adrenal corticoids tested as well as the synthetic glucocorticoid, dexamethasone, each at ~100-fold excess of the concentration of [^{3}H]triamcinolone acetonide, inhibited specific binding significantly as determined by the dextran-coated charcoal procedure. However, cortexolone, considered an antiglucocorticoid by some investigators (Turnell et al., 1974), was a much weaker competitor except at high concentrations. Surprisingly, aldosterone and progesterone demonstrated significant inhibition of binding at ~100-fold and ~500-fold excesses of the ^{3}H-labeled steroid. All the other steroids, namely the naturally occurring androgens and estrogens, showed limited or no inhibition of the binding reaction.

Kinetics of Dissociation of [^{3}H]Triamcinolone Acetonide from Binding Sites. Cytosol was allowed to bind [^{3}H]triamcinolone acetonide maximally and then excess unlabeled triamcinolone acetonide was added to the reaction and the rate of exchange of the ligands was measured as a function of time by the dextran-coated charcoal procedure (Figure 4). Using another method to estimate the rate constant of dissociation, cytosol which had bound [^{3}H]triamcinolone acetonide maximally was treated with a suspension of dextran-coated charcoal and the quantity of bound [^{3}H]triamcinolone acetonide was measured as a function of time (Figure 5). Both procedures indicated that specific association of [^{3}H]triamcinolone acetonide with binding sites in cytosol was essentially irreversible over a 5-hr period at 3°. Computation of the half-life of the complexes between ^{3}H-labeled steroid and binding protein using a K_d of 3 × 10^{-9} M and a rate constant of association of 3 × 10^5 M^{-1} min^{-1} gave a value of 11–12 hr, however. Kinetics of dissociation of labeled ligand at higher temperatures was not measured. From the data shown in Figure 5 it was also evident that treatment of reactions with dextran-coated charcoal for 10 min was sufficient to remove "free' [^{3}H-labeled] ligand. Hence, in all subsequent binding determinations a 10-min incubation period with the dextran-coated charcoal was employed.

Molecular Characteristics of [^{3}H]Triamcinolone Acetonide-Binding Proteins from Mammary Tumors. Centrifugation of [^{3}H]triamcinolone acetonide-binding protein complexes labeled in vitro on sucrose gradients of low ionic strength indicated a single major component sedimenting at 7.1 S in the cytosol of the DMBA-induced tumor (Figure 6A). However, on gradients of high ionic strength a single binding component was observed sedimenting at 4.4 S (Figure 6B). Sedimentation coefficients of the binding proteins

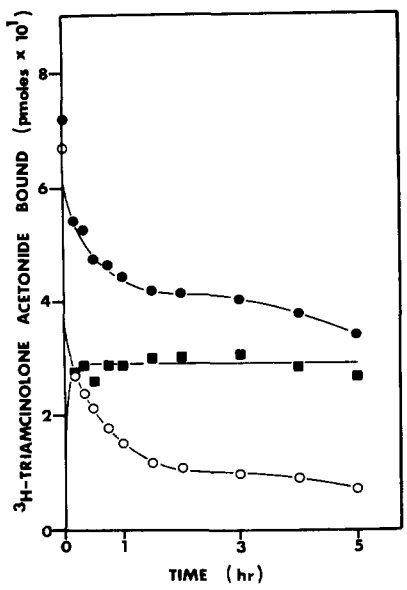

FIGURE 5: Time course of dissociation of [^{3}H]triamcinolone acetonide binding protein complexes. Cytosol was prepared from mammary gland of a 16-day postpartum rat by homogenization (1:4, w/v) in Tris-HCl buffer (pH 7.4) and incubated with 25 n*M* [^{3}H]triamcinolone acetonide either in the presence (O) or absence (●) of 2×10^{-5} *M* unlabeled triamcinolone acetonide for 16 hr at 3°. At the end of this incubation period dextran-coated charcoal (1 ml, 0.5% Norit A) was added to each reaction and incubated for various lengths of time. Triplicate determinations of binding were made at each time point shown. Specific binding (■), estimated as the difference in binding in the presence and absence of unlabeled ligand, remained constant for 5 hr.

were estimated using several marker proteins which were sedimented through similar sucrose gradients in the same centrifuge run (Martin and Ames, 1961). Radioactivity near the top of the gradient was attributed to unbound steroid. These data suggested dissociation of the 7.1S component into lower molecular weight entities in the presence of 0.4 *M* KCl. Summation of the specific binding capacities of the components sedimenting at 7–8 S and 4–5 S demonstrated that there was conservation of bound radioactivity following dissociation of the heavier component into the more slowly sedimenting species (Figure 6).

Similarly, cytoplasmic glucocorticoid-binding proteins labeled in vitro from the R3230AC tumor demonstrated two binding components of 8 S and 3.5 S when sedimented on linear sucrose gradients of low ionic strength. In the presence of 0.4 *M* KCl a single species sedimenting at 3.5 S was observed (data not shown). Conservation of specific binding capacity was also associated with the dissociation process. These data on the sedimentation properties of glucocorticoid binding components in the R3230AC tumor and the DMBA-induced tumor of the rat are consistent with those described previously for the normal mammary gland (Gardner and Wittliff, 1973a).

It has been shown that the sedimentation characteristics of the glucocorticoid binding proteins labeled in vivo in the lactating mammary gland and in the R3230AC adenocarcinoma were similar to those observed by procedures in vitro (Boyd and Wittliff, 1973). Likewise, the molecular properties of glucocorticoid binding proteins from the cytosol of DMBA-induced tumors which were labeled in vivo with [^{3}H]triamcinolone acetonide were correlated with those observed in vitro. Shown in Figure 7, the cytoplasmic triamcinolone acetonide-binding protein complexes sedimented at 8.6 S on linear 5–40% sucrose gradients of low ionic strength (profile A). Nuclear extracts which were centrifuged on linear 5–20% sucrose gradients containing 0.4 *M* KCl indicated a single binding component sedimenting at 4.1 S. The data indicated that within 1 hr after subcutaneous injection of [^{3}H]triamcinolone acetonide 75% of the specifically bound steroid was recoverable in the nuclear fraction.

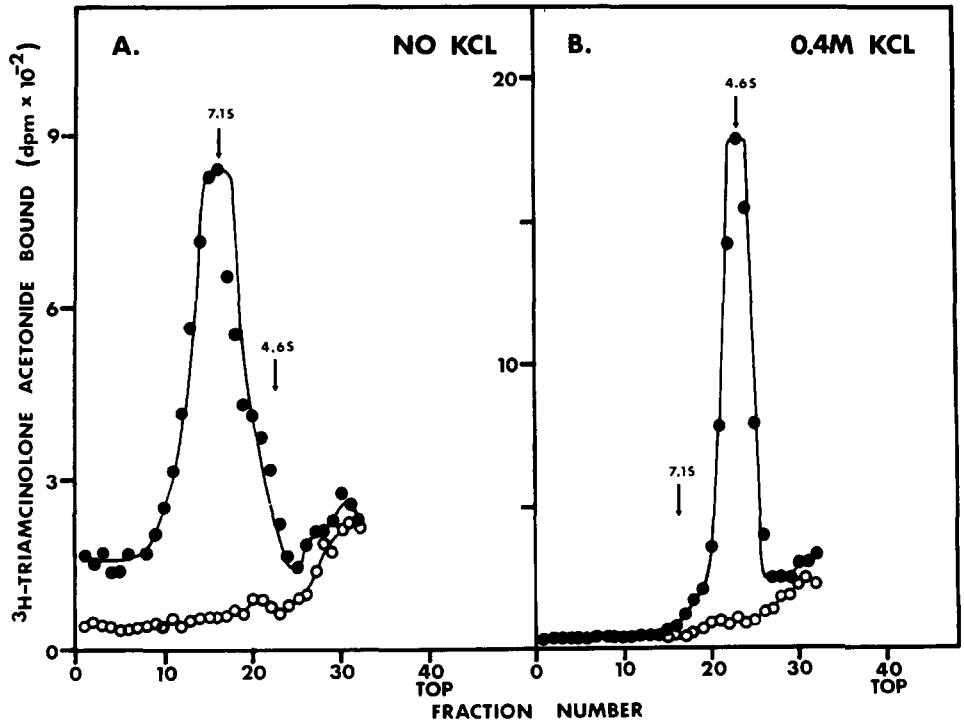

FIGURE 6: Sedimentation characteristics of cytoplasmic glucocorticoid binding proteins labeled in vitro from DMBA-induced mammary tumor. Cytosol prepared from a DMBA-induced tumor which was homogenized (1:3, w/v) in Tris-HCl buffer (pH 7.4) was incubated with 25 n*M* [^{3}H]triamcinolone acetonide alone (●) or in the presence of unlabeled triamcinolone acetonide (O) for 6 hr at 3°. Aliquots (200 μl) of the reaction were centrifuged for 15–16 hr at 3° on linear 5–40% sucrose gradients either in the absence (A) or presence (B) of 0.4 *M* KCl.

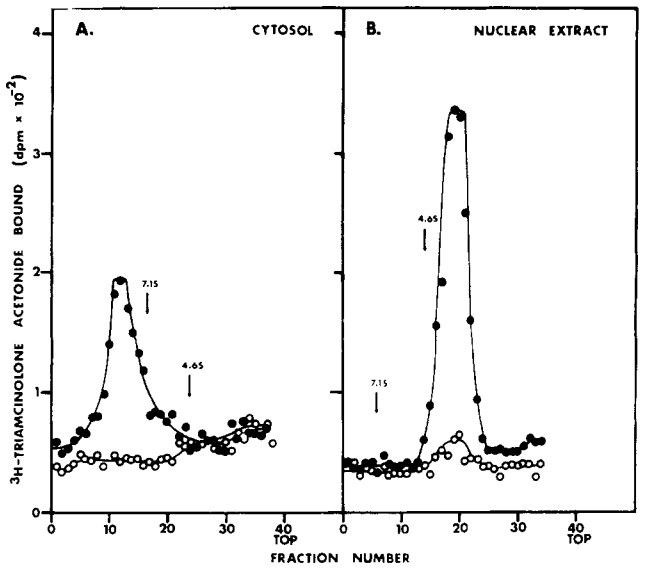

FIGURE 7: Sedimentation of glucocorticoid binding proteins labeled in vivo from DMBA-induced mammary tumors. Cytosols and nuclear extracts of tumors were prepared from pairs of rats carrying DMBA-induced tumors matched based upon tumor size and location. Each animal had been injected subcutaneously with 0.4 μCi of [^{3}H]triamcinolone acetonide alone (●) or following an injection of unlabeled triamcinolone acetonide (○) 30 min earlier. Cytosols (200 μl) were centrifuged on linear 5–40% sucrose gradients of low ionic strength (A) while nuclear extracts (200 μl) were centrifuged on linear 5–20% sucrose gradients containing 0.4 *M* KCl (B). All gradients were centrifuged for 16 hr at 3° in a SW56Ti rotor at 308,000*g*.

Discussion

Specific uptake and retention of [^{3}H]cortisol by cultured bovine mammary cells was demonstrated first by Tucker et al. (1971) who detected two types of binding sites with high affinity. Using [^{3}H]triamcinolone acetonide which does not bind significantly to transcortin, our laboratory employed sucrose gradient centrifugation to characterize a single type of glucocorticoid binding sites in the lactating mammary gland of the rat (Gardner and Wittliff, 1973a) and in a glucocorticoid-responsive mammary tumor (Gardner and Wittliff, 1973b). Similar studies have been reported for the mammary gland of the mouse using [^{3}H]dexamethasone (Shyamala, 1973). Since these later studies found only one type of binding sites using ligands containing a 9α-fluoro group, it appears that one of the types of sites with high affinity for [^{3}H]cortisol observed by Tucker et al. (1971) was contributed by transcortin.

More extensive characterization of the [^{3}H]triamcinolone acetonide binding sites required the development of a rapid, quantitative procedure permitting large numbers of binding measurements. A modification of a dextran-coated charcoal procedure described by Gardner and Wittliff (1973c) for measurements of estrogen binding met these criteria.

Time course of association of [^{3}H]triamcinolone acetonide to specific binding sites in cytosol from mammary tissues were similar to those found by Pratt and Ishii (1972) using cytosols from cultured fibroblasts. Optimal binding at 3° was achieved by 8–10 hr and remained unchanged up to 24 hr in the presence of monothioglycerol. Glucocorticoid binding by components in rat skeletal muscle was reported to reach a maximum within 2 hr (Mayer et al., 1974). In contrast, maximal binding of [^{3}H]triamcinolone acetonide to specific sites in cytosols from lung tissues required incubation periods of 18–20 hr at 3° (Toft and Chytil, 1973). At 25°, binding was maximal by 15 min followed by rapid dissociation and/or degradation of complexes from mammary tissue. Since elevated temperatures adversely affected determinations of glucocorticoid-binding capacities and the short incubation period was unsatisfactory, binding reactions were performed routinely at 3° for 8–18 hr.

Binding of [^{3}H]triamcinolone acetonide was linear over a range of 0.5–5.0 mg of protein/reaction ensuring that the estimations of specific glucocorticoid binding capacity in mammary tissues were quantitative under the conditions outlined. In comparison, association of [^{3}H]triamcinolone acetonide with binding components in cytosol from fibroblasts was linear to 1.5 mg of protein (Pratt and Ishii, 1972).

As observed in cultured hepatoma cells (Baxter and Tomkins, 1971) and in liver (Koblinsky et al., 1972), PCMB significantly reduced [^{3}H]ligand binding capacity measured in vitro for the lactating mammary gland (Gardner and Wittliff, 1973a). Additional evidence of a role for sulfhydryl groups in the binding reaction was provided by the observation that reducing agents, such as monothioglycerol, maximized [^{3}H]triamcinolone acetonide binding to specific sites in lung (Toft and Chytil, 1973) and rat skeletal muscle (Mayer et al., 1974). Likewise glucocorticoid binding measured in vitro in cytosols from the lactating mammary gland was increased twofold by the addition of this agent.

Dissociation constants calculated from Scatchard analyses of [^{3}H]triamcinolone acetonide binding data for the lactating mammary gland and the R3230AC mammary tumor of the rat were lower than those determined earlier using sucrose gradient analyses (Gardner and Wittliff, 1973a,b), i.e., 10^{-8}–10^{-9} *M* compared to 10^{-7}–10^{-8} *M*. However, our estimates by Scatchard analysis using the dextran-coated charcoal procedure were in agreement with values reported by Shyamala (1973, 1974) for the normal and neoplastic mammary gland of the mouse and by others using lung (Ballard and Ballard, 1972; Giannopoulos, 1973; Toft and Chytil, 1973), thymocytes (Kaiser et al., 1973; Schaumberg and Crone, 1971), liver (Koblinsky et al., 1972), and rat skeletal muscle (Mayer et al., 1974). Binding capacities of the lactating mammary gland measured by the dextran-coated charcoal procedure varied considerably (70–200 fmol/mg of cytosol protein) presumably due to the stage of differentiation of the gland as was suggested earlier (Gardner and Wittliff, 1973a,c). These were comparable to those of glucocorticoid binding capacities of the lactating gland of the vole (Turnell et al., 1974) and mouse (Shyamala, 1973), in which the number of sites ranged from 100 to 300 fmol/mg of cytosol protein. The binding capacity determined for the R3230AC mammary tumor (250–500 fmol/mg of cytosol protein) was 2–5-fold higher than previously reported (Gardner and Wittliff, 1973b). The number of glucocorticoid binding sites in the DMBA-induced mammary tumor also varied considerably over a range of 50–400 fmol/mg of cytosol protein. The nature of the variation in binding may be the result of changes in the levels of circulating glucocorticoid since these experiments were conducted on animals with intact adrenal glands.

Association rate constants (k_1) for [^{3}H]triamcinolone acetonide binding were 2–4 $\times$ 10^5 M^{-1} min^{-1} in cytosols from the lactating mammary gland and the mammary tumors described. These estimates compared well with those determined in fibroblasts (8 $\times$ 10^5 M^{-1} min^{-1}) by Pratt and Ishii (1972) and those in thymocytes (2 $\times$ 10^5 M^{-1}

min^{-1}) by Schaumberg (1972). [^{3}H]Triamcinolone acetonide did not appear to be bound covalently, but rather dissociated with a long half-life (11–12 hr), confirming observations in other systems that unlabeled triamcinolone acetonide does not exchange readily with previously bound ^{3}H-labeled steroid (Pratt and Ishii, 1972; Schaumberg, 1972; Toft and Chytil, 1973).

Ligand specificity studies using cytosols of mammary tissues indicated that [^{3}H]triamcinolone binding sites also bound corticosterone, cortisol, and dexamethasone readily. Aldosterone and the anti-glucocorticoid, cortexolone, had intermediate competitive properties while androgens and estrogens were poor competitors even at high concentrations. These results were consistent with competition studies of glucocorticoid binding in cytosols from lung (Ballard and Ballard, 1974), hepatoma (Baxter and Tomkins, 1971), and leukemic blast cells (Lippman et al., 1973). Progesterone was also a good competitor in the lactating mammary gland system and to a lesser degree, except at high concentrations, in the tumor preparations. Similarly, progesterone was an effective competitor of glucocorticoid binding in cytosols from other glucocorticoid target tissues (Lippman et al., 1973; Rousseau et al., 1972, Schaumberg, 1972; Shyamala, 1973; Mayer et al., 1974). In contrast Toft and Chytil (1973) observed that progesterone and aldosterone at low excesses (ca. tenfold) of the ^{3}H-labeled ligand showed essentially no competition for glucocorticoid binding in lung cytosols.

The cytoplasmic glucocorticoid binding components from the R3230AC and DMBA-induced tumors sedimented at 7–8 S on sucrose gradients containing low salt and dissociated into components sedimenting at 4–5 S on high ionic strength sucrose gradients. These properties are consistent with those of glucocorticoid receptors reported for the normal mammary gland (Gardner and Wittliff, 1973a; Shyamala, 1973; Turnell et al., 1974) and certain mouse mammary tumors (Shyamala, 1974).

Perhaps most important was the demonstration that binding in vivo of [^{3}H]triamcinolone acetonide to cytoplasmic and nuclear components from the DMBA-induced tumor was similar to that described for the lactating mammary gland and R3230AC tumor (Boyd and Wittliff, 1973). These results confirmed studies in vitro and indicated that [^{3}H]triamcinolone acetonide binding components translocated from cytoplasmic to nuclear compartments. Nuclear translocation of glucocorticoid–receptor complexes has been observed consistently in other glucocorticoid target tissues as well (Baxter and Tomkins, 1971; Boyd and Wittliff, 1973; Giannopoulos, 1973).

The data in this report suggest that characteristics of glucocorticoid binding in the normal and neoplastic mammary gland of the rat are similar, although the response to or dependency upon glucocorticoid may vary considerably. It is known that glucocorticoids may promote lysis in lymphoid tissues (e.g., Baxter et al., 1971; Kaiser et al., 1973) while in organs such as the liver or muscle these hormones are stimulative (e.g., Baxter and Tomkins, 1970; Koblinsky et al., 1972; Mayer et al., 1974). Therefore, even though normal and neoplastic mammary cells may have a similar mechanism for the initial interaction with glucocorticoids, each may proceed through a different sequence of events leading to the biologic response, be it alterations in cellular proliferation, formation of endoplasmic reticulum, or cell lysis. Currently we are examining this sequence in dissociated cells of each tissue.

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Heavy Metal–Nucleoside Interactions. Binding of Methylmercury(II) to Inosine and Catalysis of the Isotopic Exchange of the C-8 Hydrogen Studied by ^1H Nuclear Magnetic Resonance and Raman Difference Spectrophotometry†

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ABSTRACT: Raman difference spectrophotometry reveals that CH_3Hg^{II} binds quantitatively to N(1) of inosine at pH 8, substituting for the proton. When N(1) is saturated, binding occurs at a second site. Measurements of the ^1H nuclear magnetic resonance spectra of both inosine and of CH_3Hg^{II} are in agreement with the N(1) binding and indicate that the second site for mercuriation is N(7). This second binding reaction is observed to increase the rate of exchange of the C(8) hydrogen with solvent, consistent with results observed for alkylation at N(7). Coordination of the electrophilic CH_3Hg^{II} to N(7) increases the acidity of H(8), facilitating OH^--catalyzed proton abstraction and reprotonation by the medium. For comparison, the reaction of CH_3Hg^{II} with [8-^2H]inosine has been studied. Displacement of the N(1) hydrogen upon mercuriation of inosine causes a significant electron delocalization into the ring, increasing the basicity of N(7), and accounting for the synergic effect in metal binding observed originally by Simpson. In contrast, 1-methylinosine interacts only slightly with CH_3Hg^{II} at pH 8. Coordination appears to be at N(7), since H(8) again is observed to exchange rapidly with solvent protons. In acidic solution, pH <2, binding to inosine is almost quantitative and exclusively to N(7). The behavior of CH_3Hg^{II} is compared with that of Pt(II) and with Ni(II), Co(II), and Zn(II). A brief comparison is made among ultraviolet absorption spectrophotometry, nuclear magnetic resonance (NMR), and Raman difference spectrophotometry for studying reactions of nucleosides and nucleotides.

Recently, we used Raman difference spectrophotometry to study the binding of CH_3Hg^{II} to GMP in H_2O and D_2O at pH 2 and 8.5 (Mansy and Tobias, 1974a). The methylmercury(II) cation makes an ideal probe for determining the binding sites of heavy metals. At pH 8.5, a quantitative substitution of the proton bound to N(1) occurred up to a 1:1 metal:base mole ratio, and at higher ratios a second, almost quantitative reaction occurred with additional binding of CH_3Hg^{II} to the base moiety. There was no indication of any coordination to the phosphate. The changes in the GMP vibrational spectrum suggested that the electron distributions of the base in [$CH_3HgGuoH_{-1}$-5′-P] and [$GuoH_{-1}$-5′-P]$^-$ [1] were quite similar, i.e. that binding of CH_3Hg^+ does not prevent extensive delocalization of the lone electron pair into the ring system. The second binding site could not be assigned definitely, although Simpson (1964) had suggested that binding could occur at N(1), N(7), and the C(2) NH_2 group by analogy with protonation. A recent Raman study of the binding of CH_3Hg^{II} to the amino groups of cytidine and adenosine (Mansy et al., 1975) indicates that this type of reaction will be unimportant under the conditions of the GMP binding studies. At low pH, the Raman spectra suggested that binding occurred with displacement of the N(7) proton of [GuoH-5′-P]$^+$ which is the predominant species at pH 2.

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[1] Since Guo refers to the neutral molecule which is a dibasic acid, we have used $GuoH_{-1}$ to describe the conjugate base which has lost the N(1) hydrogen. The methylmercury complex of this ligand then is written $CH_3HgGuoH_{-1}$. Mercuriation at N(7) of guanosine that is protonated at N(1) gives CH_3HgGuo^+. This emphasizes the pH dependence of the formation of these complexes.