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Correlation between Hormone Binding and Growth Response of Rat Mammary Tumor[1]

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SUMMARY

Dimethylbenzanthracene-induced rat mammary tumors were defined as either prolactin responsive or prolactin independent on the basis of growth response to prolactin administration. There was no difference in tumor binding of prolactin between the two groups when tumors were biopsied before treatment. Prolactin binding was, however, significantly higher in responding tumors when biopsies were obtained following treatment. By contrast, when tumors were defined as responsive or independent on the basis of response to suppression of serum prolactin with bromoergocryptine, there was significantly higher prolactin binding in the responsive than in the independent group both before and after treatment. During serial treatment with prolactin followed by bromoergocryptine, there was a progressive decline in prolactin binding to tumor biopsies, particularly in prolactin-independent tumors.

Prolactin binding to pretreatment tumor biopsies thus did not predict which tumors would respond to administration of prolactin but, for the total group, did indicate tumors likely to regress with prolactin withdrawal. However, the correlation between prolactin binding and tumor regression following hormone withdrawal was not sufficiently strong to permit reliable prediction of behavior for individual tumors. Prolactin-independent growth was associated with decreased prolactin binding to tumor tissue, particularly following manipulation of serum prolactin levels.

INTRODUCTION

It has recently become apparent that the content of hormone binding sites in normal tissues may vary under both physiological (12) and pathological (10) conditions. This has led to the concept that hormone responsiveness may in part be related to tissue content of hormone binding sites. There is some evidence that hormone binding by neoplastic tissue might similarly indicate the endocrine responsiveness of a tumor. Thus the presence or absence of specific estrogen-binding proteins in animal (17) or human (9) mammary carcinomas has been held to give some indication of underlying sensitivity to estrogen stimulation and, in the human, to predict the probable response to estrogen withdrawal. There is some evidence that polypeptide hormones share a similar relationship with tumor tissues. The DMBA[3]-induced rat mammary tumor, an experimental neoplasm known to be responsive to polypeptide hormones including prolactin (20) and insulin (7), contains appreciable quantities of binding sites specific for prolactin (11). Several groups have demonstrated that the hormone responsiveness of such tumors is correlated with tumor binding of labeled prolactin in samples obtained after endocrine manipulation (6, 11). However, in these studies the possibility exists that the hormone treatment itself may have influenced the receptor content of tumor tissue; for instance, an increase in circulating levels of prolactin can induce prolactin receptors in liver tissue from male rats (18). The present investigation was thus designed to test whether hormone binding to tumors measured prior to treatment might predict the growth response to endocrine manipulations.

MATERIALS AND METHODS

Animals and Experimental Tumors. Female Sprague-Dawley rats were obtained from North American Breeding Laboratories, Manitoba, Canada. The rats were housed under 12-hr light-dark conditions and maintained on Teklad rat diet. At 55 days of age, animals received a single injection of 5 mg DMBA emulsion by tail vein. When mammary tumors appeared they were measured weekly, and length, breadth, and depth were summed to give a size equivalent.

Buffers, Standard Materials, and Labeled Hormones. The standard assay buffer was 0.025 M Tris-HCl, pH 7.6, containing 10 mM $MgCl_2$ and 0.1% bovine serum albumin. Ovine prolactin (NIH-P-S-10) for treatment of animals, labeling, and use as an assay standard was kindly supplied by the Endocrine Study Section, Institute of Arthritis, Metabolic and Digestive Diseases, Bethesda, Md. Rat prolactin for labeling and radioimmunoassay was obtained as a kit from the National Institute of Arthritis, Metabolic and Digestive Diseases. Porcine insulin (22 units/mg) was obtained from Connaught Laboratories, Toronto, Ontario, Canada. Nafoxidine and DMBA were kindly supplied by Upjohn Co., Kalamazoo, Mich., and 17β-[6,7-^{3}H]estradiol (40 mCi/ml) was obtained from New England Nuclear, Boston, Mass. Bromoergocryptine was supplied by Sandoz Ltd., Basel, Switzerland. Labeling of ovine and rat prolactin with ^{125}I (New England Nuclear) was carried out using a soluble

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[3] The abbreviations used are: DMBA, 7,12-dimethylbenz(a)anthracene; bromoergocryptine, 2-bromo- α-ergocryptine.

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lactoperoxidase method (21), and ^{125}I insulin was prepared by chloramine-T oxidation (5).

Treatment of Animals. Seventeen weeks after injection of DMBA, 25 tumors in 12 rats were biopsied under ether anesthesia to obtain tissue for binding studies. About 5 cu mm of tissue were removed; biopsies did not appear to disturb the growth curve of individual tumors. After 10 to 14 days were allowed for healing, animals were given s.c. injections of 1 mg prolactin daily for 10 days. Growth measurements were made every 4 days over the treatment and immediate posttreatment period. Five days after the final injection, tumors were again biopsied for binding studies.

In a 2nd experiment, 12 rats from the prolactin-treated group and 4 fresh animals, comprising 45 tumors in all, were treated with bromoergocryptine. Biopsies were obtained 2 to 4 weeks before treatment, and in the case of rats previously treated with prolactin the postprolactin treatment biopsies served as the prebromoergocryptine biopsy. At 23 weeks of age animals were given injections of an emulsion of 400 μg (150 μg/100 g weight) bromoergocryptine in oil daily for 3 weeks. Blood samples were taken before the 1st treatment and 24 hr after the final injection to check the degree of prolactin suppression. Four days after the final injection animals were sacrificed and segments of liver and tumor were removed for binding studies. Tumor dimensions were measured weekly over the treatment period.

Definition of Hormone-responsive and Hormone-independent Tumors. Tumors showing no change in size or a net change opposite to that expected during treatment (*i.e.*, tumors that decreased in size during prolactin administration or grew during prolactin suppression) were defined as hormone independent. Tumors that at the end of treatment exceeded (in the case of prolactin administration) or were less than (in the case of prolactin suppression) pretreatment measurements by 10% or more were defined as hormone responsive.

Binding of Polypeptide Hormones. The binding of polypeptide hormones to biopsy samples was performed using ultrathin (20 μm) slices of tissue obtained in a cryostat. Fresh biopsies were trimmed into 5-cu mm blocks and snap frozen in *n*-hexane maintained at −70° in an ethanol-dry ice chilling bath. Tissue was kept at −20° for up to 4 weeks before sectioning, and under these conditions the binding capacity remained constant for at least 8 weeks of storage. The segments were mounted in the frozen state on chilled cryostat chucks; sections 20 μm thick were taken and, while still frozen, were transferred with cold forceps to 12- x 75-mm chilled glass test tubes. Four slices/tube provided adequate tissue for hormone binding to be easily measurable. About 30 tubes could be prepared from a single tissue block. The protein content of slices was measured (15) after digestion in 0.1 M sodium hydroxide, and slices were also collected onto preweighed aluminum pans, dried, and weighed in a microbalance.

The binding of ^{125}I-prolactin was measured in tubes containing 4 tumor slices (approximately 200 μg protein or 400 μg dry weight/tube). Quadruplicate tubes received either 100 μl assay buffer or 100 μl buffer containing prolactin, 10 μg/ml, followed by 100 μl of buffer containing 0.5 ng ^{125}I-prolactin (approximately 50,000 cpm). The samples were shaken for 6 hr at room temperature, and under these conditions binding reached a plateau, whereas 5% or less of the labeled hormone was degraded by tissue enzymes. At the end of the incubation period, 3 ml of cold assay buffer were added to each tube and the samples were centrifuged at 3000 rpm for 20 min. Further wash steps were found to be unnecessary, and after decanting the supernatants the tissue pellets were counted in an automatic gamma counter. Insulin binding was carried out by the same technique but using 24 hr of incubation at 4°. The lower temperature and consequent longer incubation time were found to be necessary in order to prevent excessive degradation of the labeled hormone. Specific binding was calculated as the difference between mean counts bound in the absence and presence of excess unlabeled hormone and expressed as a percentage of total counts. Since there was a linear relationship between binding and tissue weight or protein content over the range 100 to 500 μg and 100 to 350 μg, respectively, binding was adjusted to 200 μg tissue protein or 400 μg tissue dry weight for comparative purposes.

Estradiol Binding. Approximately 3 g of tissue, obtained at the time that animals were sacrificed, were homogenized and centrifuged at 100,000 × *g* according to the method of McGuire and De La Garza (16). The cytosol fraction was either measured directly or stored at −20° for a short period until assay by a charcoal adsorption method (13) using very low concentrations of labeled estradiol (0.5 nM). Binding was expressed according to the method of Leung *et al.* (14) as specific binding (total binding minus binding in the presence of 10 μM nafoxidine) divided by total binding, expressed as a percentage. This value was occasionally negative when the binding in the presence of inhibitor exceeded "total" binding.

RESULTS

Specificity and Precision of Microslice Assay. The displacement of labeled ovine prolactin from slices of rat mammary tumor by 4 dose levels of a number of unlabeled hormones is shown in Table 1. Unlabeled ovine prolactin has been arbitrarily assigned a potency of 100%. Nonspecific binding, that is, counts remaining in tubes containing

Table 1
Specificity of binding of labeled ovine prolactin by ultrathin slices of rat mammary tumor

Hormone	Potency (%)
Ovine prolactin	100
Bovine prolactin	120
Human prolactin	100
Human growth hormone	100
Rat prolactin	60
Human placental lactogen	55
Ovine growth hormone	1
Rat growth hormone	all <0.1
Bovine growth hormone	all <0.1
Ovine luteinizing hormone	all <0.1
Bovine thyroid stimulating hormone	all <0.1
Porcine insulin	all <0.1

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excess unlabeled prolactin expressed as a percentage of total counts added, was 3 ± 1.1% for prolactin and 1.8 ± 0.9% for insulin (mean ± S.D., $n = 43$). Approximately one-half of this figure represents counts bound to the tube, *i.e.*, nonspecific binding in the absence of tissue. The precision of measurement, assessed as the mean coefficient of variation for total binding measured in quadruplicate tubes, was ±6.7% for prolactin and ±5.7% for insulin. The reproducibility of binding of ^{125}I-prolactin and ^{125}I-insulin to multiple biopsies from different areas of a large mammary tumor is shown in Table 2. The coefficient of variation for hormone binding per 200 µg protein was ±18% for prolactin and ±24% for insulin.

Prolactin Binding and Tumor Response to Prolactin Treatment. There was no significant difference in the binding of labeled prolactin between hormone-responsive and hormone-independent tumors when the binding study was performed before treatment with prolactin (Chart 1). By contrast, after treatment a significant difference appeared between the 2 groups, with those tumors that had responded to prolactin administration binding significantly

Table 2

Variation in specific binding of prolactin and insulin in 7 biopsies from a rat mammary tumor

	% specific binding/200 µg protein	% specific binding/ 400 µg dry tissue wt
Prolactin	7.4 ± 1.3[a] (18)[b]	7.9 ± 1.3 (16)
Insulin	3.3 ± 0.8 (24)	3.5 ± 0.7 (20)

[a] Mean ± S.D.

[b] Numbers in parentheses, coefficients of variation (%).

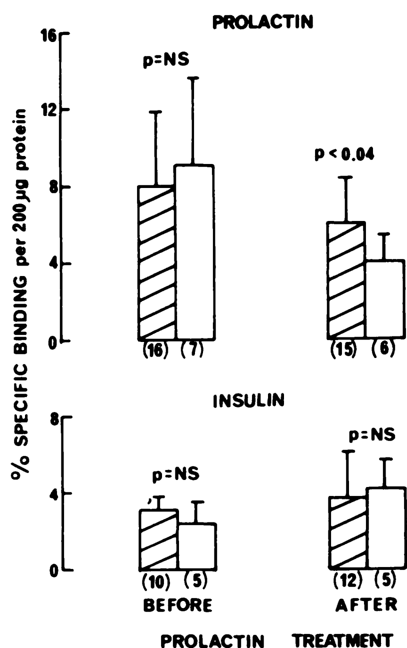

Chart 1. Mean specific binding of ^{125}I-prolactin (*upper panel*) and ^{125}I-insulin (*lower panel*) to hormone-responsive (*shaded columns*) and hormone-independent (*open columns*) rat mammary tumors, in biopsies obtained either before or after treatment of rats with prolactin. *Numbers in parentheses*, number of tumors in each group; *vertical bars*, 1 S.D.; *NS*, not significant.

more prolactin than did nonresponders (Chart 1). However, if binding and growth response of individual tumors was compared by regression analysis, there was no significant correlation either before or after prolactin treatment. Treatment with prolactin was associated with an increase in binding of insulin ($p < 0.01$) and a fall in binding of prolactin ($p < 0.01$) in hormone-independent tumors, whereas hormone binding to the prolactin-responsive group was not significantly altered.

Response of Serum Prolactin to Bromoergocryptine Treatment. There was a pronounced fall in serum prolactin in all animals treated. The mean prolactin level ±2 S.D. 24 hr after the final injection of bromoergocryptine was 8.1 ± 7.9 ng/ml ($n = 11$) compared with a mean level of 27.8 ± 16.2 ng/ml ($n = 14$) before institution of treatment ($p < 0.001$).

Prolactin Binding and Tumor Response to Prolactin Suppression. The binding of labeled prolactin to biopsies obtained before prolactin suppression was significantly higher in tumors that later responded to prolactin suppression than in the independent group (Chart 2). This difference was maintained when binding was restudied in tissue obtained at the end of bromoergocryptine treatment. The results were similar both in rats previously given prolactin by injection in the initial part of the study and in those animals receiving bromoergocryptine as their 1st endocrine treatment. When individual results were correlated by regression analysis, there was a significant relationship between binding in pretreatment biopsies and growth inhibition with prolactin suppression (Chart 3; $r = 0.67$), although there was considerable scatter of results. Binding of insulin and prolactin was significantly reduced ($p < 0.03$) in both responsive and independent groups following prolactin suppression.

Of 22 tumors in 11 animals treated with both prolactin and bromoergocryptine, 3 of 6 tumors that were nonresponsive to prolactin administration later responded to prolactin suppression, and 2 of 5 tumors that failed to regress with prolactin suppression had been previously responsive to prolactin administration. Thus the populations of hormone-independent tumors in Charts 1 and 2 are not identical. However, when the data were analyzed using only independent tumors common to both treatments, the values for specific binding and significance in Charts 1 and 2 were not altered.

Estrogen Binding following Prolactin Suppression. Binding of labeled estradiol to cytosol preparations obtained from tumor and liver tissue removed following bromoergocryptine treatment is shown in Chart 4, compared with results from tissues in control rats not exposed to hormone manipulation. There was significant reduction of estrogen binding in tumors following 3 weeks of prolactin suppression, with no significant change in specific estrogen uptake by liver. In the treated group there was no significant difference in estrogen binding of responsive compared with hormone-independent tumors; however, the numbers involved were too small to permit adequate statistical evaluation.

Change in Hormone Binding during Treatment. As seen in Chart 5 there was a significant fall in specific binding of prolactin to both responsive and independent tumors during the progress of the experiment. Insulin binding in-

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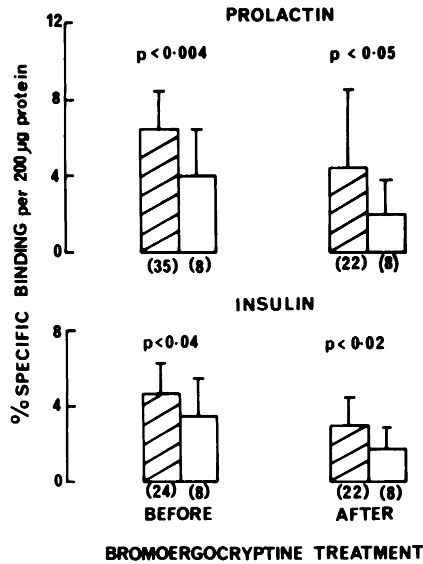

Chart 2. Mean specific binding of ^{125}I-prolactin (*upper panel*) and ^{125}I-insulin (*lower panel*) to biopsies of rat mammary tumors obtained before or after treatment of rats with bromoergocryptine. *Numbers in parentheses*, number of tumors in each group; *vertical bars*, 1 S.D.

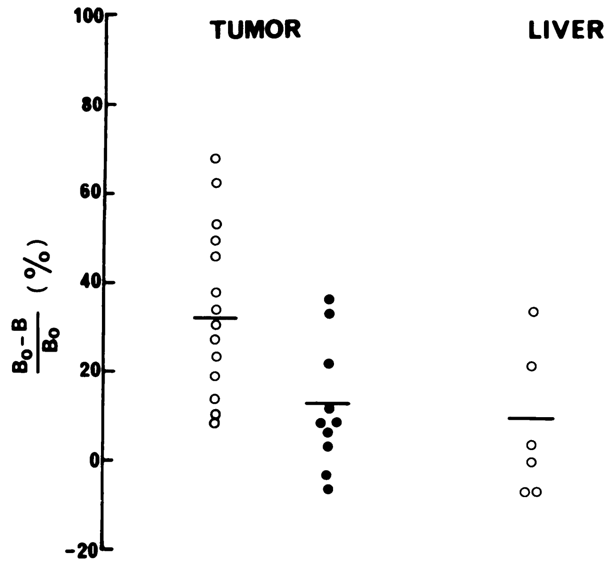

Chart 4. Binding of [^{3}H]estradiol by tumor and liver cytosol from control rats (○) and animals treated with bromoergocryptine (●). *B*, *Bo*, counts bound in presence and absence of 10 μM nafoxidine, respectively. *Bar*, mean.

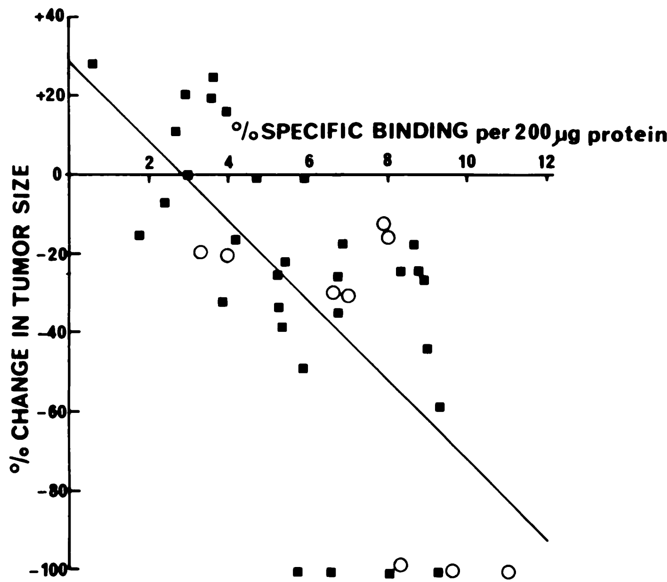

Chart 3. Relationship between inhibition of tumor growth with bromoergocryptine treatment and specific binding of ^{125}I-prolactin to biopsies obtained before treatment. ■, tumors from rats previously treated with prolactin; ○, tumors from animals with no previous hormone treatment. $r = 0.67$.

creased significantly after prolactin treatment but at the end of the experiment was not significantly different from the starting value.

DISCUSSION

In this study binding of labeled hormones to tumor tissues has been followed during manipulation of circulating prolactin levels in the rat. Animals received 2 treatments, prolactin administration and prolactin suppression, sequentially in the same order. This could be considered a weakness of experimental design since the 1st treatment could

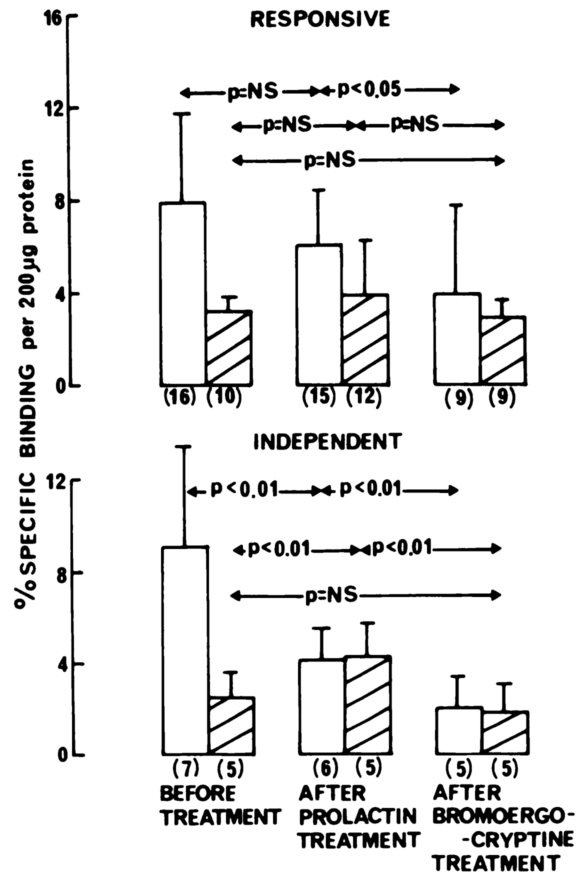

Chart 5. Specific binding of ^{125}I-prolactin (*open columns*) and ^{125}I-insulin (*shaded columns*) to serial biopsies of responsive (*upper panel*) and hormone-independent (*lower panel*) rat mammary tumors. Only tumors from animals receiving both treatments are included. *Numbers in parentheses*, number of tumors in each group; *vertical bars*, 1 S.D.; *NS*, not significant.

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have influenced later results; in particular, prolactin administration may have induced a group of tumors particularly responsive to changes in circulating prolactin levels. However, this seems unlikely since a fresh group of animals having received no previous endocrine treatment was introduced into the 2nd part of the study, and tumors in these rats yielded results identical to those from animals previously treated with prolactin. Also, tumors responding to prolactin were not necessarily those regressing with hormone suppression, showing that the response to the 2 treatments could be independent. It is possible that, with prolactin administration, hormones may have occupied binding sites and interfered with measurement *in vitro*, but since tumors were biopsied 4 days after the final injection, serum levels should have been close to normal. In addition, we have evidence that endogenous prolactin does not interfere with *in vitro* binding estimates until extremely high levels are attained.[4]

Bearing in mind these reservations, it appears that binding of labeled prolactin by pretreatment biopsies of DMBA-induced rat mammary tumors does not predict which tumors will grow in response to prolactin administration (Chart 1). A significant difference in the prolactin binding of responsive and independent tumors is, however, apparent after prolactin administration (Chart 1), due to a depression of binding in nonresponding tumors. Since prolactin binding in the hormone-responsive group was not significantly increased by treatment, it seems unlikely that prolactin administration induced binding sites in these tumors.

In contrast to the results of prolactin administration, tumor biopsies obtained either before or after suppression of prolactin showed a clear difference in binding between responsive and independent groups (Chart 2). There was a significant correlation between binding in pretreatment biopsies and response to treatment (Chart 3), although the trend was not sufficiently strong to permit reliable prediction of behavior for an individual tumor.

A progressive decrease in mean prolactin binding was observed during the experiment in serial biopsies of both responsive and independent tumors (Chart 5), particularly in the independent group. However, the observation that insulin binding was the same at the beginning and end of the study (Chart 5) argues against any nontreatment influence such as age as a cause for this decrease. Since the reduction in prolactin binding occurred particularly with prolactin suppression (Chart 5), the maintenance of these binding sites may depend upon maintenance of adequate levels of circulating prolactin. Also the striking reduction in prolactin binding in hormone-independent tumors suggests that autonomy may be associated with a low concentration of hormone binding sites, as already suggested by other investigators (4). Three "hormone-independent" tumors decreased in size with prolactin administration and then grew, sometimes dramatically, during prolactin withdrawal. These tumors could be redefined as responsive to the absence of prolactin; they all demonstrated extremely low binding of prolactin following both hormone administration and withdrawal.

[4] I. Holdaway, M. Deegan, and H. Friesen, manuscript in preparation.

We have no explanation for the increased insulin binding in hormone-independent tumors following prolactin administration (Chart 1), although the finding that a proportion of DMBA tumors regress following ablation of the pancreatic islets (3) suggests that insulin plays an important role in tumor maintenance *in vivo*.

The DMBA-induced rat mammary tumor is known to possess specific intracellular estrogen-binding proteins (17), levels of which can be increased *in vitro* by prolactin administration (19). Conversely, estrogen treatment can increase binding of prolactin to rat liver both *in vivo* (18) and *in vitro* (1). In the present study a decrease in estradiol binding to tumors was observed after a period of prolactin suppression (Chart 4). Binding of estradiol and prolactin by rat tissues thus appears to be interrelated, with the possibility that maintenance of binding sites for either hormone depends on adequate serum levels of the other.

The pathophysiological significance of hormone binding to tumor tissue remains uncertain. This study and other studies (6, 12) suggest that, in the DMBA-induced rat tumor, changes in growth following endocrine treatment may be reflected by changes in prolactin binding. There is also evidence that loss of hormone dependence in some tumor sublines is associated with a loss of prolactin and estradiol binding sites (4). In human mammary tumors the absence or presence of estrogen receptor may indicate the probable response to endocrine therapy (9). This study suggests that binding of prolactin to human mammary tumors could be investigated as a basis for a similar predictive test in tumors possessing a measurable content of prolactin binding sites (8).

Finally, we suggest that the use of ultrathin sections of frozen tissue may be useful for performing binding studies when only limited amounts of material are available, *e.g.*, from small biopsy specimens. The specificity and precision of the method are satisfactory when applied to rat tumor tissue, and the reproducibility of binding in repeated biopsies appears reasonable (Table 2). Samples can be stored for up to 2 months without loss of binding activity, enabling biopsies obtained at different times to be assayed together. The technique has already been applied to the study of binding of growth hormone to human liver (2).

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