NUCLEAR BINDING OF GLUCOCORTICOID RECEPTORS

by

Ernest Bloom

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The abbreviations used in this paper are: HTC, hepatoma tissue culture; TAT, tyrosine aminotransferase; complex, glucocorticoid-receptor complex; acceptor, nuclear acceptor site; PBS, phosphate buffered saline; CPM, counts per minute; uM, μm.

SUMMARY

Glucocorticoid receptors when complexed with an active hormone bind to chromatin. An "activation" of the complex is apparently required for this. In the cell the kinetics of activation, the nature of the nuclear binding sites, and their relationship to the hormone response are poorly understood. In the current studies, we have examined a number of aspects of these processes in the intact cell.

When intact cells are incubated in the presence of dexamethasone at 0^{o} cytosol binding of the steroid occurs readily but nuclear binding proceeds very slowly. Heating at 37^{o} for 40 seconds is apparently sufficient to induce activity; after this 30% of the cytoplasmic steroid-receptor complexes will bind to the nucleus within 30 minutes. Thus, in the intact cell at 0^{o}, like cell free systems, activation and not nuclear binding is rate-limiting. These observations also further support the notion that activation does occur in the intact cell, and that it is probably not rate-limiting at 37^{o}.

Since about 20% of the nuclear-bound complexes are resistant to extraction with 0.5 M KCl, the possibility of fundamental thermodynamic differences between the salt-extractable and salt-resistant complexes was examined. We explored their kinetics of formation at varying dexamethasone concentrations and times (40 minutes vs 16 hours). No difference between the

proportion of salt-extractable and salt-resistant nuclear-bound receptors was found with either variable. These results suggest that the salt-labile and salt-resistant nuclear-bound receptors are not associated with sites that have fundamental differences in their thermodynamics of receptor association. It is possible, therefore, that the incomplete extraction of the receptors reflects some property of the solubilization process rather than two types of nuclear acceptors.

Other studies were performed to examine the relationship between nuclear binding in the intact cell and glucocorticoid responses, in this case the induction of tyrosine aminotransferase. Binding was measured at 37° at 30-40 minutes, and also at 16 hours, the time at which tyrosine aminotransferase was measured. No difference in the level of nuclear (or cytosol) binding was observed between 40 minutes and 16 hours. Thus, the homologous hormone does not appear to regulate the level of its receptors. Tyrosine aminotransferase induction was linearly related to the content of nuclear bound receptors. The latter result suggests that the number of glucocorticoid receptors, and not some distal event, is the limiting factor in glucocorticoid hormone response and that "spare receptors" are not present. The data also suggest that in these cells, a physiological "acceptor" that binds receptors with very high affinity, "high affinity operators", (i.e., with a Kd much less than 10 nM) does not mediate glucocorticoid action. These

hypothetical sites would have been saturated at the receptor concentrations achieved. Instead, the data suggest that a nuclear acceptor population with an affinity that does not vary greatly from the nuclear binding observed is responsible for the glucocorticoid hormone response. The data are discussed in terms of a model in which receptors bind to a population of acceptors present in excess. By this model, the probability of obtaining a glucocorticoid response is proportional to the chance association of receptors with those acceptors located at important loci.

INTRODUCTION

Glucocorticoid hormones bind to specific receptor proteins inside the cells of responsive tissues (1). Active steroids appear to influence the receptor in such a way that the receptor-steroid complex can undergo an "activation" step which then allows it to bind to chromatin. This latter interaction in some way influences the transcription of specific mRNAs. Very little is known about the nature and number of these nuclear binding sites. Further, it is not established whether the nuclear binding which is observed either in the intact cell or in cell-free systems is that which results in the glucocorticoid response. Studies in other steroid-responsive systems have resulted in considerable controversy as to whether the observed binding is all nonspecific and is masking detection of a relatively few "specific" sites (of much higher affinity) or whether it is "specific" and involved in the hormonal response.

Only the activated form of the glucocorticoid-receptor complex binds to nuclei(2). Activation probably involves a conformational change, although a significant change in sedimentation behavior has not been demonstrated (3). At 0^{o}, in low salt conditions, (less than .02 M), receptor-steroid binding is perhaps 10,000 times faster than activation (4,5,by calculation). In the intact cell (at physiological salt concentration) studies suggest that less than 5 minutes at 37^{o} is sufficient to reach equilibrium values of activation, i.e. as shown by

nuclear binding (2). Some investigators have reported inhibitors of activation to be present in cells (6,7,8). The receptor has not yet been sufficiently purified to permit clear understanding of what activation is.

Evidence that nuclear acceptor sites in the intact cell are not saturated with receptor-steroid complexes at saturating concentrations of glucocorticoid comes from the finding that complexes bound in the intact cell did not inhibit subsequent cell-free binding of receptor steroid complexes (8). One hypothesis is that the receptor-glucocorticoid complexes are binding nonspecifically anywhere there is "open" DNA. This idea would be supported by findings that the glucocorticoid receptor is a DNA-binding protein, and by findings that both the binding of an active glucocorticoid to the receptor and the activation step are necessary for optimal DNA binding. However, other studies suggest that the affinity of nuclei or chromatin for binding complexes is higher than is that of pure DNA. These findings suggest that proteins or other influences in the chromatin render to portions of the chromatin a higher affinity for receptor-glucocorticoid complexes than free DNA (at least as it exists in solution). These considerations suggest that there are a limited number of acceptors in the intact cell, even though the total content of these acceptors may be quite large compared to the number of receptors. The idea of a

limited number of nuclear binding sites is also suggested by studies by Milgrom (9). Whereas the receptor-dexamethasone concentration dependency studies using crude cytosol did not point to a saturable binding capacity, sequential binding studies suggest that liver nuclei may be saturable. The total content of nuclear sites exceeded by several-fold the binding achievable using a single incubation with crude cytosol.

An attempt to estimate the number of nuclear sites in the intact HTC cell has not been made. However, the data (mentioned above) that complexes bound in the intact cell do not inhibit subsequent cell-free binding suggests perhaps the nuclear sites are not fully saturated in the intact cell even though there is saturation of the receptor by steroid. Estimates of the nuclear binding capacity in intact cells in other systems have been made. Williams and Gorski found that about 85% of the estrogen receptors in the uterus were associated with the nucleus at hormone concentrations which saturated the receptors (10). They also found that as the fractional saturation of the sites progressed, the ratio of nuclear and cytosol complexes remained constant. From these data they suggested that it is likely that the nuclear acceptor capacity exceeds by several-fold the cytosol receptor capacity. Similar estimates have been made using dexamethasone binding by rat kidney; it was found that there

was a linear relation between the amount of nuclear binding and the concentration of cytosol receptor-dexamethasone complexes. Milgrom has had similar findings using dexamethasone binding and rat liver.

Nuclei with bound steroid-receptor complexes retain only about 20% of the complexes after a 30 minute extraction in 0.5 M KCl. In the uterus-estradiol system with similar extraction characteristics it has been suggested that only the salt-resistant acceptors are involved in the hormonal response (11). This comes from studies in the intact animal showing that the salt-labile sites are not filled for longer than 6 hr compared to 18 hr for the salt-resistant sites. The salt-labile sites are not believed to be required to be filled for "true" uterine growth. However, another study seems to contradict this. A careful study of the extraction characteristics of uterine nuclei implies that there is no evidence for differences of affinity of the two classes of acceptors, all acceptor sites are extractable under proper conditions, and that the observed non-extractable complexes are perhaps physically entrapped in the nuclear chromatin (12). Also, Scatchard analysis failed to reveal two classes of sites with different affinities. The characteristics of the two classes of sites have not previously been thoroughly investigated in the HTC system.

An important question is whether or not the receptor is inducible by its hormone. Some investigators have suggested this with the glucocorticoid system. Data from the uterus-estradiol (11), and liver-estradiol (13) systems imply that it is. This had not been previously investigated in the HTC-glucocorticoid system.

A final question regarding nuclear binding is whether any of the observed binding actually mediates the biological response. Reasoning behind this question is based on analogy with the bacterial lactose repressor which in spite of its extremely high affinity for the operator site at which it represses, is mostly bound "nonspecifically" by non-operator DNA which has a much lower affinity for the repressor, but which, due to its large quantity in relation to operator DNA, can extensively bind the repressor. Thus, it has been proposed that hidden under the "nonspecific" binding sites which account for most of the observed receptor-estradiol complex binding in the uterus are "operator" sites which bind the complexes more tightly and which mediate estrogen action. Such a model makes certain predictions as to the extent of the hormone response as a function of fractional saturation of the receptors with estrogen. In most instances it is predicted that this relation is nonlinear. Unfortunately, this model is difficult

to test since for the uterine system it is difficult to precisely quantify the nuclear binding and the estrogen response. Further, because of the kinetics of nuclear binding in the intact uterus--in which the biological response is measured--the amount of nuclear receptor-estradiol complexes at 30 min are markedly greater than at several hours (11), it is difficult to decide which parameter to compare. Finally, most of the data comes from experiments done by different laboratories under markedly varying experimental conditions and the extent of "activation" (or "transformation") has not been studied.

The concept of high affinity operator DNA binding masked by low affinity "nonspecific" DNA binding, although true for the lactose repressor and other proteins such as RNA polymerase, is not always the case. A range of variations is observed. For example, the "nonspecific" binding by the lambda repressor is sufficiently low that in comparable circumstances, "operator" binding is mainly observed. On the other hand, with the cyclic AMP-binding protein of Escherichia coli, the affinity of this protein for DNA at the promotor sites where it acts is only slightly higher than for non-promotor DNA. Thus, a number of variations can be observed and a priori any of these circumstances could be observed.

Cultured hepatoma cells offer a good system in which to compare the quantitative aspects of receptor binding with the glucocorticoid response. Previous correlations with intact cell binding were crude, and the more detailed comparisons were made between binding by isolated cytosol at 0^{o} and induction in the intact cell at 37^{o}. The present studies were undertaken to analyze the quantitative relationship between the amount of nuclear binding in intact HTC cells and tyrosine aminotransferase (TAT) induction.

In the HTC system we: 1) investigated the properties of receptor activation in the whole cell; 2) estimated a lower limit for the total number of nuclear acceptors; 3) determined the quantitative relationship between salt-estractable and salt-labile acceptors as a function of time and steroid concentration ; 4) asked whether the receptor is an inducible protein; and 5) measured a glucocorticoid response, TAT induction, as a function of nuclear binding to see if there was a linear relationship.

MATERIALS AND METHODS

Materials- Buffers and media composition have been described previously (2). Briefly, PBS contained salt at physiological salt strength, homogenization buffer contained tricine with salt less than 0.02 M, induction medium was a defined mixture of nutrients designed for tissue culture use, and growth medium was induction medium plus 5-10% calf serum.

Purity of [^{3}H] dexamethasone was determined as described previously (2).

30 Minute Cell Binding Experiments- Cultured hepatoma cells were grown in spinner cultures, in growth medium, as described previously (2). The experiments were performed using cells in the logarithmic phase of growth at a cell density of between 300,000 and 1,100,000 per ml. Cells were harvested by centrifugation at 3000 x g. The supernatant medium was discarded and the cells were taken up (in approximately 1/5 of the original volume) in fresh growth medium (to which 0.1% sodium bicarbonate had been added) containing varying concentrations of radioactive dexamethasone (Amersham-Searle, specific activity 22-35 Ci/mmole) with or without 10 uM nonradioactive dexamethasone for determination of "background" binding(2). The cells were then incubated at 37^{o} for 30 min to allow maximal binding (14) in a gyrotory shaker bath at 100 rpm. Following incubation, the cell suspensions were transferred to conical tubes and were

centrifuged at 3000 x g for 2 min. The supernatant medium was assayed for radioactivity from which the free dexamethasone concentration was determined. The pellet was immediately washed with 30 ml of ice-cold phosphate buffered saline (PBS) and the supernatant medium was discarded. All remaining procedures were performed at 0-4^{o}. Then the cells were resuspended in 2 ml of PBS, were transferred to 12 x 75 mm tubes and were pelleted by centrifugation for 5 min at 600 x g. The supernatant medium was discarded and the pellet was frozen in liquid nitrogen. Then 0.7 ml of homogenization medium was added. The pellet was thawed, resuspended with a glass rod, and the mixture was centrifuged at 600 x g for 5 min.

The volume of the supernatant medium was recorded and 0.5 ml of it was filtered over a Sephadex G-25 5 x 70 mm column, and eluted in medium A. Radioactivity in the excluded volume (macromolecular-bound steroid) was assayed.

The pellet from the 600 x g centrifugation of the cell homogenate was resuspended in 2 ml of buffer A and was transferred to small homogenizer tubes. The tubes were then washed with 2 ml of buffer A and these washes were added to the homogenization tube. These suspensions were homogenized (6 strokes at 2000 rpm) and then centrifuged

at 2500 x g for 5 min. The pellet was washed twice with 10 ml of buffer A. These "partially purified nuclei" finally resuspended in 0.6 ml of water. Portions were taken for determination of radioactivity as described above and for measurement of DNA (15) and protein (16). The specifically bound radioactivity in the nuclei was measured by subtracting from the total bound at a given concentration the amount of radioactivity bound in the presence of excess dexamethasone (nonspecific binding). Induction of tyrosine aminotransferase was measured with the Diamondstone assay (17).

Whole Cell Activation Experiments

Hepatoma cells were harvested in the log phase, washed 3 times in induction media and suspended in induction media, 0^{o}, at 5,000,000 cells/ml. At time 0 hours, 10 nM [^{3}H] dexamethasone was added to the cells. A parallel experiment with 10 nM [^{3}H] dexamethasone plus 10 uM cold was run to determine nonspecific binding. At time 1 hr 45 minutes the cells were aliquoted, 1 ml per tube (5,000,000 cells) into 9 test tubes. At time 2 hours four tubes were heated at 37^{o} for 45-60 seconds. Thereafter, at timed intervals, 2:00, 2:15, 2:30, 3:00, and 4:00 hours, cells were washed 3 times in PBS, frozen 10 minutes on dry ice, thawed at 4^{o}C, homogenized in 3 ml of homogenization medium, and centrifuged 5 min at 1200 x g.

The pellet was washed three times in induction medium and the supernatant was run over a 5 x 70 mm G-25 Sephadex column. Both the nuclear pellet and the G-25 exclusion volume were counted for radioactivity as previously described (2).

We found it important to adhere closely to the outlined procedure to obtain consistent results.

Salt-Extraction of Nuclear Bound Receptor Experiments

The procedure was as in the 30 minute cell binding experiments except as follows: Cells were incubated up to 16 hours in induction medium without bicarbonate added. Nuclear pellets were split into 2 aliquots, one was extracted for 30 minutes in homogenization medium and the other in homogenization medium plus .5 M KCl added. Other details have been described previously (2).

RESULTS

Steroid Concentration Dependency on Cytosol and Nuclear Binding

Nuclear and cytosol binding of dexamethasone at different concentrations of the hormone is shown in Fig. 1. Data are from intact cells incubated at 37°. Both fractions become relatively saturated with steroid at similar concentrations. In this experiment, about 40% of the maximal binding is nuclear and 60% is cytoplasmic. However, the maximal nuclear-bound steroid varied in different experiments from 40% to 60% of the total binding. A Scatchard analysis of these data is shown in Fig. 2. As indicated, the slopes are similar, suggesting that the extent of relative saturation of cytosol and nuclear binding is similar at a wide range of steroid concentrations. From these data and from other experiments we estimate that the "apparent" equilibrium dissociation constants for cytosol, nuclear or total cell binding of dexamethasone is approximately the same and around 10 nM.

Cytosol Receptor-Dexamethasone Complex Dependency on Nuclear Binding

To understand the nature of the nuclear binding, the relationship between the cytosol complex and

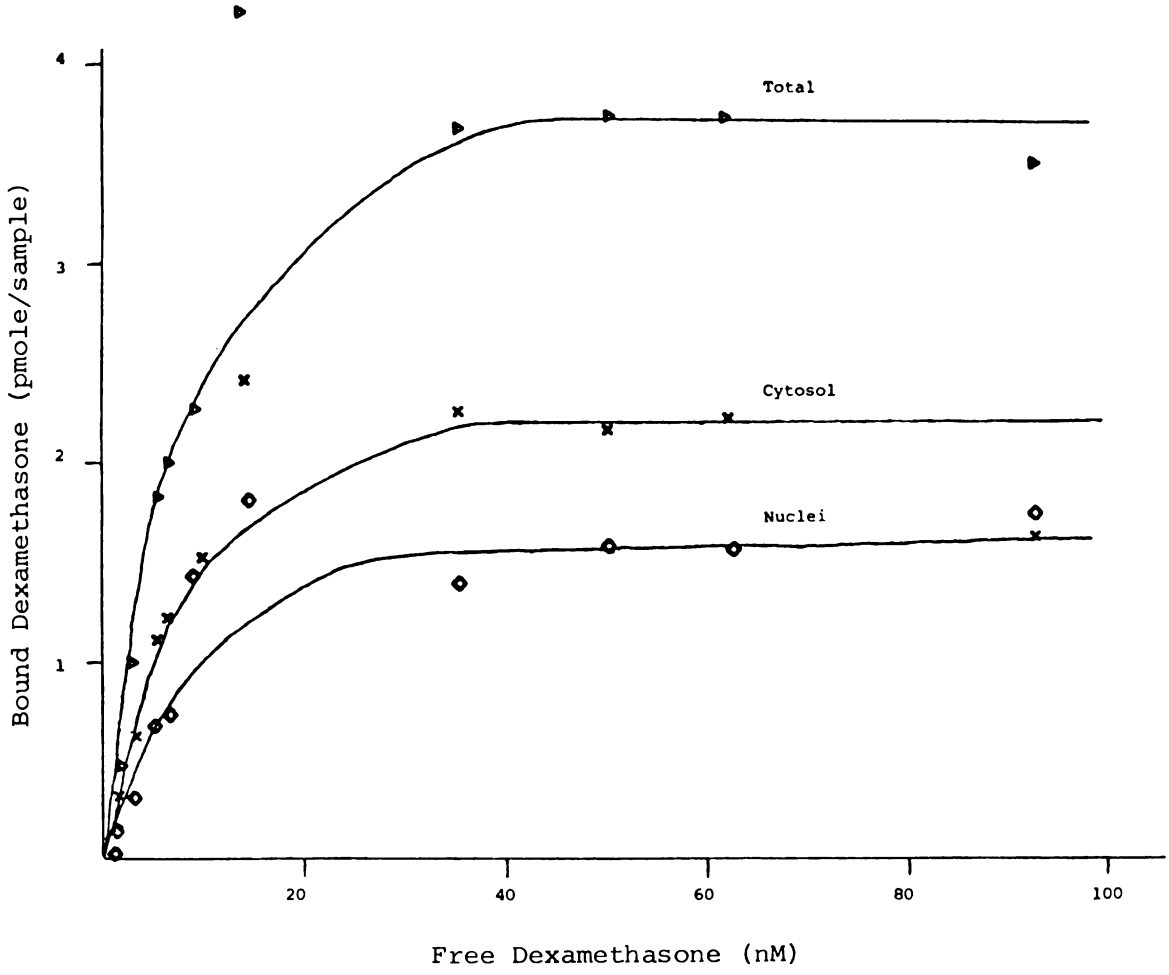

Fig. 1: Dexamethasone concentration dependency on total, nuclear, and cytosol binding in intact HTC cells at 37°. Binding was performed as described in the methods. Each 20 ml incubation contained 8×10^7 cells. Maximal total binding corresponds to about 20,000 binding sites per cell.

Fig. 2

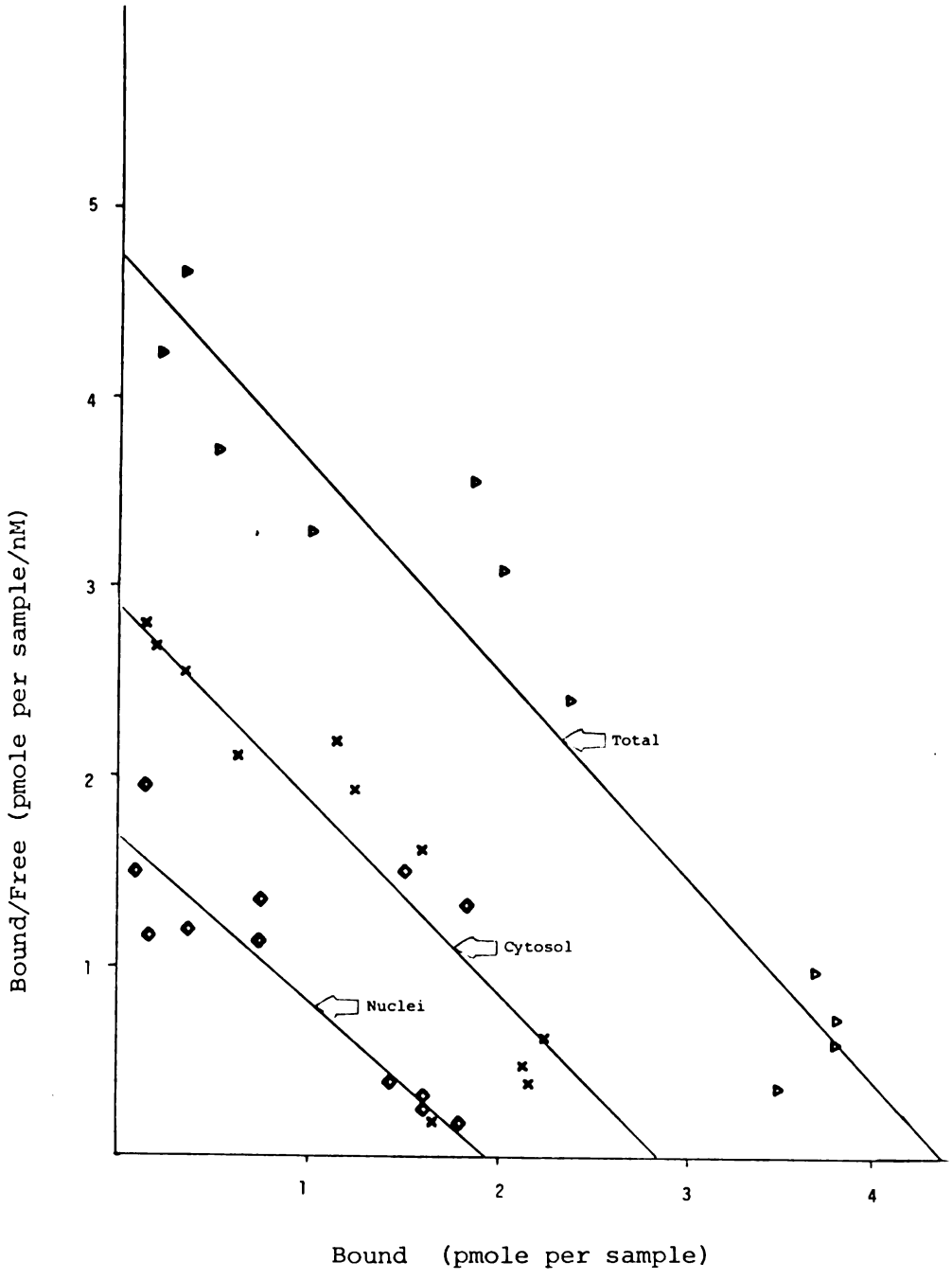
Bound/Free (pmole per sample/nM)
5
4
3
2
1
Total
Cytosol
Nuclei
1
2
3
4
Bound (pmole per sample)

Fig. 2: Scatchard plot of the data shown in Fig. 1. The intercept on the abscissa corresponds to 4.0 pmole/mg DNA for nuclear binding and 0.3 pmole/mg protein for cytosol binding. In four experiments, the extrapolated maximal nuclear binding varied from 2.3 to 4.9 (mean = 4.0) pmole mg/DNA and from 0.2 to 0.4 (mean, 0.3) pmole/mg protein.

nuclear binding was investigated. As might be anticipated since slopes of the cytosol and nuclear binding shown in Fig. 2 are identical, this relationship appears to be linear (Fig. 3). The nuclear binding shows no signs of beginning to plateau; these data suggest that at maximal concentrations of cytosol receptor-dexamethasone complexes (i.e. when the receptors are near-saturated with steroid) the nulcear acceptors are not saturated with receptor-dexamethasone complexes.

A Scatchard analysis of the nuclear binding of receptor-dexamethasone complexes, as related to the concentration of cytosol receptor-dexamethasone complexes, (data pooled from four separate experiments) is shown in Fig. 4. The proportion of receptors in the nuclei and cytosol varied somewhat in different experiments. Thus, the data were normalized to mean the ratio of nuclear to cytoplasmic binding for all the determinations in a given experiment (taken as 100%) and this ratio is plotted as a percentage of this value as a function of fractional saturation. As shown, although there is considerable scatter, at all levels of fractional saturation (up to nearly 90%), the ratio remains constant and does not appear to approache the abscissa. These data therefore do not provide any evidence that the nuclear acceptor sites are saturated or are approaching saturation

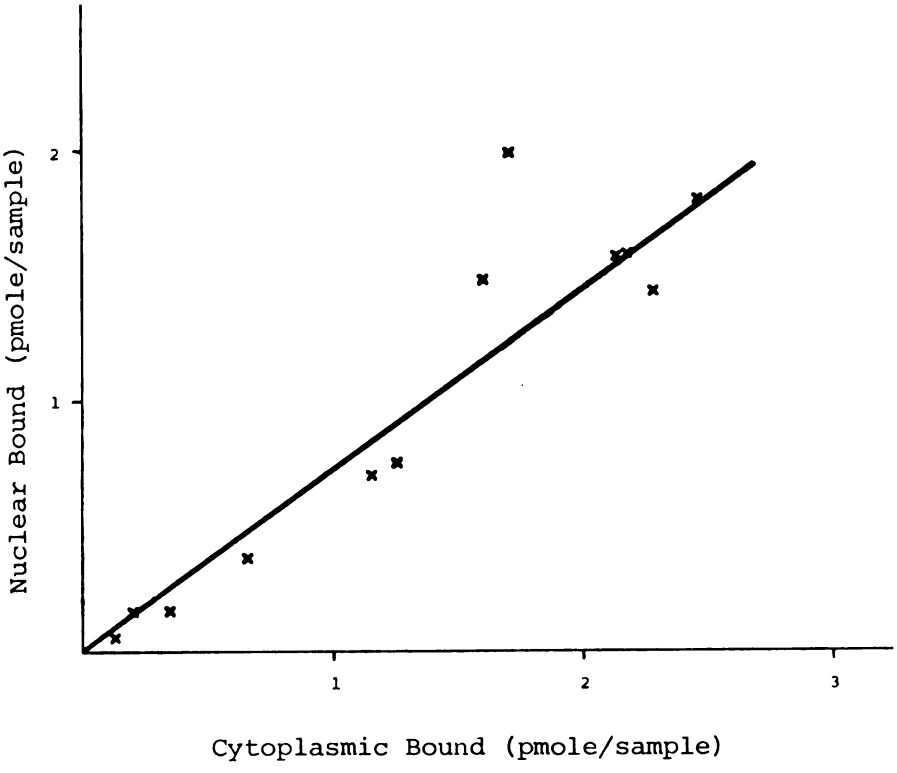

Fig. 3: Nuclear binding as a function of cytosol binding. Data are taken from the experiment shown in Fig. 1.

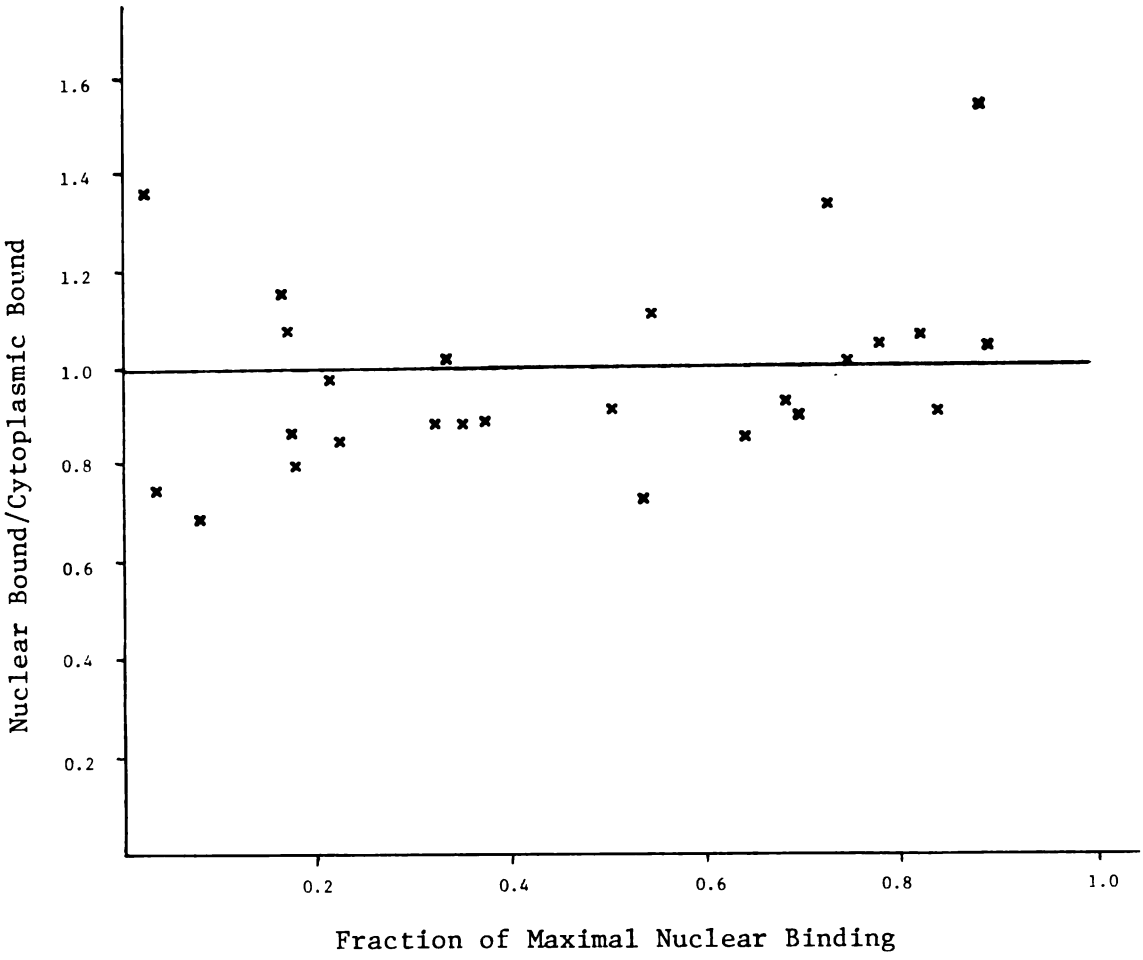

Fig. 4: Scatchard plot of the receptor-steroid complex binding to the nucleus. Influence of saturation of the receptors with dexamethasone on the ratio of nuclear to cytoplasmic binding. See text.

with receptor-dexamethasone complexes as the receptors are becoming saturated with steroid. If the data are extrapolated to the abscissa, it is likely that it does so at a distance considerably farther out on the axis. Thus, the acceptor capacity may exceed (probably by several-fold) the maximal nuclear binding. Further, since about half of the total receptors are nuclear-bound, it is therefore likely that the acceptor capacity exceeds perhaps by several-fold the content of the cytosol receptors.

Activation in the Whole Cell

Cytosol (40,000 g x 10 minute supernatant) incubated with 10 nM ^{3}H-dexamethasone, 0°, did not begin to reach equilibrium values of binding before 6-8 hours (data not shown). In the intact cell a 45-60 second heating at 37° was sufficient to cause the transfer of about 40% of the complexes to the nucleus in less than 30 minutes (Fig. 4A). Nuclear binding (after heating) was very fast compared to cytosol binding. For the cells left at 0°C cytosol binding was about 5 times faster than nuclear binding.

Salt Labile Compared to Salt-Resistant Acceptors

We found no difference between 40 min and 16 hr in total cell, cytosol, nuclear, salt-labile, or salt-resistant acceptor binding (Fig. 6). On the average, about 55% of the receptors were in the nucleus for these experiments. The receptor level was not regulated under our conditions, but remained constant.

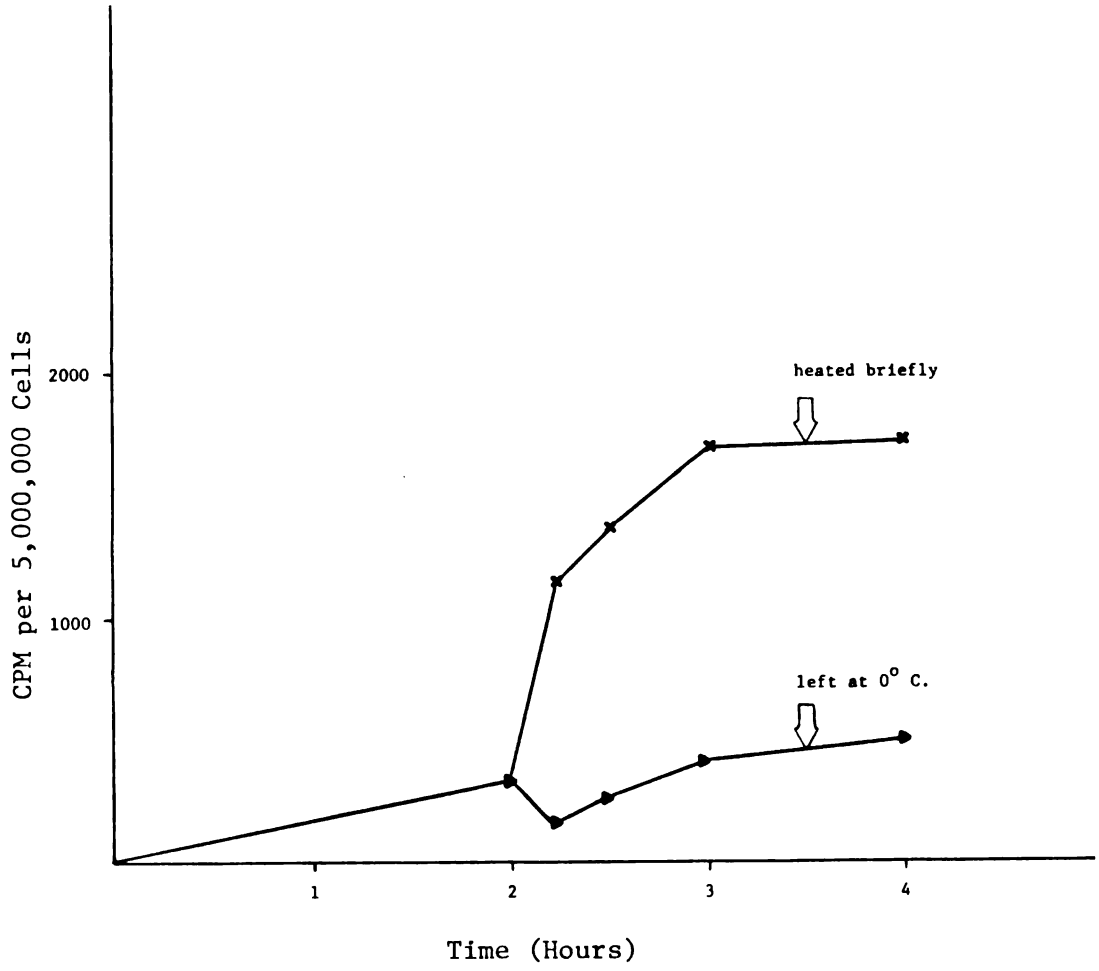

Fig. 5a: Nuclear bound receptor-hormone complex after "activation" in the intact cell. Cells were incubated at 0° with 10^{-8} M dexamethasone. At 2 hours half of the samples (X) were heated 45-60 seconds at 37° C, and half (▷) were left at 0°. At timed intervals cell samples were washed, disrupted, and centrifuged. Samples contained about 5 million HTC cells, data averaged from three experiments.

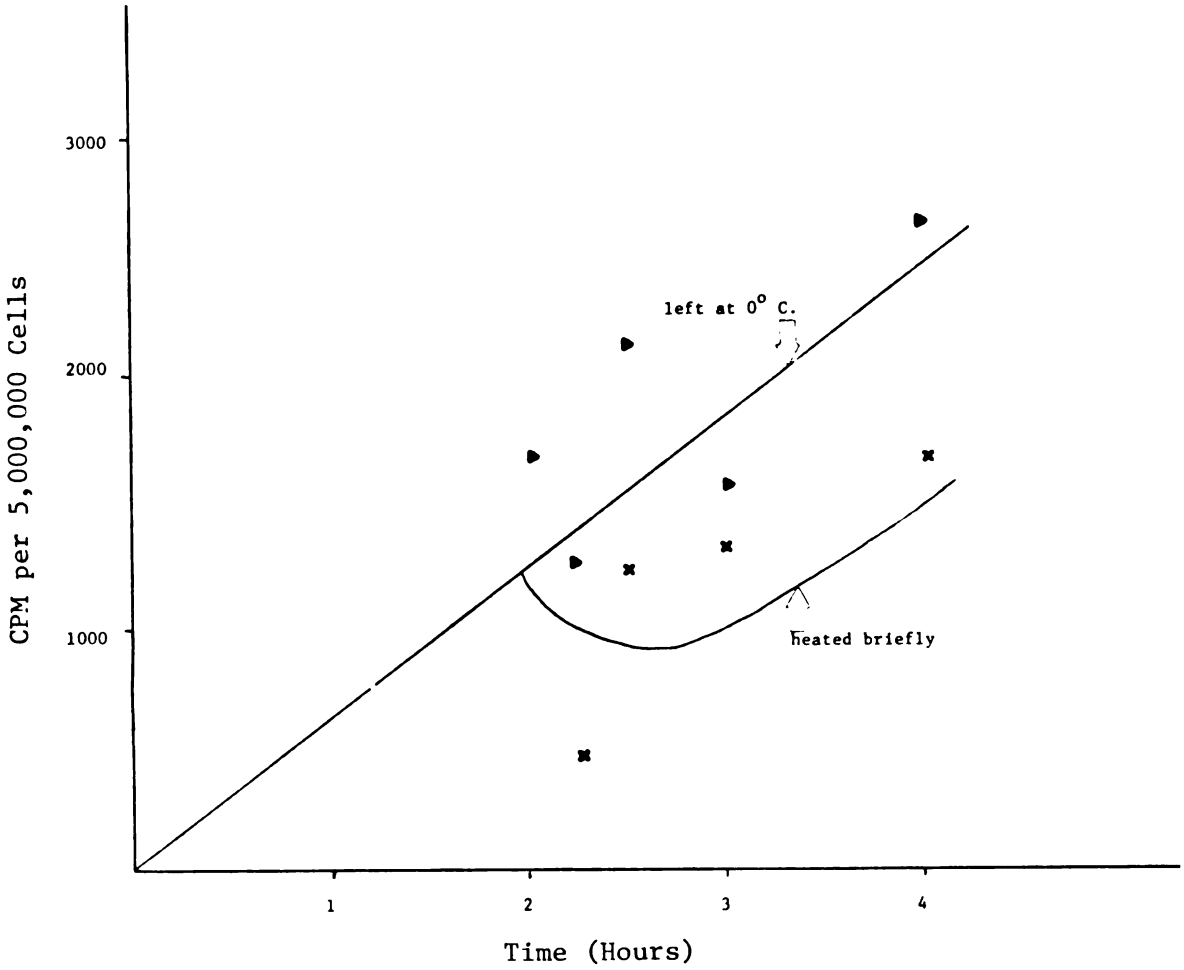

Fig. 5b: Cytosol receptor-hormone complex. Data is from two experiments shown in Fig. 5a. (X) were heated 45-60 seconds at 37° and (▷) were left at 0°.

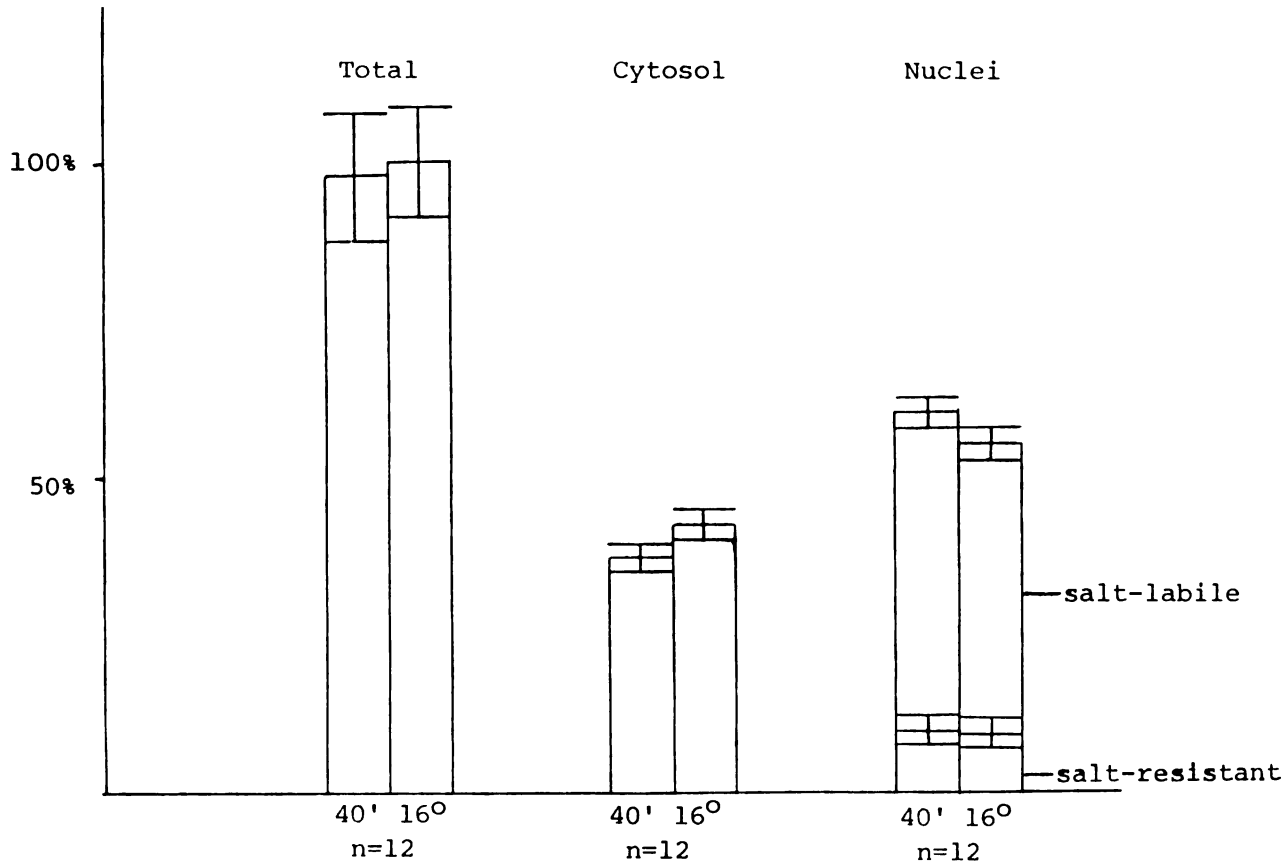

Fig. 6: Distribution of receptor-steroid complexes at 40 minutes compared to 16 hours. Cells were incubated with about 10 nM dexamethasone. After harvesting, disruption, and centrifugation the nuclei were split and one half were incubated in homogenization medium and one half in homogenization medium with .5 M KCl added. Cytosol was gel filtered to remove bound from free steroid. The average total binding for these 12 experiments was about 13,000 molecules of dexamthasone specifically bound per cell.

In Fig. 7 using the same data from Fig. 6 we find no change in acceptor ratio as a function of hormone concentration.

Relation Between Nuclear Binding and the Glucocorticoid Response

We found a close correlation between nuclear binding and tyrosine aminotransferase induction (Fig. 8a). TAT induction was half maximal at about 15 nM, also, nuclear binding was at about half the maximum achievable at this hormone concentration. Although these data are from separate experiments the correlation is excellent as shown in Fig. 8b.

Fig. 7

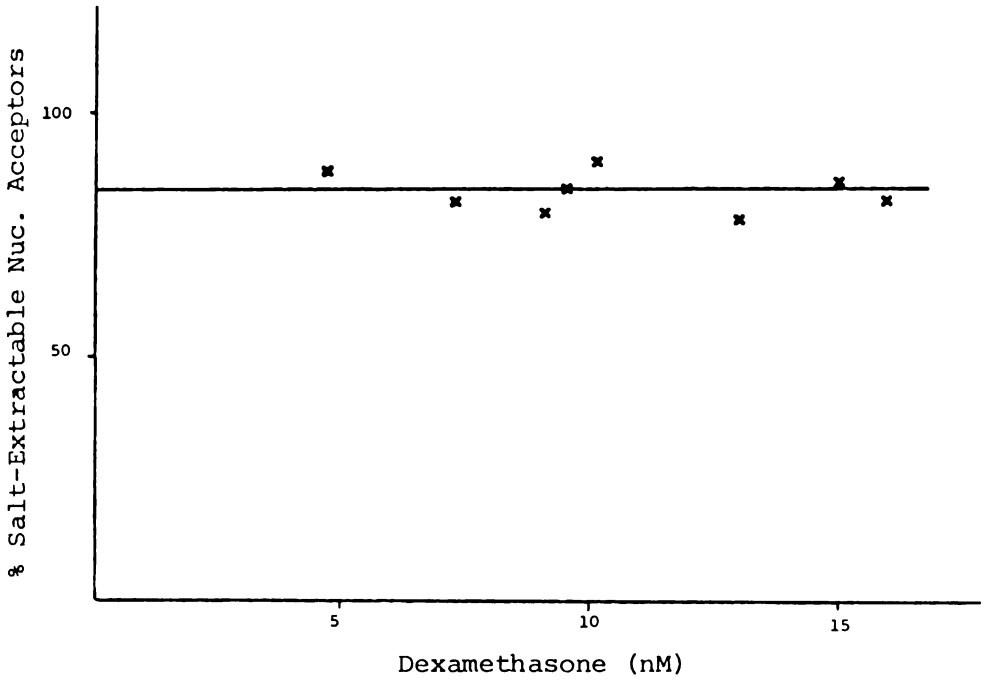

Fig. 7: Salt-labile nuclear-bound receptor-steroid complexes as a function of hormone concentration (at 16 hours). Data were taken from experiments shown in Fig. 6.

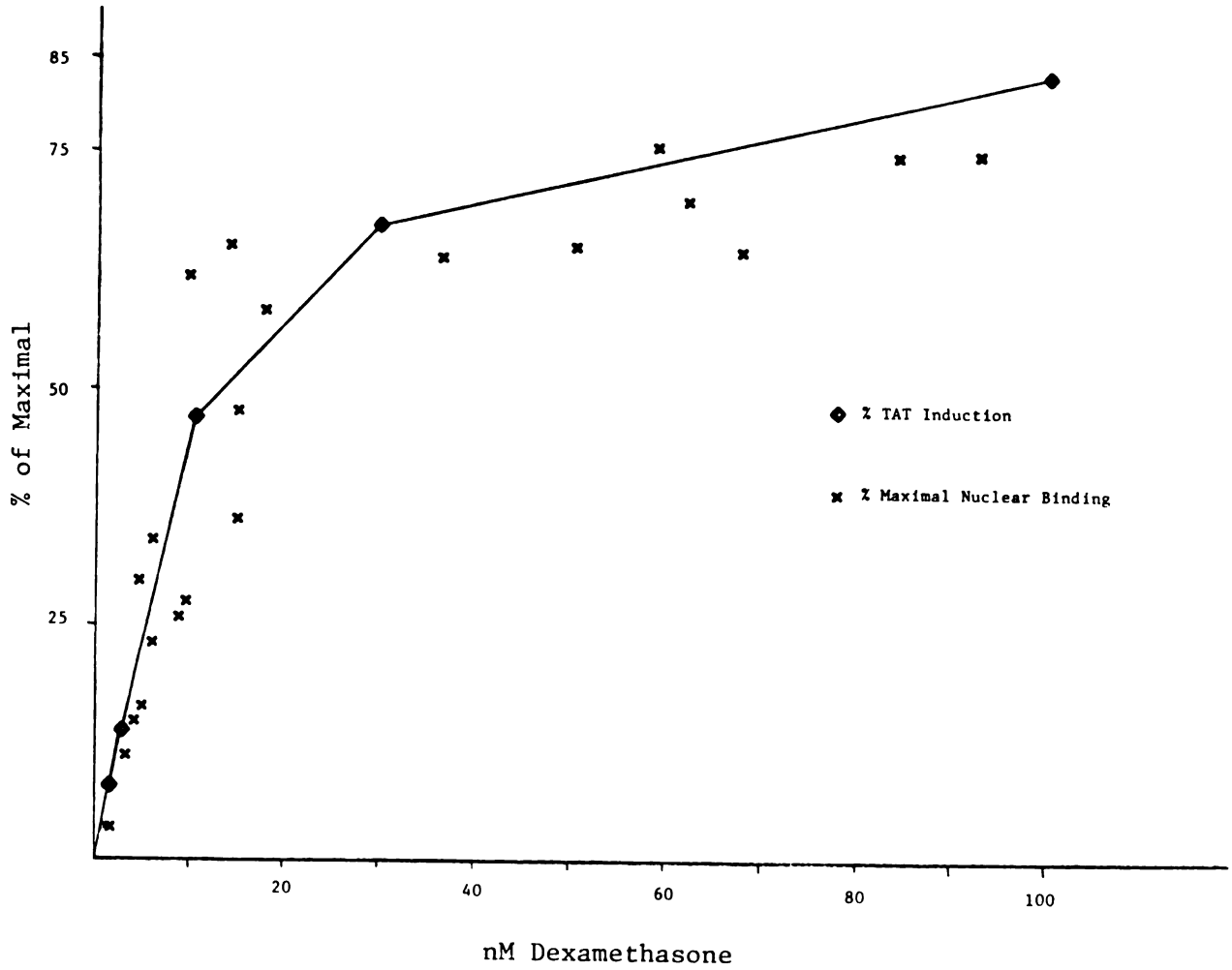

Fig. 8a: Relation between relative nuclear binding and tyrosine aminotransferase induction. The nuclear binding data (x) (pooled from four separate experiments) are plotted as a percent of the maximal nuclear binding (taken from the abscissa intercept of a Scatchard plot analogous to Fig. 2). The induction data (◇) represent the mean of three separate experiments performed with duplicate incubations in each.

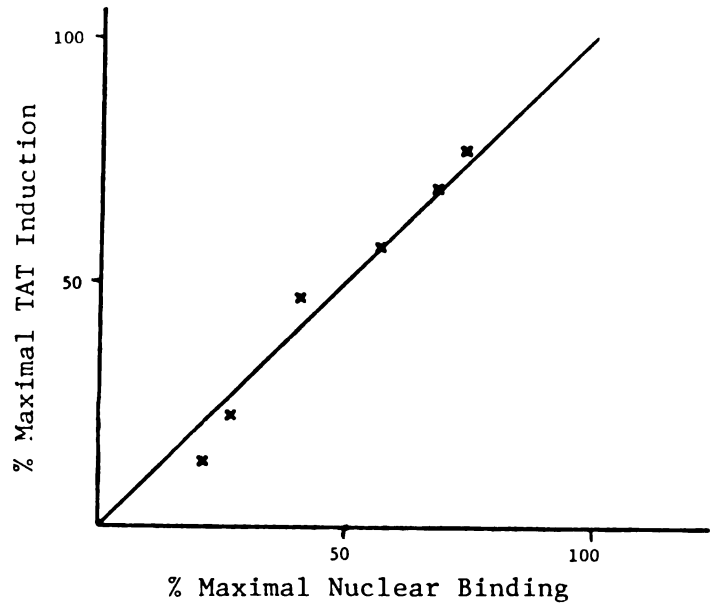

Fig. 8b: Data from Fig. 8a. Points are taken from approximated curves drawn for nuclear binding and TAT induction.

DISCUSSION

The present studies using intact cultured hepatoma cells have focused on the quantitative relations between formation of cytosol receptor-dexamethasone complexes, nuclear binding of the complexes and the glucocorticoid response of induction of tyrosine aminotransferase. In the intact cell the apparent equilibrium dissociation constant for receptor binding by dexamethasone is around 10 nM. Further, the constant is roughly the same whether cytisol, nuclear or whole cell binding is measured. A plot of nuclear binding as a function of the concentration of cytosol receptor-dexamethasone complexes was linear. Further, the ratio of nuclear bound to cytosol complexes as a function of the relative saturation of the nuclear sites was parallel to the abscissa and did not show an indication of approaching the abscissa (Fig. 4). These data suggest that the nuclear capacity exceeds probably by several-fold the quantity of cytosol receptors. Based on the experiment shown in Fig. 4 it appears that the plot would intersect the abscissa at least five times further out from the points of highest nuclear binding shown. This suggests there are more than 50,000 nuclear acceptors per cell. Thus, even though nuclei bind receptor-dexamethasone complexes rather tightly (they are retained after cell fractionation and multiple washes of nuclei), this nuclear binding is not saturated

in the intact cell.

Activation may be an important process in the whole cell at 0^{o}. 45-60 seconds of heating at 37^{o} was sufficient to cause the eventual transfer of about 40% of the cytosol complexes to the nucleus. Using thermodynamic data on activation from Atger and Milgrom (4), (and Baxter and Tompkins (20)), we estimate that at 0^{o} receptor-glucocorticoid binding is roughly 10,000 times faster than activation and that at equilibrium under their conditions about 60% that of cytosol receptors should be in the activated form. These calculations are based on data from low salt conditions, (about .01M) with 100,000 x g cytosol. Under our conditions--in the whole cell--we estimate that cytosol binding is only 5 times faster than nuclear binding. Though the conditions are markedly different, using their thermodynamic data we predict that if cytosol were suddenly heated and allowed to reach equilibrium values of activation, and then quickly cooled, roughly 35% of the receptors should eventually bind to the nucleus. This assumes that the ratio of free activated complex to nuclear bound is 1:2. However, Atger and Milgrom's data indicate that at 37^{o} under low salt conditions at least 6 minutes are needed to reach equilibrium and about 1 1/2 minutes to reach half-equilibrium. Possibly the rate constant, k, for activation is faster under physiological salt conditions (150 mM). Except for rates, our in vivo experiments are in rough conformity

with their in vitro data, though as noted above the conditions are markedly different and not directly comparable.

Clearly, in our system, there is no difference between 40 minutes and 16 hours in receptor content for any compartment measured; total cell cytosol, total nuclear, salt-labile acceptors, or salt-resistant acceptors. Possible regulation of the glucocorticoid receptor by glucocorticoids has been suggested by some investigations (18). Also observations on the liver-estradiol system imply that the receptor can be induced (13). These studies are done on intact animals and are extremely difficult to interpret.

Since nuclear binding of the receptor-glucocorticoid complex probably initiates subsequent events, it seemed important to precisely quantify the relations between nuclear binding and the response. Three possibilities exist, assuming that nuclear binding is a step in glucocorticoid action. First, all of the observed nuclear binding may be "nonspecific" and the biologically important binding is to a much smaller number of much higher affinity nuclear sites. Thus, the extent of "important" binding would be small and not observed within the large background of "nonspecific" binding. At first glance, this would be analogous to the binding of the lac repressor to total E. coli DNA in which most of the binding is nonspecific in spite of the fact that the repressor binding to the operator site is orders of magnitude higher in affinity (but nevertheless a small proportion of the total binding). However, the situation with E. coli has an important difference. The

DNA binding of the repressor is so extensive that most of the receptor molecules are ordinarily bound to DNA. This results in a marked decrease in molarity of the receptor. Thus, even though the affinity of the repressor for DNA is high, the effective molar concentration of repressor is low. By contrast, in HTC cells only about half of the total receptors are bound to the nucleus even at concentrations of dexamethasone which essentially saturate the receptor. We estimate that at saturation of the receptor with steroid, the cytosol receptor-steroid complex concentration is perhaps around 6 nM. It is not known in what proportion of these complexes are in the "activated" form. For instance, if half of the receptors are "activated" and can bind to the nuclear sites, then (assuming noncooperative associations) the receptor would at least half saturate nuclear sites which bound the complex with an affinity of 2 nM (equilibrium dissociation constant) or higher. Thus, if important nuclear sites were present which bound receptor-steroid complexes with an apparent affinity (equilibrium dissociation constant) in the range of 1-100 pM, then these would be approaching saturation with receptor-glucocorticoid complexes at dexamethasone concentrations below those required for maximal receptor saturation. If this were the case, then one would expect that the relative glucocorticoid response would precede relative receptor saturation as concentrations of steroid were increased.

This was not observed.

A second possibility is that most of the observed binding is "nonspecific" but a subset of sites with similar binding affinity is responsible for the response. Perhaps only 10 proteins are regulated by glucocorticoids in HTC cells (19). Thus, the response would be initiated by a "chance" interaction of receptors with important sites. If this were the case, then the total receptor concentration and thus saturation of the receptor would be limiting in the glucocorticoid response since higher concentrations of receptor-steroid complexes would result in a higher probability for such chance interactions. If this possibility were true, then we might expect a very close parallel between the amount of receptor steroid complexes in the nucleus and the amount of enzyme induction.

A third possibility would be that most or all of the nuclear binding observed is important. If this were true, because of the large number of complexes bound, then many binding sites would be involved in the response. However, there are data showing that glucocorticoids alter the structure of nuclear chromatin by increasing the number of RNA polymerase initiation sites about 10 times more than the number of receptors bound (21).

As shown in Fig. 8, the relative nuclear binding and

enzyme induction at different concentrations of dexamethasone appears to be identical. This makes the first possibility seem quite unlikely. Therefore, it does not appear that hidden in the large amount of observed nuclear binding are sites with a markedly higher affinity for the receptor-steroid complex.

These data do not allow us to make a distinction between the second 2 possibilities. However, we speculate that the third possibility is not unreasonable although it is the more complex of the two. Gene regulation in eukaryotes is now thought to be much more complex than the lactose operon model from prokaryotes. The second possibility is the simplest.

Both binding and induction by dexamethasone follows an approximately hyperbolic function with respect to dexamethasone. Thus, these reactions do not appear to have a high degree of cooperativity. There appears to be some element of cooperativity in the glucocorticoid response when the effect of anti-inducer steroids is examined (Rousseau, G.G. and Baxter, J.D., unpublished observations). However, these influences are not a major factor when the dose-response of an inducer such as dexamethasone is examined. Thus, the response appears to be a linear function of the concentration of cytosol complexes and thus of the nuclear-bound complexes. This suggests that in fact nuclear binding may be limiting in the response

and thus that some biologically-important nuclear sites are not occupied under conditions of saturation of the receptor with steroid. It seems unlikely, however, even though the nuclear sites are in excess, that receptors are binding indiscriminately to the DNA as proposed elsewhere. At concentrations of complex and at salt concentrations achieved in the intact cell, DNA binding is extremely low. By contrast, under the same conditions, isolated chromatin or nuclei bind complexes tightly. Thus, chromatin confers on the receptor some specificity for binding. Therefore, it is likely that the observed nuclear binding is "specific" even though its capacity exceeds that of the cytosol receptors.

In sum, it is most likely that nuclear binding similar to that observed is involved in the glucocorticoid response. The nuclear sites involved in the response are probably not filled in the intact cell at saturating concentrations of hormone. Therefore, probably the concentration of receptors per cell is the limiting factor in the glucocorticoid response.

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