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THERMODYNAMIC AND KINETIC ANALYSIS OF GLUCOCORTICOID HORMONE RECEPTOR INTERACTIONS

by

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DISSERTATION

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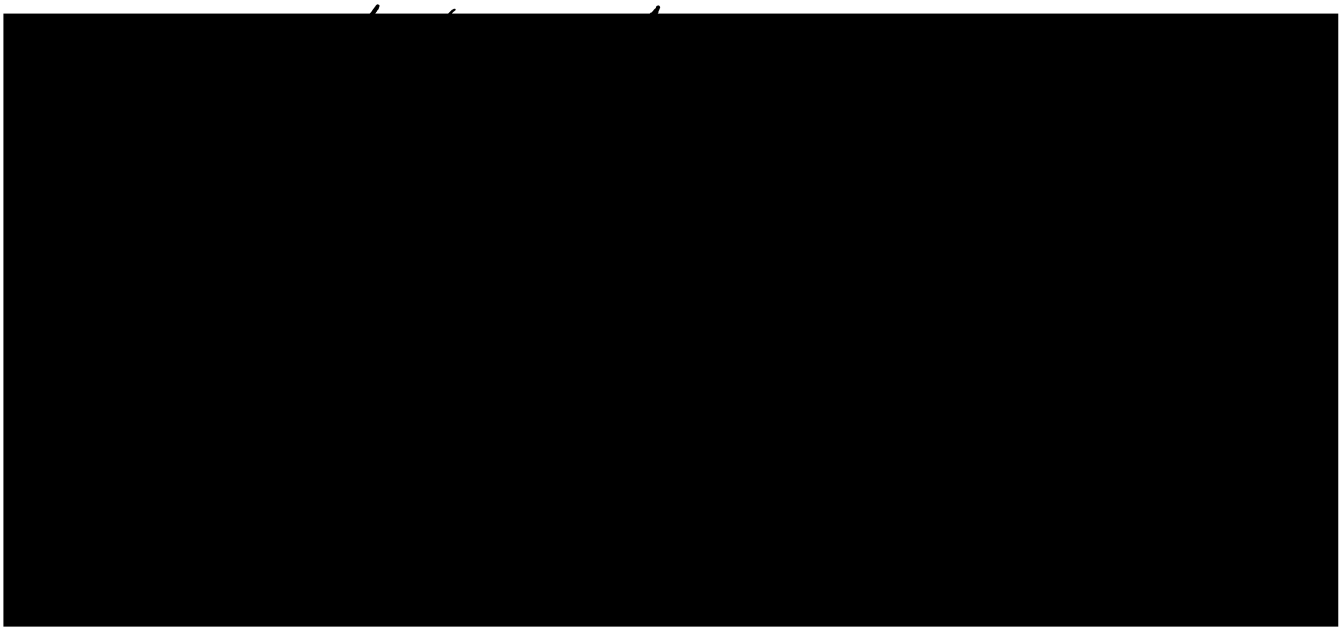

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I wish to dedicate this result to:

My parents, brothers, sisters and relatives in Vietnam with all my soul and heart.

My wife and my child-to-be with love.

*

Công cha như núi Thái-Sơn,
Nghĩa mẹ như nước trong nguồn chảy ra.
Một lòng thờ mẹ kính cha,
Cho tròn chữ hiếu mới là đạo con.

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PART I

THERMODYNAMIC ANALYSIS OF GLUCOCORTICOID HORMONE RECEPTOR INTERACTIONS

Part I of this thesis is arranged into four major sections:

1. The general introduction and sections 1.0 to 1.7 present an introduction of the properties, uses, models of action etc. of glucocorticoid hormones and receptors.

2. The second section (2.0 to 2.2.4) describes kinetic measurements of glucocorticoid-receptor binding using Scatchard analyses.

3. The third section includes subsections 3-6 and describes a comparison of the binding of ^{3}H-dexamethasone by cultured hepatoma (HTC) cell cytosol receptors and intact cells. Each subsection describes a set of experiments including Materials, Methods and Results. Subsections 3.0 and 4.0 describe the thermodynamics of the receptor-glucocorticoid binding in cytosol and in intact cells. Subsections 5.0 and 6.0 discuss an analysis of the nuclear binding as related to cytosol binding.

4. The discussion (7.0 - 8.0) reviews the thermodynamic analysis of the entropy, enthalpy and heat capacity changes and of the hydrophobic properties of the hormone-receptor interaction. The discussion also analyses the nature of the binding of the receptor-steroid complexes to the nucleus. Finally, section 8.0 provides an analysis of the hydrophobicity of the interaction as well as the overall features of the glucocoricoid hormone-recpetor interactions.

INTRODUCTION TO PART I

In 1960, Jensen's early use of tritiated estrogen to study the properties of hormone binding to the chick oviduct receptors (71) opened an expanding field of molecular research: the study of the binding of small molecules (drugs, hormones, etc.) to macromolecules (enzymes, receptors, DNA, etc.). This field has rapidly become the backbone of many research diciplines at the molecular level, e.g. molecular biology, molecular pharmacology, etc. Understanding the nature of the interaction between small molecules and macromolecules is therefore extremely important. In drug design, the understanding of the binding of a drug to a specific protein, such as an enzyme or a receptor, will define the basic structural and physiochemical requirements of that drug. In molecular biology, such an understanding of the interaction of a hormone with its receptor will provide crucial insight into the complicated mechanism of hormone action.

This is the reason for our present study. Many glucocorticoids are drugs (as discussed in section 1.3); like many other therapeutic agents, however, they elicit certain effects that are not beneficial to treatment. A study of the binding of glucocorticoids with its receptor will define the structural and physiological properties of the drug required for those activities induced by its binding to the receptor.

Various disciplines have been applied to investigate the different mechanistic aspects of the hormone-receptor interactions. For example, the active binding site of the receptor can be defined by comparative binding or biological studies (Westphal 1958, 1959, 1962; Rousseau et. al. 1972; Smith et. al. 1974, Kontula et. al. 1975) or through affinity labelling of the receptor (Wolff et. al. 1975; Marver et. al. 1976; Chin and Warren 1978, 1970; Katzenellenbogen et al 1973, 1977; Liarakos May 1969; Solo and Gardner 1968, 1971; Steve Nordeen et al., 1982). Although less popular, thermodynamic analysis have been increasingly applied to understand the nature of the interaction of the hormone and receptor in glucocorticoid-responsive and other (e.g. insulin) systems. Late in 1970, Schaumberg and Bojensen (84) reported three Scatchard plots for corticosterone binding to glucocorticoid receptors in intact thymocyte cells, plotted the $\ln k_A$ of the reaction versus $1/(t + 273)$, and generated a Van't Hoff plot. They obtained a negative entropy change (ΔS = 187 e.u.) and concluded that this resulted from a change in the conformation of the receptor upon interacting with the steroid. In 1972, Koblinsky et al. (101) studied the binding of dexamethasone and corticosterone to different components of rat liver cytosol including proteins G, A and B. Van't Hoff plots generated for three temperatures (4°, 17° and 37°C) revealed negative entropy changes (ΔS = -43 to -30 e.u.) in the case of corticosterone interacting with protein A and B, but positive entropy changes (ΔS = 18 e.u.) with dexa-

methasone binding to protein G (the presumed receptor). Because only a few temperatures were studied, it is difficult to interpret or to accept these results.

Earlier in 1964, Westphal reported temperature dependent influences on the affinities of progesterone for α-1-acid glycoprotein (102), human serum albumin (HSA) (103) and corticosterone-binding globlin (CBG). Late in 1978, Wolff et al. examined Westphal's data and concluded that both the entropy and enthalpy of the system are temperature dependent. At low temperature (0°C), the changes in entropy (from 35 to 50 e.u.) and heat capacity (ΔC_p) are positive, implying hydrophobic interactions between progesterone and its carrier proteins. Recently, Wolff et al. studied the thermodynamics of the interactions of corticosterone with glucocorticoid receptors in hepatoma tissue culture (HTC) cell cytosol. A Van't Hoff plot (lnkA vs. $1/(t + 273)$ of the data from eight temperatures (-2°C to 16°C) was curvilinear. Enthalpy changes (ΔH) determined from the slope of the curve increased as temperature decreased; similarly, the entropy changes (obtained from the free energy of the binding) also decreased as temperature increased. The heat capacity (ΔC_p) change led Wolff et al. to conclude that the major driving force in the glucocorticoid hormone-cytosol receptor interaction is hydrophobic. Similarly, the insulin-receptor interaction also appears to be hydrophobic in nature (76) reported by Waelbroeck et al. in 1979.

The above studies could be criticized because measurement may be altered by factors such as receptor-protein denaturation, especially at high temperature and long incubation times. Receptor denaturation has also limited the range of temperatures that have been studied (-2°C to 16°C). Further, the binding was studied only in cell-free conditions in order to examine only the initial receptor-steroid interaction. However it would be of interest to compare results obtained in this way with those from studies in which the steroid is incubated with intact cells; in this way the thermodynamic importance of the initial hormone-receptor interaction can be compared with other steps in steroid action such as membrane uptake, conformational changes associated with the activation of the receptor-steroid complex and nuclear binding of the complex.

In the present study, we have established conditions to study the glucocorticoid receptor interaction that do not suffer from the above disadvantages and that allow a comparison of the data obtained, by incubating the steroid with either isolated cytosol or intact cells. Two major systems have been used in our studies:

a. Glucocorticoid receptor-containing cytosol: Similar to the previous work of Wolff et al., hepatoma tissue culture (HTC) cell cytosol was incubated with the steroid. However, the receptor protein was stabilized by 3 mM dithiothreotol (DTT) and 10 mM sodium molybdate. This allowed us to study the binding at higher temperatures (up to 25°C) than were utilized before. In addition sodium molybdate was

used to block receptor activation; this allows the present analysis with isolated cytosol studies to focus only on the initial receptor-steroid interaction.

b. Hepatoma tissue culture (HTC) cells: By incubating intact cells with the steroid and measuring total, nuclear and cytosol binding under conditions in which there must be steroid penetration of the cell, steroid-receptor binding, and activation and nuclear binding of the complex, an assessment of the thermodynamics of the entire system can be obtained. This can be compared to the results with isolated cytosol in order to understand the contribution of processes other than the intial steroid-receptor binding to the overall thermodynamics of the system. The use of intact cells has also allowed us to extrapolate the temperature studied to 37°C without significant loss of receptor binding sites due to receptor protein denaturation.

Van't Hoff analysis generated from the affinities of dexamethasone binding to HTC cell cytosol and intact HTC cells have shown that both enthalpy and entropy changes are temperature dependent. The enthalpy and entropy changes decrease as temperature increases. At high temperature, the reaction is driven by both enthalpy and entropy; at low temperature positive enthalpy change works against the reaction, however, the large entropy change at this low temperature becomes the major force that drives the reaction. These observations support the notion that the removal of water on the surface of the hormone and the receptor is a major element in the binding.

Additionally, calculations of the free energy (ΔG) obtained from the removal of hydrated water molecules on the surface of both the hormone and the protein receptor suggest that both sides of the hormone are engulfed by the receptor.

The shape of Van't Hoff plots from data in which the steroid was incubated with isolated cytosol and with intact cells were identical, suggesting that under physiological conditions the glucocorticoid receptor interactions in the intact cell are driven primarily by the hydrophobic interactions of the initial steroid receptor interaction and that other steps in the process such as steroid uptake, hormone-induced conformational changes associated with activation of the hormone-receptor complex and the nuclear binding of the activated complex do not contribute substantially to the overall binding. By fractionating the cells into cytosol and nuclei after allowing maximum binding with ^{3}H-dexamethasone, it is shown that activation does occur inside intact cell and at the concentration that ^{3}H-dexamethasone saturates all the cell cytosol receptors, the nuclear acceptor sites are still far from saturation since Scatchard analysis of nuclear-bound over cytosol bound steroid vs. nuclear-bound steroid reveals a line that is parallel to the abscissa. Thus we conclude the nuclear acceptor sites exist in a very large concentration that exceeds the number of the cytosol receptors.

1.0 History and General Properties of Glucocorticoid Hormones and Their Receptors

1.1 Discovery of Glucocorticoids

Glucocorticoids are steroid hormones secreted by the adrenal cortex. The function of these glands was unknown until 1855 when Thomas Addison described a wasting disease now known as Addison's disease associated with destruction of the suprarenal glands (1). By 1932, Harvey Cushing identified the syndrome of glucocorticoid excess which bears his name (2). It was also shown in 1927 that crude extracts of adrenal tissue could maintain life in adrenalectomized animals, suggesting that the extracts contained hormones.

Beginning in the early 1930's, four groups of investigators lead by Kendall (at the Mayo Clinic), Wintersteiner and Pfiffner (at Columbia University), Reichstein (at the Swiss Federal Institute of Technology), and Corland and Kinzengar (at Upjohn Laboratories) undertook research programs to isolate and identify these compounds and encountered great technical problems. Reichstein obtained 75 mg of cortisone and 55 mg of cortisol from 450 kg of bovine adrenal glands (3). By December of 1944, Lewis Sarrett at Merck had succeeded in synthesizing small quantities of cortisone by a 36-stage process, with an infinitesimal yield of 0.0015%. Fortunately, this was followed in 1948 by a more practical synthesis ultimately producing 938 g of cortisone. At this point, a second dramatic development occurred. A rheumatologist, Phillip Hensch, working at the Mayo Clinic in asso-

ciation with Kendall, had been speculating for years on the causes of rheumatoid arthritis (4). Hensch found that arthritic patients sometimes experienced remission when pregnant or jaundiced and suspected profound hormonal changes, he theorized that the hormones produced in greater abundance during pregnancy, particularly those originating in the adrenal gland, might be useful in the treatment of rheumatoid arthritis. The same hormones might escape metabolic destruction in a liver impaired by the factors that lead to jaundice. Cortisone, the corticoid first isolated at the Mayo Clinic by Kendall, was an obvious trial choice for Hensch. In 1948, he administered the compound to a woman hopelessly crippled by rheumatoid arthritis. Three days later she walked almost normally. A second patient was later treated with equal success. In 1949, Hensch published his findings, and their impact was underscored by the award of the Nobel prize to Hensch, Kendall and Reichstein in 1950. Cortisone was hailed as being "among the greatest advances that medicine has ever made in one leap." As is frequently the case, the initial enthusiasm was followed by a subtler evaluation as the serious side effects of glucocorticoid therapy became apparent. Despite the problems, the number of conditions for which glucocorticoids are beneficial has grown (Table 1) to the point that as many as five million Americans receive some form of glucocorticoid therapy annually. This covers a large therapeutic range from simple skin rashes to critical leukemia (5). Figure 1 shows the major known adrenal glucocorticoids and synthetic glucocorticoid analogs.

Addison's syndrome — replacement therapy	Eye diseases
Adrenal hyperplasia due to enzymatic defects (e.g., 11-, 17- and 21-hydroxylase syndromes)	Acute uveitis
Allergic diseases	Allergic conjunctivitis
Angioneurotic edema	Choroiditis
Bee stings	Optic neuritis
Contact dermatitis	Gastrointestinal diseases
Drug reactions	Inflammatory bowel disease
Hay fever	Nontropical sprue
Serum sickness	Regional enteritis
Urticaria	Subacute hepatic necrosis
Arthritis, bursitis, and tenosynovitis	Ulcerative colitis
Inflammatory complications of a variety of types of arthritis	Hypercalcemia
Collagen vascular disorders	Malignant exophthalmos
Giant cell arteritis	Neurologic diseases
Lupus erythematosus	Pulmonary diseases
Mixed connective tissue syndromes	Aspiration pneumonia
Polymyositis	Bronchial asthma
Polymyalgia rheumatica	Infant respiratory distress syndrome (antenatal)
Rheumatoid arthritis	Sarcoidosis
Temporal arteritis	Renal diseases
Blood dyscrasias	Certain nephrotic syndromes
Acquired hemolytic anemia	Transplantation — prevention of rejection
Allergic purpura	Infections — occasionally helpful to suppress excessive inflammation
Autoimmune hemolytic anemia	Skin conditions
Idiopathic thrombocytopenic purpura	Atopic dermatitis
Lymphoblastic leukemia	Dermatoses (see above)
Multiple myeloma	Lichen simplex chronicus (localized neurodermatitis)
	Mycosis fungoides
	Pemphigus
	Seborrheic dermatitis
	Xerosis

Table 1: Selected clinical conditions for glucocorticoids uses (18).

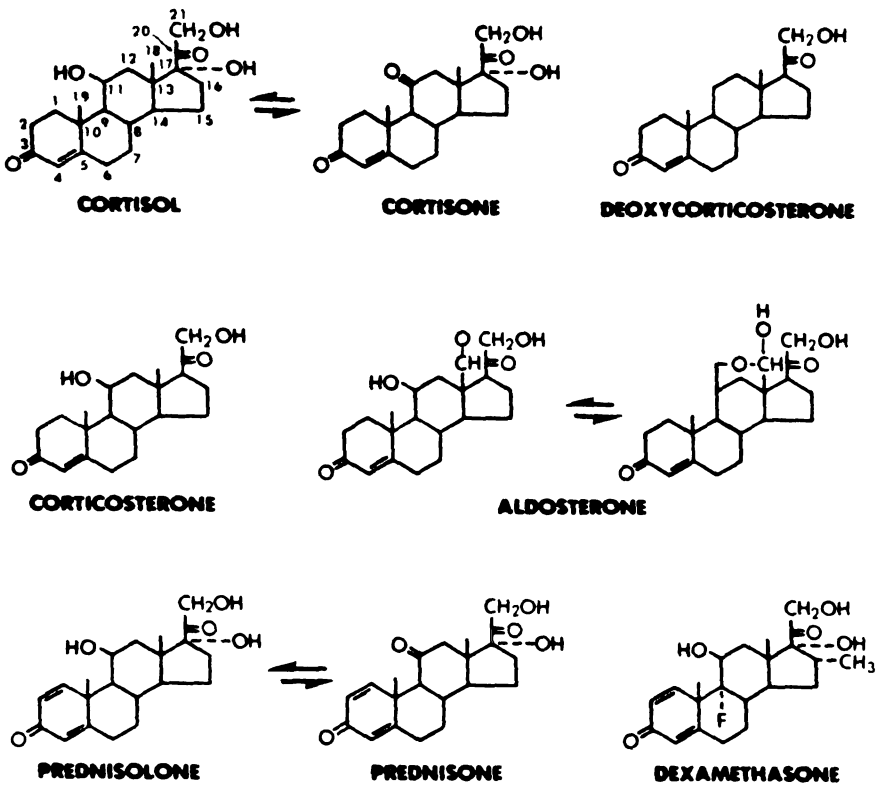


Figure 1: Structures of some adrenal steroids with glucocorticoid and/or mineralocorticoid activity, and the synthetic glucocorticoid analogs prednisolone, prednisone, and dexamethasone (18).

1.2 Regulation of Glucocorticoid Production

The synthesis and secretion of adrenal steroids are controlled by adrenocorticotropin (corticotropin; ACTH) from the pituitary (6,7). The secretion of ACTH is regulated, in turn, by corticotropin-releasing factor (CRF) released from the hypothalamus during stress (6,7).

After stimulation of the gland, there is a rapid decline in the concentration of cholesterol within the adrenal. This and other evidence indicates that ACTH has its effect at a step involving conversion of cholesterol to pregnenolone.

It is still unclear whether the specific action of ACTH is to increase the initial 20-hydroxylation or oxidative cleavages of the cholesterol side chain employing NADPH as cofactor (Mulrow (1972)). ACTH specifically increases the release of cortisol within 2 or 3 minutes of contact with the adrenal gland (6-8).

Large amounts of ascorbic acid are found in the adrenal cortex. The role of this is not known. Such amounts may act to provide reducing equivalents for the NADPH-dependent hydroxylations required for steroid synthesis mentioned below. Ascorbic acid is not synthesized in the adrenal but is concentrated there from extraadrenal sources. ACTH reduces ascorbic acid uptake by the gland. The measurement of cholesterol or ascorbic acid depletion in the adrenal glands of hypophysectomized animals after the injection of ACTH was an early method of assay for the effects of the tropic hormone.

Stimulation of steroid synthesis and release by ACTH may be mediated through cyclic AMP since the level of this substance is increased in adrenal slices within minutes by the tropic hormone and since cyclic AMP itself can directly stimulate ACTH action. The resulting cyclic AMP activates protein kinase, which phosphorylates a number of proteins (6) that are responsible for steroidogenesis.

Stimulation of steroid synthesis is usually associated with alterations in structure of the adrenal mitochondrial membrane and also is dependent on the presence of calcium ions. The ultimate effect of ACTH and cyclic AMP, therefore, may involve changes in ionic flux across adrenal cell membranes. As in most reactions stimulated by cyclic AMP, ATP is inhibitory.

The secretion of ACTH is under feedback control by circulating steroids; in man, cortisol is the most important regulator. Thus, when cortisol levels decrease, there is a concomitant rise in ACTH. Figure 2 briefly describes the regulation of cortisol production.

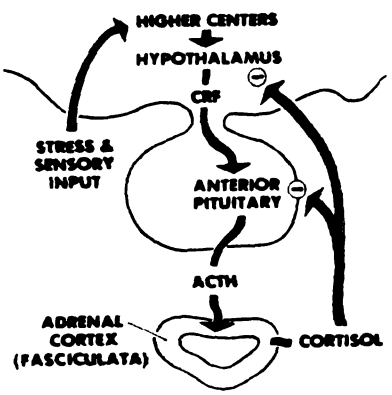


Figure 2: Regulation of cortisol production (18).

Aldosterone is secreted by the zona glomerulosa and its production is controlled by the renin angiotensin system, by the deprivation of sodium, administration of potassium, ACTH, serotonin, or by any decline in the normal volume of the extracellular fluid; this last circumstance is attributed to the presence of what are termed "volume receptors" (Bartter, 1956). It follows that activities resulting from an increase in aldosterone production-sodium retention, potassium excretion, and an expansion of extracellular fluid volume would serve to reduce secretion of this hormone by a type of "feedback regulation." There is evidence that the regulatory effect of each of the above-mentioned factors is exerted independently of the others; however, questions of interdependence and the relative importance of each remain unsettled. Other factors are also reported to affect aldosterone biosynthesis. For instance, the dopamine agonist, bromocryptine, inhibits the response of aldosterone to angiotensin II and to ACTH (9). Glucocorticoids can also inhibit the production of aldosterone (10) via ACTH.

1.3 "Catabolic" and "anabolic" affects of glucocorticoid hormones

In the peripheral tissues (muscle, adipose, and lymphoid tissue), the steroids are catabolic and tend to "spare" glucose. Glucose uptake and glycolysis are depressed. Protein synthesis is depressed, whereas protein degradation is increased. In muscle, there may be tissue-wasting as protein stores are depleted. In adipose tissue,

glucocorticoids increase lipolysis. The impairment of glucose metabolism in this tissue decreases the available glycerol phosphate, thereby impairing fat synthesis. In Cushing's disease (hyperadrenocorticism), a centripetal redistribution of fat occurs without change in total body fat as lipid is mobilized from steroid-sensitive tissue and redeposited in the neck, face and trunk. It is not known why fat is mobilized. However, fat deposition in certain areas may be due to lipogenic actions of the increased plasma insulin concentration (11).

In the liver of animals treated with adrenal steroids, all processes which help remove amino acids are increased. Thus, total protein synthesis, gluconeogenesis, glycogen deposition, amino acid conversion to CO_2, and urea are all enhanced. An increase in RNA synthesis occurs within minutes after glucocorticoid administration (12), indicating that some of these effects may result from direct action of the glucocorticoids on liver. Many of the gluconeogenic effects in the liver are caused by glycerol (from triglyceride) and amino acid mobilization from peripheral tissues.

In particular, the adrenal steroids increase the amount of hepatic enzyme involved in amino acid metabolism such as tyrosine transaminase as well as tryptophan pyrrolase. The key enzymes in the regulation of gluconeogensis (pyruvate carboxylase, phosphoenolpyruvate carboxykinase, fructose-1,6-diphosphatase, and glucose-1-6-phosphatase) are also increased. This seems to be a comparatively specialized

action of the adrenal steroids since many other hepatic enzymes are not increased. In liver, adrenal steroids not only increase amino acid conversion to glucose but also conversion of CO_2 to glucose, suggesting that they may act on CO_2 fixation, particularly at the level of pyruvate carboxylase, a key enzyme involved in gluconeogenesis. Conversion of fructose or glycerol to glucose is not specifically increased in vitro, thus supporting the concept of an action at a stage lower than the entry of these metabolites into the gluconeogenic pathway. In vivo, hyperglycemia, particularly during later periods of treatment, is a result of increased gluconeogenesis in the liver and decreased glucose uptake in peripheral tissues induced by glucocorticoids.

Glucocorticoids increases glycogen storage by stimulating glycogen synthetase activity. As shown in Figure 3, this enzyme exists in inactive (b) form and is promoted to active (a) form by glucocorticoids (13). This may be a result of blocking of the inhibitory action of glycogen phosphorylase (a) on glycogen synthetase phosphatase, which converts glycogen synthetase from the (b) to (a) form (13). Stalmans and Laboux have suggested that the steroid induced protein which inhibits the action of phosphorylase (a).

Although the primary source of the glucose moiety in the progess of gluconeogenesis is usually considered to be amino acids, the amount of glucose produced cannot be entirely accounted for by amino acid breakdown. It is possible that lactate and glycerol derived from

muscle and adipose tissue, respectively (the latter is a product of the increased lipolysis), can also serve as sources of carbon for hepatic glucose synthesis.

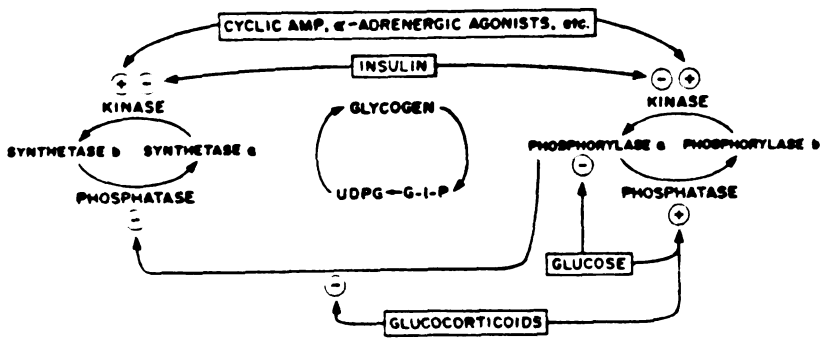


Figure 3: Action of glucocorticoid on glycogen accumulation (114).

Other effects of the glucocorticoid can be extremely important:

Anti-inflammatory effects. At high concentration, glucocorticoids decrease cellular protective reactions and in particular retard the migration of leukocytes into traumatized areas. Thus, cortisol is an anti-inflammatory agent and is used in this capacity in the so-called collagen diseases such as rheumatoid arthritis. One possible way that glucocorticoids exert their action by inhibiting the production of phospholipids required for the biosynthesis of prostaglandins, which are responsible for the inflammatory effect (14). This inhibitor (macrocortin) is a peptide or protein. This protein exerts its action by inhibiting the action of phospholipase A_2, which is responsible for the synthesis of lipid mediators of inflammation (including prostaglandins) from membrane phospholipids. Fig. 4 depicts the above mechanism.

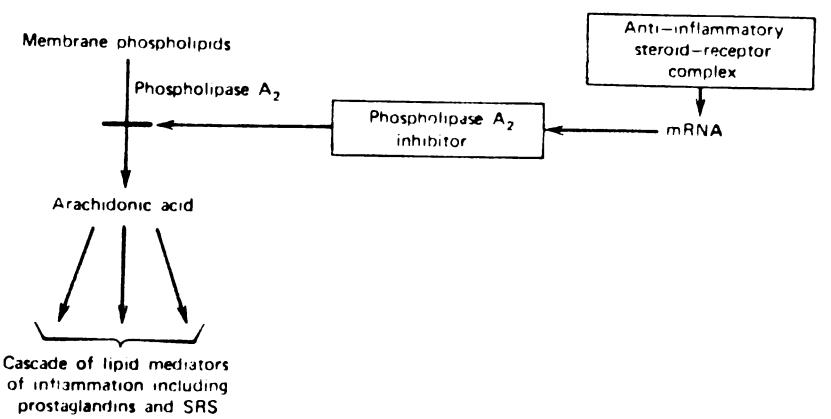


Figure 4: Mechanism of action of anti-inflammatory steroids (14).

Immunosuppressive effects. Cortisol decreases immune responses associated with infections, allergic states, and anaphylaxis. Indeed, glucocorticoids may be used for the purpose of repressing antibody formation when, as in organ transplantation procedures, it is essential to prevent rejection of the transplanted tissue or organ.

Glucocorticoids suppress virtually every phase of the immunologic and inflammatory response. The mechanisms of this suppression are unknown, but they undoubtedly include inhibition of metabolic function, membrane changes, synthesis of new inhibitory protein, and interference with binding of numerous factors (such as antibodies or complements to specific receptors on the cell surface). In addition, a major potential mechanism in the suppression of various functional capabilities, particulary of lymphocytes engaged in immunologic reactivity, is by inhibiting the availability of different cell types partaking in the cell-to-cell cooperation necessary for optimal activitation, differentiation, and effector function of individual populations of cells (15).

Exocrine secretory effects. Chronic treatment with glucocorticoids causes increased secretion of hydrochloric acid and a pepsinogen by the stomach, and trypsinogen by the pancreas; this may enhance the formation of gastrointestinal ulcers.

Effects on bone. Glucocorticoids reduce the osteoid matrix of bone, thus favoring oteoporosis and excesive loss of calcium from the body. Osteoporosis is a major complication of prolonged adrenal steroid therapy.

Cyclic AMP. In some tissues, the glucocorticoids decrease phosphodiesterase activity, thereby increasing cyclic AMP (cAMP) levels. However, it is unlikely that steroids act primarily to increase cAMP since their action are mostly on chromatin. Although there is no evidence that cAMP is directly involved in the mechanism of glucocorticoid hormone action (16), glucocorticoids amplify the effects of the peptide hormones that presumably act through cAMP, suggesting that there is some similarity in the pathways regulated by glucocorticoids and cAMP. In fact, in bacteria lacking glucocorticoids, cAMP plays a dominant role in the conservation of glucose by a mechanism similar to that of glucocorticoids (e.g., binding to a cytoplasmic receptor that in turn bind to the nucleus), which also results in the production of specific mRNAs (17).

1.4 A definition of a "glucocorticoid" hormone

Like other drugs proposed by Langley and Ehrlich (1878, 1905, 1906), the initial and crucial event for glucocorticoid hormone action is binding with specific receptor molecules. Because of the ultimate role of the receptor in the glucocorticoid system, the glucocorticoid needs a new definition like that described by Baxter, J.D. and Rousseau, G.G. (18): A glucocorticoid is a compound that acts through

its binding to a class of receptors, termed "glucocorticoid" receptors, which act as a mediator for glucocorticoid action. By this criterion, any glucocorticoid binding protein that does not bind certain potent glucocorticoids, such as dexamethasone, is not assigned the designation "glucocorticoid receptor." No physiologic effects have been demonstrated that are elicited by corticosterone or cortisol but not by other glucocorticoids, such as dexamethasone. Receptors are, thus, binding proteins that specifically bind glucocorticoids. However, their specificity includes some but not all glucocorticoids (as in the case with mineralocorticoid receptors). Of course, all steroids that bind to glucocorticoid receptors are not "glucocorticoids," since antgonists also bind. The use of receptors to classify glucocorticoid hormone action is simpler and more precise than other more descriptive approaches.

1.5 Physical and chemical properties of glucocorticoid steroids

Most of the glucocorticoid hormones are rather hydrophobic substances, owing to their basic steroid structure. The very potent synthetic glucocorticoid, dexamethasone (9α-fluro-16α-methyl prednisolone), is practically insoluble in water (0.0001 g/ml). Such steroids have higher solubility in weakly polar solvents like chloroform and in vegetable oils. More polar glucocorticoids with hydroxy groups, such as cortisol (having four hydroxy groups), have less solubility in chloroform (1.0 g/100 ml) but higher solubility in ethanol (2.5 g/100 ml) and water (0.01 g/100 ml). If a highly polar ionic

moiety is introduced into cortisol, as in the 21-phosphate sodium salt, the compound becomes insoluble in chloroform and oils, retains solubility in ethanol (1.0 g/100 ml), and has high water solubility (75 g/100 ml). The hormones generally form white crystals and are polymorphic, having definite melting points and solubilities (3).

1.6 Properties of glucocorticoid receptors

A hormone receptor is the locus to which the hormone is bound in order to elicit its effect. Since the first binding studies of Jensen in early 1960, showing the existence of chick oviduct receptors for ^{3}H-estradiol, there has been overwhelming evidence to identify the presence and physiochemical properties of glucocoritocid receptors in the human HeLa cells (19), mouse L929 fibroblasts (20), thymocytes, lymphosarcoma P 1978 (21), pituitary tumors (22), chick embryo retina (23), cultured rat mammary cells (24), and mouse and rat HTC (hepatoma tissue culture) cells (25). Low concentrations of receptor in the cell (less than 0.01% of the cellular protein) and the lability of the steroid-binding site (26-31) have been identified as the crux for obtaining purified glucocorticoid receptors. Nevertheless, information from studies with crude extracts of systems containing glucocorticoid receptor has revealed a number of chemical and physical properties of the receptor.

1.6.1 Chemical nature of glucocorticoid receptor

The receptor is an amphoteric protein. According to evidence provided by Nielsen and co-workers, dephosphorylation inactivates unbound

but not bound glucocorticoid receptors (32). Nielsen suggested from these observations that the receptor may be a phosphoprotein. This important finding could explain the dependence of the nuclear-cytoplasmic cycle of the receptor on ATP and provides a mechanism for regulation of the level of active receptor in the cell. The conversion of inactive receptors (released from the nucleus) to active receptors after nuclear binding of the hormone receptor complex (in cytoplasm) requires energy. Active receptors are phosphorylated receptors that bind glucocorticoids, whereas the inactive form represents the dephosphorylation of the receptor and cannot bind glucocorticoids. Thiol groups are important, since binding is abolished by sulfhydryl reagents such as N-ethylmaleimide, mercurials, and iodoacetamide. Receptor-bound steroid protects against inactivation by their reagents (33).

1.6.2 Physical properties of glucocorticoid receptors

Different techniques have been applied to isolate and characterize the physical properties of glucocorticoid receptors. However, attempts to purify glucocorticoid receptors have encountered technical problems due to receptor instability and low concentration (26-31, 34, 25). Different results have been reported about the properties of glucocorticoid receptors. By most recent reports, the molecular weight of the receptor varies (45,000 [36], 89,000 [37], 90,000 [38], 87,000 [39]). Govindan (36) purified two dexamethasone-binding components from rat liver cytosol by protamine sulfate precipitation,

affinity chromatography on Sephadex, and ion exchange chromatography. The two components eluting from DEAE-cellulose columns at 0.12 M NaCl and 0.20 M NaCl are single polypeptides of 45,000 mol wt and 90,000 mol wt, respectively. The 45,000 mol wt component is believed to be the proteolytic fragment. The Gustafsson group (37) found a molecular weight of 89,000 with 85% homogeneity when rat liver cytosol was chromatographed sequentially on phosphocellulose, DNA cellulose and Sephadex G-200. The steroid receptor complex was also found to have a Stokes radius of 6.0 nm and a sedimentation coefficient of 3.4 S in 0.15 M KCl. In the absence of KCl, the sedimentation coefficient was 3.6 S.

Photoaffinity labeling was also applied for purification and identification of glucocorticoid receptors from cultured rat hepatoma (HTC) and mouse lymphoma (S49) cell cytosol with synthetic progestin R5020 (39). The covalent bound receptor-progestin identified by polyacrylamide gel electrophoresis revealed a single band molecular weight of 87,000 for both HTC and S49 cell cytosol (39).

Other physical properties of the receptor such as thermolability (40), sedimentation coefficient (41), and Stokes radius (42) may differ depending on the type of steroid bound to the receptor, suggesting two receptor conformations.

1.6.3 Factors Affecting Glucocorticoid-Binding Activity

In the absence of steroid, the receptor is very unstable in cell-free cytosol at 0°C. It is inactivated by Sephadex gels, probably because of dilution and removal of salt. Unbound receptor can be stabilized by reducing agents such as dithiothreitol and 2-mercaptoethanol and by low concentrations of phosphorylated sugars, which might act as competitors for the dephosphorylation mechanism mentioned above (32). Corticoid-bound receptor is more stable at 0°C but is still labile at higher temperatures, binding capacity being lost in a few minutes at 37°C. Bound receptor is stabilized by high concentrations of glycerol or glucose (20-40% vol/vol). The protective effect of glycerol has been ascribed to stabilization of hydrophobic bonds because glycerol increases the rate of inactivation of the receptor at higher temperatures. Optimal pH is around 7.4. Binding is not wholly dependent upon divalent cations, but it is inhibited when their concenrations are reduced to 20 mM and it is stabilized by EDTA. Although optimal conditions for receptor preparation have to be determined for each cell type, a basic standard procedure for preparing cytosol is to homogenize the tissue in no more than 1-3 volumes of 20 mM Tris-HCl buffer, pH 7.4, containing 50 mM KCl. 20% glycerol, 20 mM 2-mercaptoethanol, 1 mM glucose 7-phosphate, 2.5 mM EDTA and 10 mM sodium molybdate (43).

1.7 Mechanism of glucocorticoid hormone action

The fact that glucocorticoids result in involution of lymphoid cells was discovered by Dougherty and White in 1944, and the idea that immunologic responses are affected by these hormones became generally appreciated by 1950. By the mid-1950s it became apparent that glucocorticoids could regulate the activity of a number of specific enymes (44). Since many of these enzymes are involved in the metabolic steps affected by the steroids, it was thought that the steroid regulation of metabolism could be due to effects on enzyme induction. Thus, the question emerged regarding the basic mechanism of glucocorticoid hormone action, i.e., how enzyme induction actually occurs.

Over the past decades more information has been accumulated concerning the mechanism of glucocorticoid hormone action. This, generally, may be described as follows. At the target cell, glucocorticoids, by simple or facilitated diffusion, penetrate the cell membrane (45-47) and bind specifically and with high affinity to cytoplasmic receptors (48-50), forming the steroid-receptor complex. This complex will, as a requirement for subsequent steps in the response, undergo a transformation process referred to as "activation" (49-51) so that the complex can bind to its acceptor site in the nuclear chromatin (52-55). This process initiates changes in the expression of specific genes in some cases by stimulating transcription, which results in changes in the levels of particular mRNAs. The protein translational products of these are responsible for mediating

the steroid hormone response (56, 57). A schematic representation of these steps is shown in Fig. 5.

2.0 Kinetics of the glucocorticoid hormone-receptor interaction

In the present study, thermodynamic data such as free energy (ΔG), heat or enthalpy change (ΔH), entropy change (ΔS) and heat capacity change (ΔC_p) of hormone interaction with receptors are derived from the Van't Hoff plot. This plot describes the temperature dependency of the affinities of the hormone for the receptor measured by Scatchard plots of the binding at different temperatures. It is thus technically important to understand elements in the binding reaction as well as the conditions that might influence the measurement of the affinity of the hormone for the receptor. The following are some kinetic properties of the observed interactions as illustrated by examples from our present experimental data.

2.1 Theoretical aspects

2.1.1 Specific, non-specific and total binding

2.1.1.1 Definitions

Like most of the target cell preparations, the preparations of HTC cell cytosol which were used in the current studies contain, in addition to glucocorticoid receptors, other substances that bind steroids that are not believed to be related to the actions of hormones. Such components usually are "non-specific" as they have a relatively low affinity and high capacity for binding steroid hormones. They are

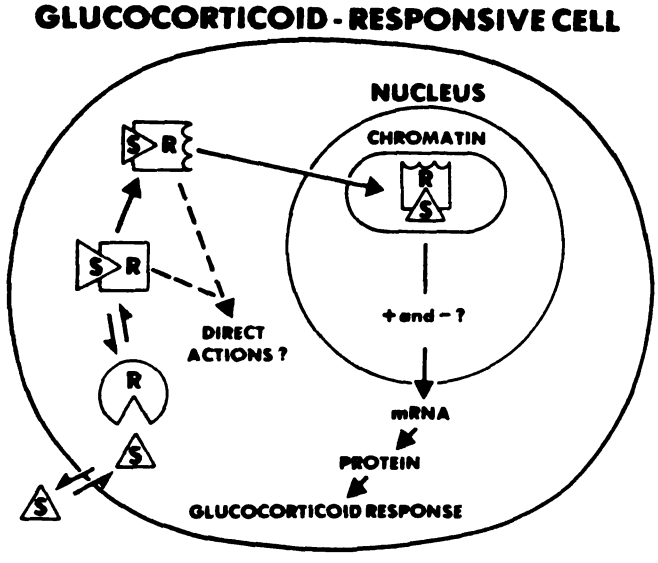


Figure 5: Steps in glucocorticoid action. S=steroid, R=receptor. Different shapes of R indicate different conformations. The direct action indicates that it is not has been excluded that the receptor steroid complex has effects other than those in the nuclear chromatin (18).

also more stable to heat than are the receptor molecules (58). Thus, to assess the specific binding of hormone to the receptor it is necessary to correct for the amount of "non-specific binding." Most of the procedures make use of the binding capacity difference between specific steroid receptors and "non-specific" components since, in most cases, receptor site concentrations are usually several orders of magnitude lower than those of the "non-specific" binding components. Thus, at high ligand concentrations the "non-saturable" components can be estimated while the proportion of ligand bound to receptors is negligible.

The high ligand concentrations necessary for estimating non-specific binding can be achieved in different ways. The single radioactive ligand method uses a single radioactive ligand, and increasing concentrations of radioinert species are added to cover a wide range of concentrations. The results, usually plotted according to Scatchard analysis, allow resolution into two or more classes of binding sites and calculation of receptor concentrations and the dissociation constant. In our present study, we used increasing amounts of radioactive ligand in the presence of an excess amount of radioinert ligand (from 500- to 1000-fold) to estimate non-specific binding.

Below is a basic theoretical calculation of the methods used to measure total and non-specific binding to assess specific binding.

2.1.1.2 Kinetic aspects

The kinetic aspects of glucocorticoid hormone receptor interaction relates to the concentration changes of free ligand, bound ligand, and free binding sites for a single binding system of $R + S \rightleftharpoons RS$, where R is unfilled binding sites, S is free ligand, RS is bound ligand, given by the relationship $K_d = (R)\ (S)/(RS)$. K_d is the apparent equilibration dissociation constant. In a simple binding system, the total number of binding sites is a constant, R_t, and it is equal to the sum of bound and unbound sites.

$$(R_t) = (R) + (RS)$$

In the equation $K_d = (R)\ (S)/(RS)$, the mass action law contains three variables. Since we know the relationship between free (R) and bound (RS), we can rewrite the equation with only two variables.

$$(R) = (R_t) - (RS)$$

$$K_d = \frac{(R_t - RS)\ (S)}{(RS)}$$

$$K_d = \frac{(R_t)\ (S)}{(K_d) + (S)}$$

A curve can be drawn relating (RS) and (S). It is the familiar rectangular hyperbolic curve shown below (named saturation curve) as an example from ^{3}H-dexamethasone binding to the HTC cytosol receptor at 0°C (Figure 6).

The slope of all binding curves defined by the above expression is, of course, the same regardless of the values of R_t and K_d. Only the values on the ordinates change when the curves are plotted. However, if we plot $(RS)/R_t$ versus $(S)/K_d$ rather than (RS) vs. (S), such binding curves have the form:

$$\frac{(RS)}{R_t} = \frac{(S)/K_d)}{(S)/K_d + 1}$$

This equation is especially useful in expanding the estimation of non-specific binding. In this case, RS becomes PrS (the complex of radiolabeled steroid binding to non-specific protein), R_t became P_t (total non-specific protein) and the K_d is several orders of magnitude higher than the concentration of radiolabeled steroid used (S). In this range, the relationship between (S) and (Prs) is essentially linear (Fig. 7), as can be seen by the fact that the above equation becomes:

$$PrS = \frac{P_t}{K_d} \times S \quad (P_t, K_d: \text{constant values})$$

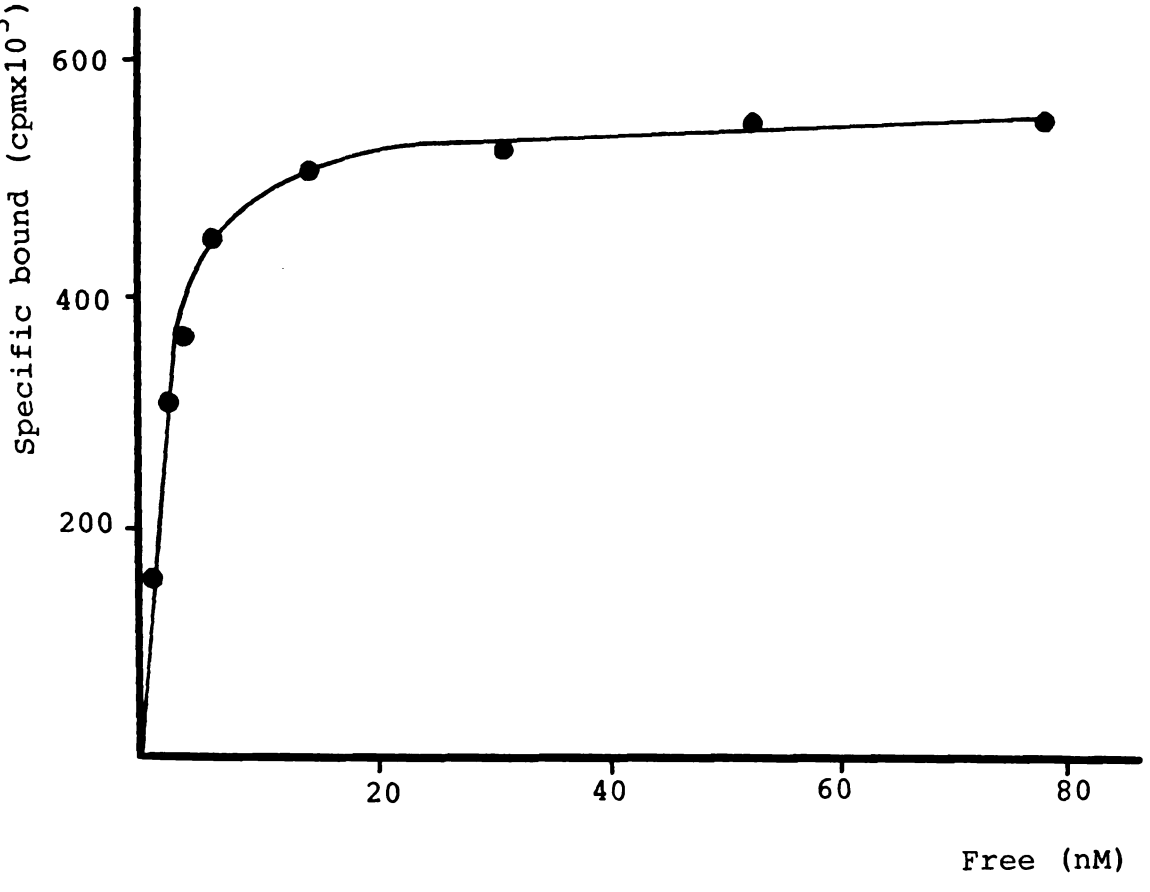


Figure 6 : Saturation curve of ^{3}H-dexamethasone binding to HTC cytosol receptors at 0^{o}C. Method and result of this data are described in section 3.0 .

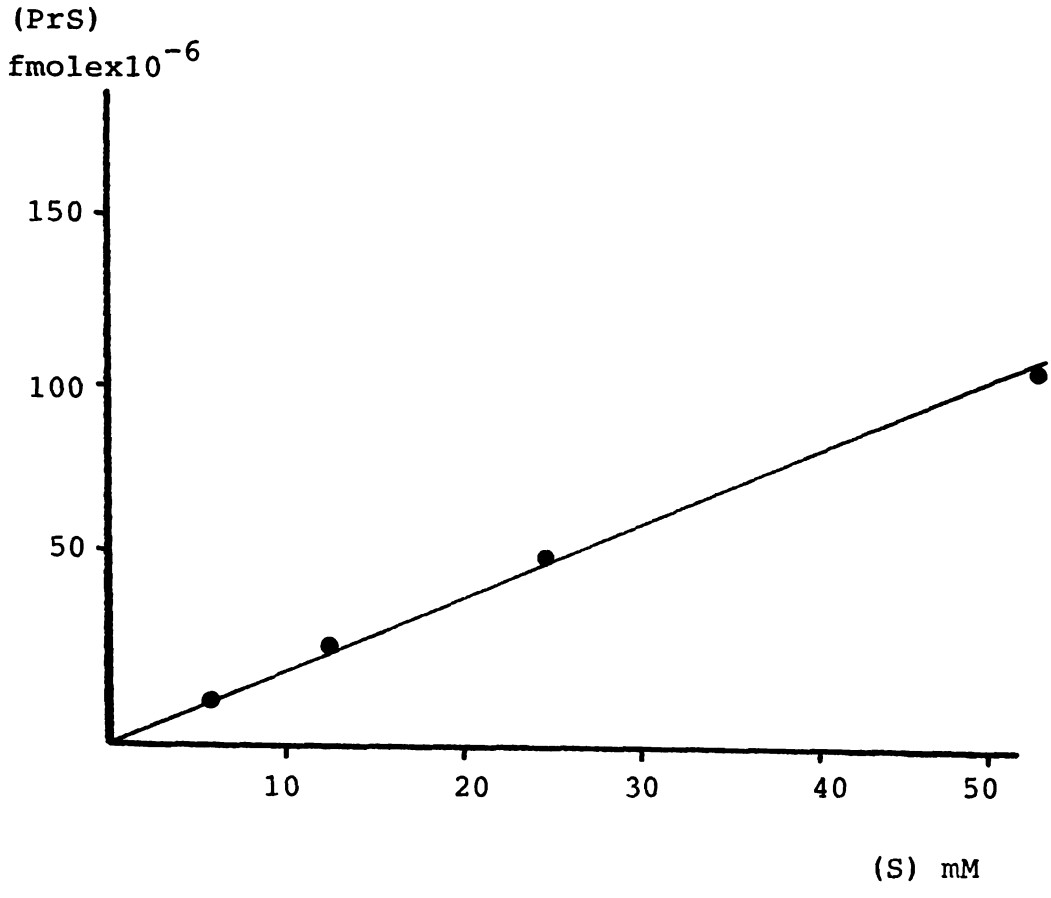


Figure 7 : Non specific proteins (Pr) bind linearly to steroid at concentrations that saturate the specific receptors. Example from ^{3}H-dexamethasone binding to intact HTC cells at 37^{o}C. Method and result are described in section 3.0 .

In our system, there are at least two types of binding that must be considered: Specific binding to high-affinity sites; and non-specific binding to low-affinity sites. When a determination of bound ligand is made, the total amount bound by both systems is measured.

Fortunately, the difference in the affinities (expressed as K_d) of the two systems is quite large (several orders of magnitude). Thus, when we are working with concentrations of free ligand in the range of the K_d of the high-affinity system, we are at the same time working with ligand concentrations several orders of magnitude below the K_d of the low-affinity system. This being the case, binding due to these low-affinity systems is linearly dependent on the ligand concentration and, due to this difference, "non-specific" binding could be achieved. Relationships of total binding, non-specific binding and specific binding are shown by an example of ^{3}H-dexamethasone binding to intact HTC cells at 37°C (Fig. 8).

2.1.2 Error in estimating non-specific and specific binding with the technique of excess radioinert steroid:

There is a limitation to the above method. If we:

(a) Assume K_{Dn} (non-specific binding) is 1000 x K_{D1} (specific binding)

(b) Experimentally analyze binding at $(S^*) = 0.1\ K_{D1} - 10\ K_{D1}$.

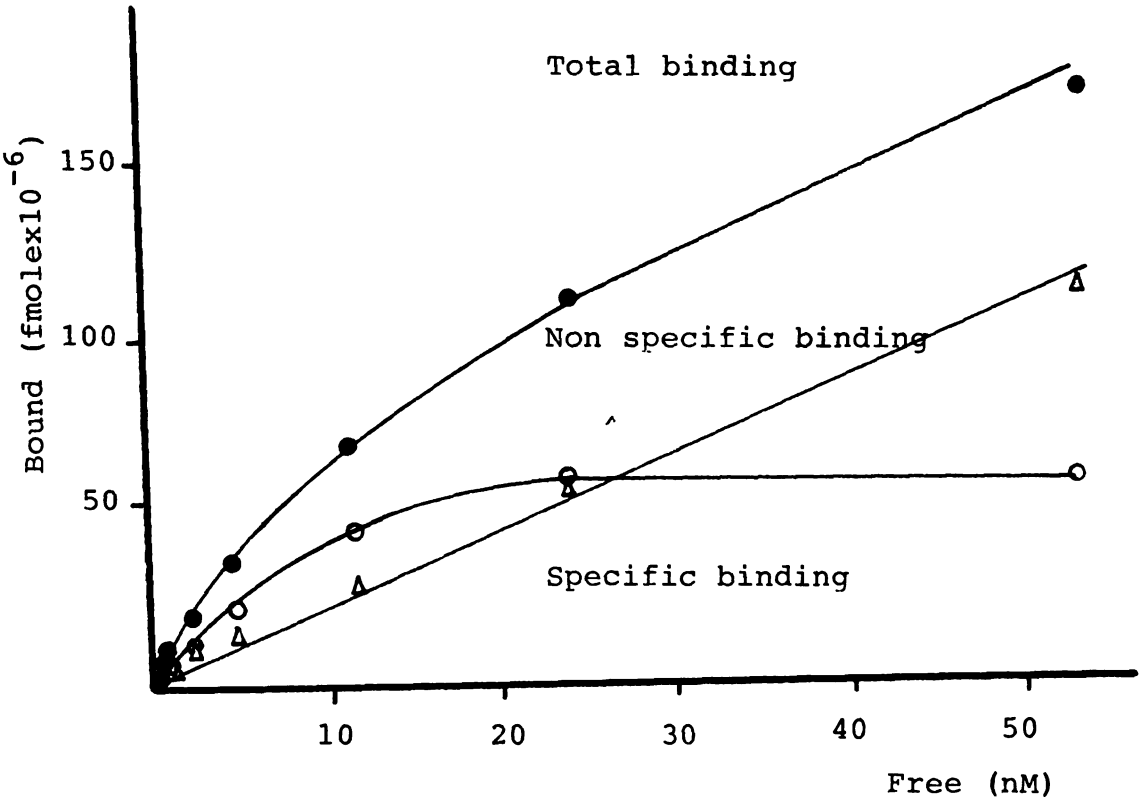


Figure 8 : Total binding, nonspecific binding and specific binding of ^{3}H-dexamethasone interaction with HTC cells at 37°C. Method and result of this data are described in section 3.0 .

In the absence of excess radioinert (S), binding of (S^*) to any protein P_r will form:

$$PrS^* = \frac{(P_t)\ (S^*)}{S^* + K_D} = \frac{(P_t)\ (S^*)}{1 + K_D^*/S^*}$$

K_D^* is the affinity of radiolabeled (S^*) to Pr in the presence of excess radioinert (S)

$$PrS^* = \frac{(P_t)\ (S^*)}{(S^*) + K_D^* + S\frac{K_D^*}{K_D}}$$

K_D is the affinity of (S) to Pr. But (S) is the same ligand as (S^*), then $K^*_D = K_D$. Thus:

$$PrS^* = \frac{(P_t)\ (S^*)}{(S^*) + K^*_D + S}$$

and by experimental design (S) = 100 (S^*)

$$PrS^* = \frac{(P_t)\ (S^*)}{(S^*) + K_D^* + 100S^*}$$

$$= \frac{(P_t)\ (S^*)}{101(S^*) + K_D^*}$$

$$PrS^* = \frac{(P_t)}{101 + \frac{K_D}{S^*}}$$

(c) Evaluate binding of (S^*) to R (specific binding) with K_{D1} and total protein (non-specific binding) with K_D = 100 K_{D1} at extremes of concentration of S^* (0.1 K_{D1} to 10 K_{D1}) in the absence (a) and presence (b) of 100-fold excess (S).

Ligand Concentration	Specific Binding to Receptor	Non-Specific Binding to Protein
$(S^*) = 0.1\ K_{D1}$	$R_t/11$ (a) $R_t/110$ (b)	protein/10,000 (a) protein/10,101 (b)
$(S^*) = 10\ K_{D1}$	Rt/1.1 (a) $R_t/101.1$ (b)	protein/101 (a) protein/202 (b)

TABLE 2: Effect of concentration of radiolabeled steroid (S^*) on the amount of specific and non-specific binding in the absence (a) and presence (b) of a 100-fold excess radioinert steroid (S).

Table 2 shows that the technique using excess radioinert (S) will eliminate binding to the high-affinity site (K_{D1}). However, non-specific binding is substantially affected at high (S^*) concentrations. The measured non-specific binding is decreased two-fold at $S^* = 10\ K_{D1}$ in the presence of excess (S) = 100 S^* compared with no (S). The non-specific binding is protein/202 in the presence of excess (S), compared with protein/101 when (S) is absent. As a result, the amount of specific binding will be overestimated at high (S^*) concentrations. In a system in which non-specific protein exists in a large amount, this will introduce considerable errors in measurement.

2.2 Measurement of affinities of glucocorticoid hormones to HTC cell cytosol receptors. Scatchard analysis

2.2.1 Theoretical aspects of Scatchard analysis

The Scatchard (1949) (59) method of analysis of binding data is frequently used in studying the binding reactions. Compared with other techniques, such as equilibrium dialysis, the Scatchard method has features that, with minimal binding data, make it possible to obtain, by extrapolation, the apparent affinity constant and binding capacity. Especially useful is the fact that a limited number of points obtained at low ligand concentrations (from 0.1 to 10 x K_D, as discussed in Section 2.1.2) can in principle be used. Furthermore, a very common practice has been to extrapolate data from competitive displacement curves (by making assumptions concerning the proportion of free and bound ligands) for use in Scatchard analysis.

However, interpretation of data by such analysis is frequently complicated and requires considerable caution. Caution must be used when aberrant binding behavior is observed or when sophisticated mechanistic interpretations are based on such data alone. In our studies, because the unlabeled and radiolabeled ligands have the same affinity, the data used to plot competitive displacement curves can be evaluated by the method of Scatchard. Estimation of affinity by Scatchard plot analysis is not invalidated by high concentrations of radiolabeled ligand or binding sites provided that the free ligands

can be accurately assessed. This method may be used in preference to estimating affinities directly from competitive binding curves whenever it is suspected that the concentration of either receptor or ligand is not small compared with the dissociation constant. However, high ligand or binding site concentrations can complicate analysis by Scatchard plots. Many of these complications are related to inaccurate estimates of the true free concentration. For example, with relatively high concentrations of labeled ligand, where only a very small fraction (e.g. about 2-5%) of the total ligand is bound, the errors in estimating small changes in the concentration of the unbound ligand may be large and magnified in Scatchard representations. Conversely, if the binding site concentration is very high, such that a very large proportion of the labeled ligand is bound, small changes in the concentration of ligand would be difficult to detect by the presence of even a small amount of unbound, labeled ligand, which may be chemically altered so that it does not bind properly. Most often, no correction for change in concentration is made for that portion of the total ligand that is bound "non-specifically," a component which in certain studies may be of substantial magnitude. The Scatchard

derivation from the two binding component systems of our study is as follows:

$$R + S \rightleftharpoons RS$$

$$K_d = \frac{(R)\ (S)}{(RS)}$$

$$= \frac{[(R_t) - (RS)]\ (S)}{(RS)}$$

$$\frac{(RS)}{(S)} = \frac{1}{K_d}(RS) + \frac{R_t}{K_d}$$

Where (R): free receptor concentration

(S): free hormone concentration

(RS): bound hormone-receptor complex concentration

(R_t): total receptor site concentration

A Scatchard analysis of the above equation gives a plot of the ratio of bound hormone-receptor complex to free hormone concentration versus bound hormone-receptor complex concentration, which gives a least-squares straight line from which the equilibrium dissociation constant of the hormone to receptor is derived from the reciprocal of

the slope of the fitted line, and the X intercept gives the total receptor binding site concentation. The linear Scatchard represents a simple class of binding site of the receptor to the ligand.

2.2.2 Artifact from "non-specific" binding in Scatchard analysis

A non-linear Scatchard plot is obtained when there is more than one class of binding sites with different affinities (Klotz and Thurston, 1971; Weder et al. 1974). In many hormone-binding studies, non-linear Scatchard plots are observed, and sometimes this can be ascribed to "two" independent classes of hormone-binding sites. However, there are other reasons for curvatures of such plots. Perhaps the most common reason for curvatures at the higher concentration range of ligand (or low RS/S ratios) is the existence of non-specific binding. The presence of heterogeneous (some saturable) sites of low affinity, or of non-specific, yet saturable, sites will produce such curvatures, as discussed in Section 2.1.2.

There also exists the cases in which the binding of a ligand to the receptor will alter the receptor in such a way that later ligands will bind with different affinities, and this will appear as a curved Scatchard plot. This effect is referred to as cooperatie binding. Decreasing binding is negatively cooperative (the case with the insulin receptor), and increasing binding is positively cooperative (oxygen binding to hemoglobin).

2.2.3 Artifacts from short incubation time and receptor instability on Scatchard analysis

Scatchard analyses can give misleading results when the incubation time of the hormone-receptor reaction is short, as well as when the receptor protein partially degrades during the course of the binding reaction. In a recent report by Arayi (60), the author shows experimentally and mathematically that, if the fixed time of incubation is less (or at least not longer) than the half-life of the hormone-receptor complex and if the total hormone concentration is not much higher than the K_d, the association function of the hormone and receptor will not simply depend on the bound hormone (B) and free hormone (H) and free receptor (R) at time (t), as usually expressed:

$$A_{(t)} = \frac{(B_t)}{[H_t]\ [R_t]}$$

Rather, the association function will substantially depend on incubation time (t) as expressed by:

$$A_{(t)} = \frac{k_1 t[1-(Ho + k_{-1}/k_1)\ k_1 t/2]}{1-Hok_1 t + Ho\ (Ho + k_{-1}/k_1)} = K_a k_{-1} t.U(H_o, t)$$

where k_1 and k_{-1} are the association and dissociation rates, respectively, of the hormone to the receptor and H_0 is the total hormone concentration at time 0. According to the above expression, function

U (H_0,t) is approximately unity for values of H_0 and t that are not too large. Moreover, the small t is at a given H_0, the closer U (H_0,t) is to unity. Therefore, for small t and H_0, as compared with k_{-1} and K_d or K_a respectively:

$$A_t \approx k_1 t = K_a k_{-1} t$$

This equation means that the association function determined from experimental data is apparently independent of the total hormone H_0 or receptor if the fixed time of incubation is short. Its value varies with the incubation time and, thus, cannot represent the real association constant of hormone and receptor. In the case of receptor protein degradation during the incubation time, the author shows that the association function $A_D(t)$ (where D denotes the denaturation situation of receptor) depends on both the incubation time (if it is short) and the denaturation function U_D (H_0,t) of the receptor:

$$A_D(t) = K_a k_{-1} t . U_D\ (H_0,t)$$

Thus, for a Scatchard analysis to be valid it is important that the incubation time be longer than the half-life of the hormone-receptor complex and that the receptor protein be stabilized during this long incubation period.

2.2.4 The Scatchard plot applied to our system

In our system, stability of the receptor is obtained by 3 mM of DTT and 10 mM Na_2MoO_4. Time courses studied show stabilization of receptor from -2°C up to 25°C (Figs. 9-12); at 30°C the receptor is quickly degraded (Fig. 12). Incubation times could be prolonged through the whole range of temperatures studied to obtain maximum binding giving an equilibrium time that is well in excess of the half-lives of the corresponding steroid-receptor complexes (dexamethasone and the HTC cell cytosol receptors in our case) as shown in Table 3.

Time of Incubation	Temp °C
260'	-2
120'	4
120'	7
110'	10
90'	14
80'	18
50'	20
30'	22
20'	25

TABLE 3: Incubation times of dexamethasone and HTC cell cytosol receptors at different temperatures.

The similarity of total binding site (from 350 to 400 fmol x 10^{-6}) in our Scatchard analyses at different temperatures and incubation times implies that the system had reached equilibrium under a stabilized receptor condition, which is also shown by the receptor stability study. The highest radiolabeled steroid concentration in the analysis is 10- to 20-fold (4-6 x 10^{-8} M) in excess of the K_d (1.0 x 10^{-9} - 7.5 x 10^{-9} M) of the glucocorticoid-receptor affinity; a single least-squares by PROPHET computer through the data of 7 to 10 points gives a correlation coefficient of R = 0.7 - 0.9; this confirms the fact that the HTC cell receptors constitute one class of binding sites. In conclusion, with radiolabeled dexamethasone concentrations in the incubations ranging from 2 x 10^{-10} to 6 x 10^{-8} M and with crude hepatoma cell cytosol stabilized by 10 mM sodium molybdate and 3 mM DTT, the Scatchard analysis can be used to study the interaction of glucocorticoid hormones with the receptors at a range of temperatures from -2° to 25°C.

3.0 Interaction of radiolabeled dexamethasone with hepatoma tissue culture cell cytosol receptors

3.1 Materials

6,7-^{3}H-Dexamethasone (47-60 Ci/mmol) was obtained from New England Nuclear and unlabeled dexamethasone from Merck (Rahway, New Jersey). Both labeled and unlabeled steroid were prepared in ethanol solution but at a concentration of ethanol that does not alter the steroid binding reaction (0.5%).

Buffers: Homogenization buffer consisted of 20 mM N-tris-(hydroxymethyl) methylglycine (Tricine), 2 mM $CaCl_2$, 1 mM $MgCl_2$, 3 mM DTT, (dithiothreitol), 10 mM sodium molybdate (Na_2MoO_4, 2 H_2O) and 10% glycerol, freshly prepared in distilled water.

Activated charcoal (Norit A, Fischer) was treated with 6N HCl to pH 4.5 and washed several times until neutral. Growth medium (pH 7.4 to 7.6) was Swim's 77 (Grand Island Biologicals, New York) supplemented with $NaHCO_3$ (0.5 g/l), 0.05 g tricine, 0.002 M glutamine and 10% calf serum in distilled water. The growth medium was bought from the Cell Culture Facility at the University of California, San Francisco, and its properties are described by Thompson et al. (61) as follows: "Swim's medium 77 (S77) has the same composition as S103 described by Swim and Barker (62) except that hydroxyproline was omitted, the serine concentration was 0.2 mM, and choline bitartrate was substituted for choline chloride." S77, when used to support growth, was supplemented with 20% bovine serum and 5% fetal bovine serum.

HTC cytosol extracts were prepared by washing HTC cells in phosphate-buffered saline (PBS) at 0°-4°C, then disrupting in one volume of the above homogenization buffer, using a Teflon-glass tissue grinder. After centrifuging at 140,000 g for one hour at 0°, the supernatant was collected for the binding study (48).

3.2 Methods

3.2.1 Time course and receptor stability study

Time courses of radiolabeled dexamethasone binding to HTC cell cytosol were studied at 4°, 12°, 18°, 20° and 25°C. Cytosol fractions in the presence of 3 mM DTT and 10 mM Na_2MoO_4 were incubated with ^{3}H-dexamethasone at the near-saturation concentration of 3×10^{-8} M in the presence and absence of 4×10^{-5} M competing non-radiolabeled dexamethasone. At time intervals shown by time course studies in Figures 9-12, varied with different temperatures, a fraction of 300 µl of cytosol was transferred to Eppendorf centrifuge tubes that contained 50 µl of the activated charcoal at 0°C. The mixture was then vortexed for 10-15 sec, allowed to stand for 1-2 min, and again centrifuged at 600 x g to remove charcoal that trapped the free radiolabeled dexamethasone. The specifically bound steroid was determined after dissolving 200 µl of the supernatant in 2 ml of an Aquasol counting solution (3 liters of toluene, 1 liter of triton, and 16 g omnifluor) to count by scintillation spectrometry. The specific binding is the difference of the total binding and nonspecific binding as discussed in Section 2.1.1.

3.2.2 Binding affinity of ^{3}H-dexamethasone to HTC cell cytosol receptor as a function of temperature

Our experiments were designed to establish the relationship of the affinities of radiolabeled dexamethasone and HTC cytosol receptors by

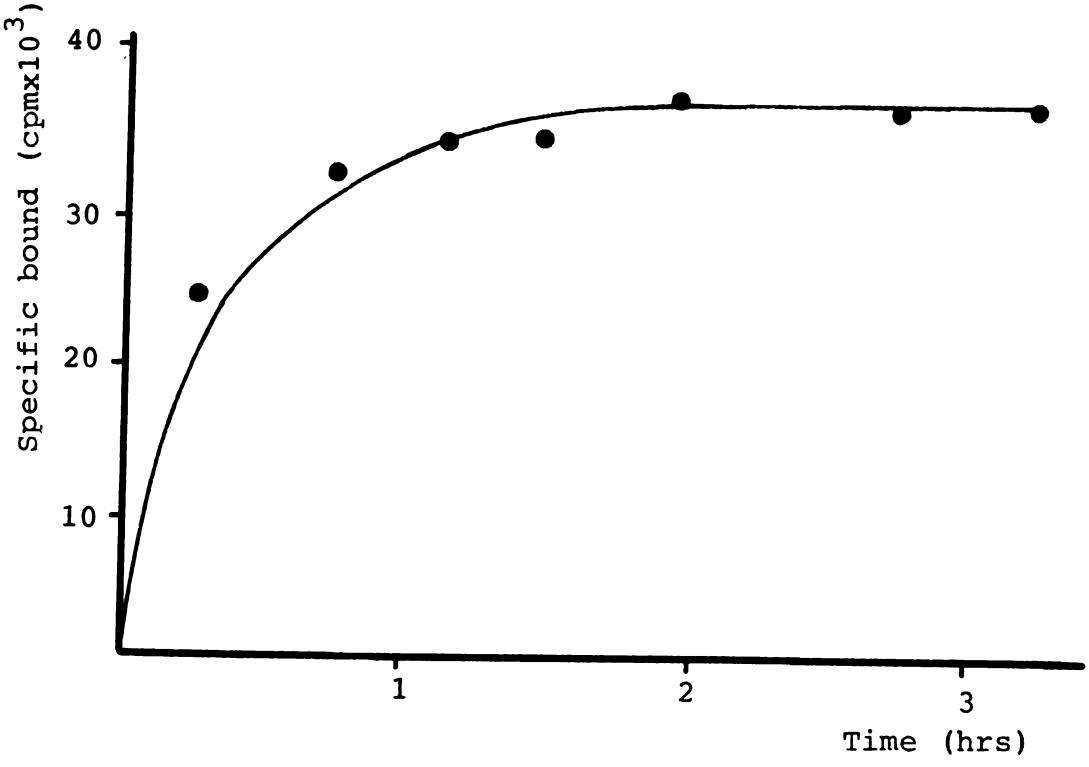


Figure 9 : Time course of ^{3}H-dexamethasone binding to HTC cell cytosol receptors at 4^{o}C. The cytosol receptor protein is stabilized by 3 mM dithiothreitol and 10 mM sodium molybdate. The labelled hormone concentration is $3x10^{-8}$M and the cytosol protein concentration is 6 mg/ml.

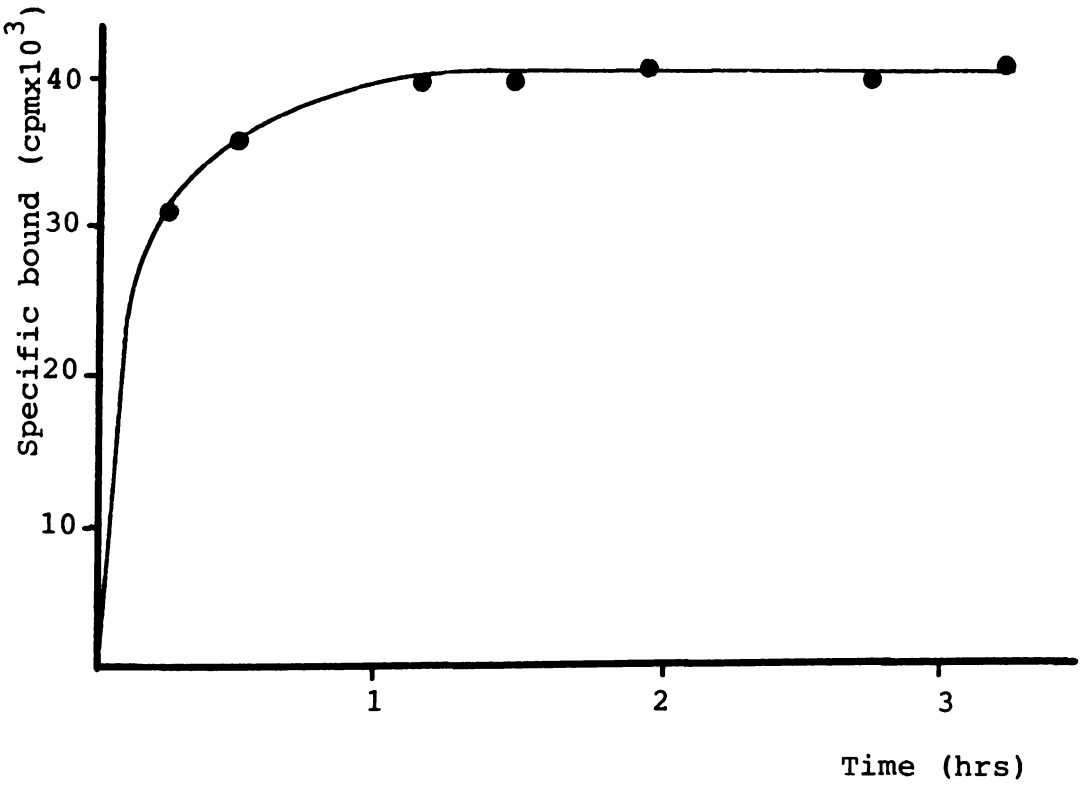


Figure 10 : Time course of ^{3}H-dexamethasone binding to HTC cell cytosol receptors at 12^{0}C. The cytosol receptor protein is stabilized by 10 mM dithiothreitol and 10 mM sodium molyddate.The labelled hormone concentration is $3x10^{-8}$M and the cytosol protein concentration is 6 mg/ml.

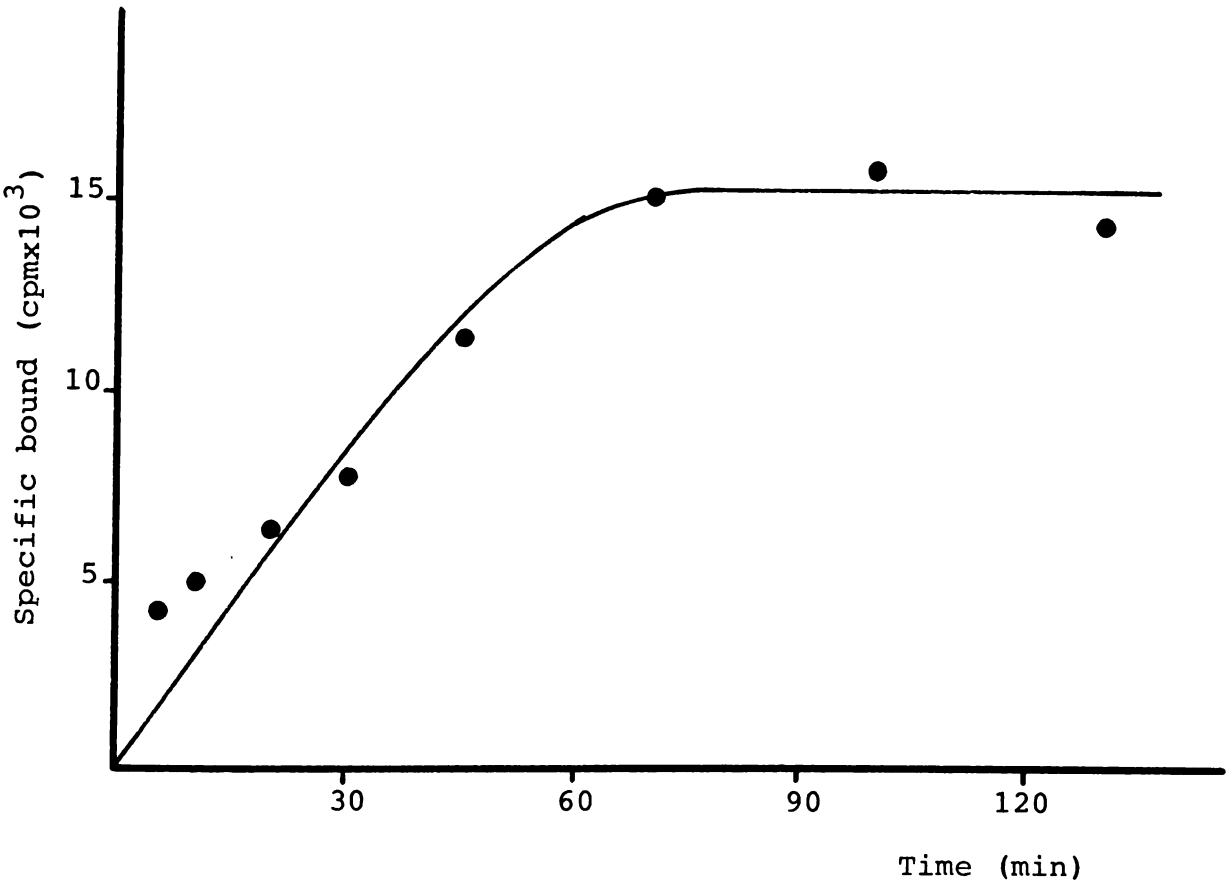


Figure 11: Time course of ^{3}H-dexamethasone binding to HTC cell cytosol receptors at 18°C. The cytosol protein is stabilized by 3 mM dithiothreitol and 10 mM sodium molybdate. The labelled hormone concentration is 3×10^{-8} M and the cytosol protein concentration is 6 mg/ml.

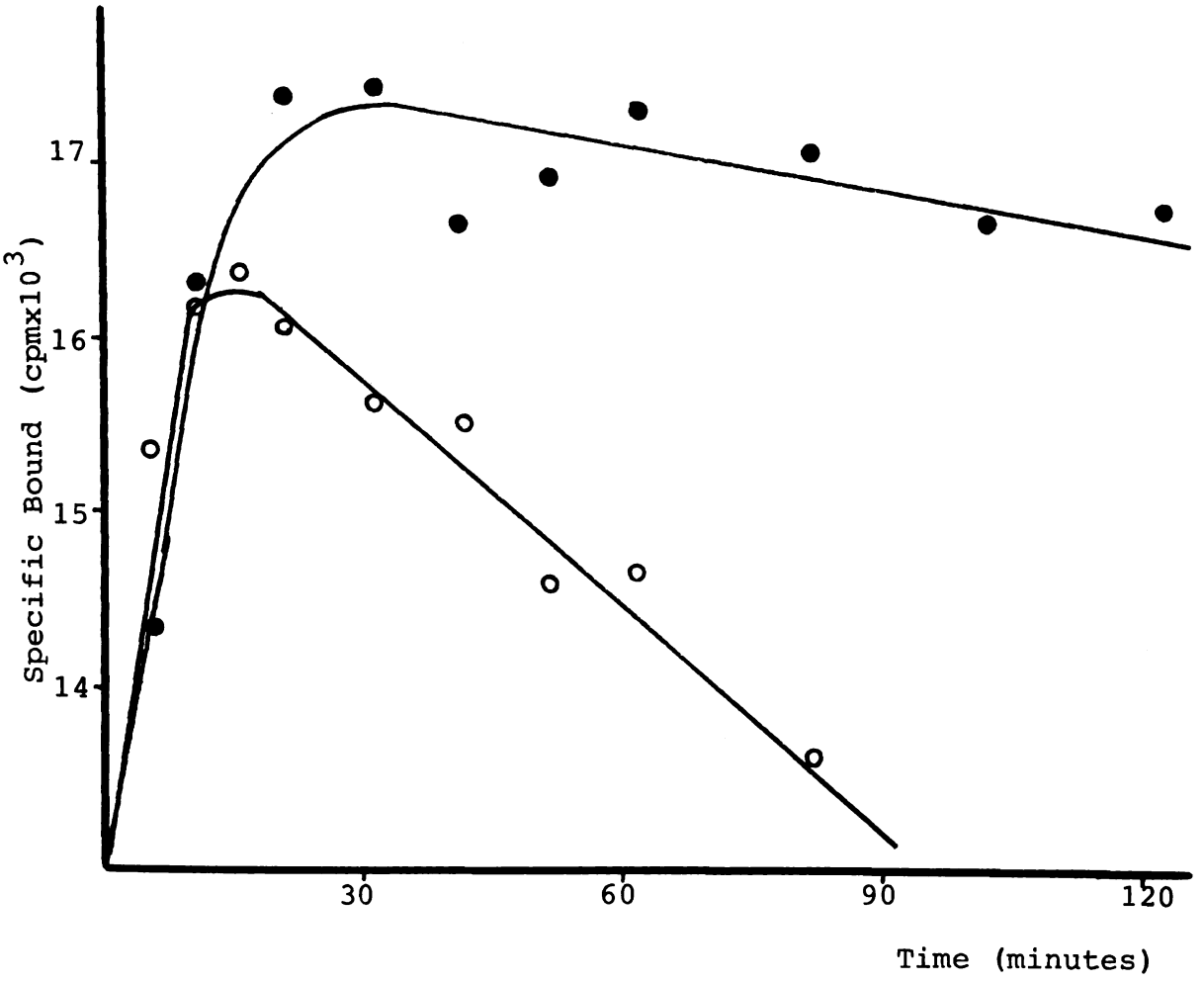


Figure 12 : Time course of ^{3}H-dexamethasone binding to HTC cytosol receptors at 25°C (●) and 30°C (○). The cytosol receptor protein is stabilized by 3 mM dithiothreitol and 10 mM sodium molybdate.

Scatchard analysis at different temperatures. In such a comparative kinetic study, it is critical to minimize the experimental variations.

It has been our experience that the best way to achieve identical conditions is to do all the experiments at the same time, with the same cytosol preparation at 0°C stabilized by 3 mM DTT and 10 mM sodium molybdate. A constant amount (120 µl) from this cytosol pool was added to tubes containing radiolabeled dexamethasone at concentrations varying from 2×10^{-10} M to 6×10^{-8} M in the presence and absence of an excess concentration of non-radiolabeled dexamethasone (1×10^{-5} M). The tubes were then equilibrated at the temperatures studied. The incubtions were allowed to reach maximum binding (or equilibrium), as shown by the time-courses studied (Figs. 9-12). The incubation times for different temperatures studied were chosen as shown in Table 3. Two aliquots of 10 µl of the incubation mixture were added to 1 ml of scintillation solution each to measure the total concentration of radiolabeled dexamethasone; the average of the two in the volume incubated in each tube was taken as the total concentration in each tube. The rest of the mixture was then treated with 10 µl of charcoal, as described. The mixture was then vortexed 10 to 15 sec, allowed to stand for 1-2 min at 0°C, and centrifuged at 20,000 rpm in an Eppendorf centrifuge to remove charcoal. Eighty microliters of the supernatant were taken from each tube and

added to 1 ml of scintillation counting solution to determine the amount of specific binding from the total hormone concentration. However, because our experiments involved 9 Scatchard analyses, it was not feasible to do all of these at the same time. Thus we performed the study in three sets of experiments and maintained identical conditions by using cytosol receptor from one harvest of HTC cells. In the first set of experiments, four Scatchard analyses were performed at temperatures of -2°, 7°, 14° and 20° (Fig. 13); in the second set, temperatures were 10°, 22° and 25° (Fig. 14); in the third set, 4° and 18° (Fig. 15). The data, represented as fmol x 10^{-6} of specifically bound steroid/100 µl aliquots of cytoplasmic protein, were plotted as a function of the free steroid concentration at equilibrium. The receptor protein concentration, estimated by the method of Lowry et al. (63), varied in the three sets of experiments from 4-6 mg/ul.

3.3 Results

3.3.1 Time course and receptor stability study: The roles of DTT and sodium molybdate

In analysis of the affinity changes of hormone and receptor at different temperatures, the greater the range of temperature extrapolated, the better the information. It is thus necessary to study the binding of hormone and receptor at temperatures as high as possible. However, the receptor protein degradation increases as temperature increases. Fortunately, various reported chemicals stabilize the

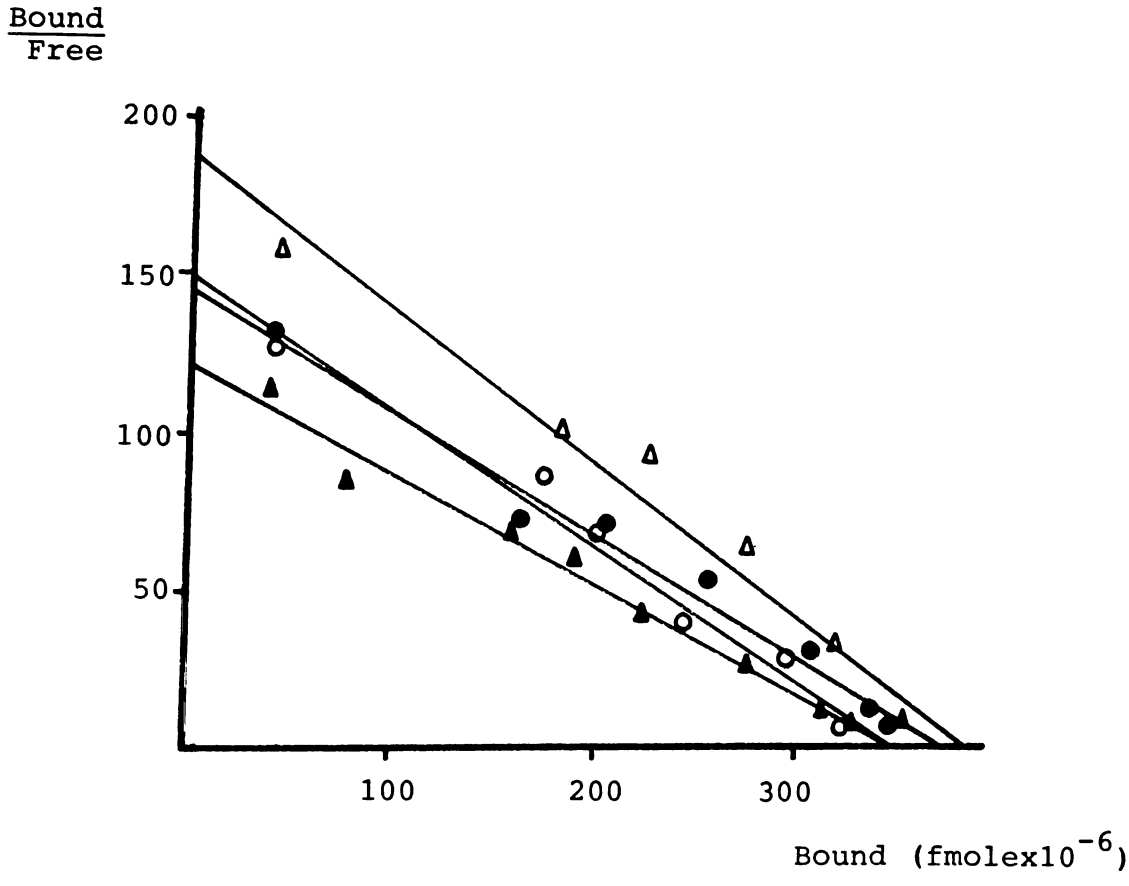


Figure 13 : Scatchard plots of ^{3}H-dexamethasone interaction with HTC cytosol receptors at -2°C (▲), 7°C (○) 14°C (Δ) and 20°C (●). The total binding site concentration is 360×10^{-6} fmole per mg of cytosol receptor protein. The cytosol protein is stabilized by 3mM dithiothreitol and 10mM sodium molybdate.

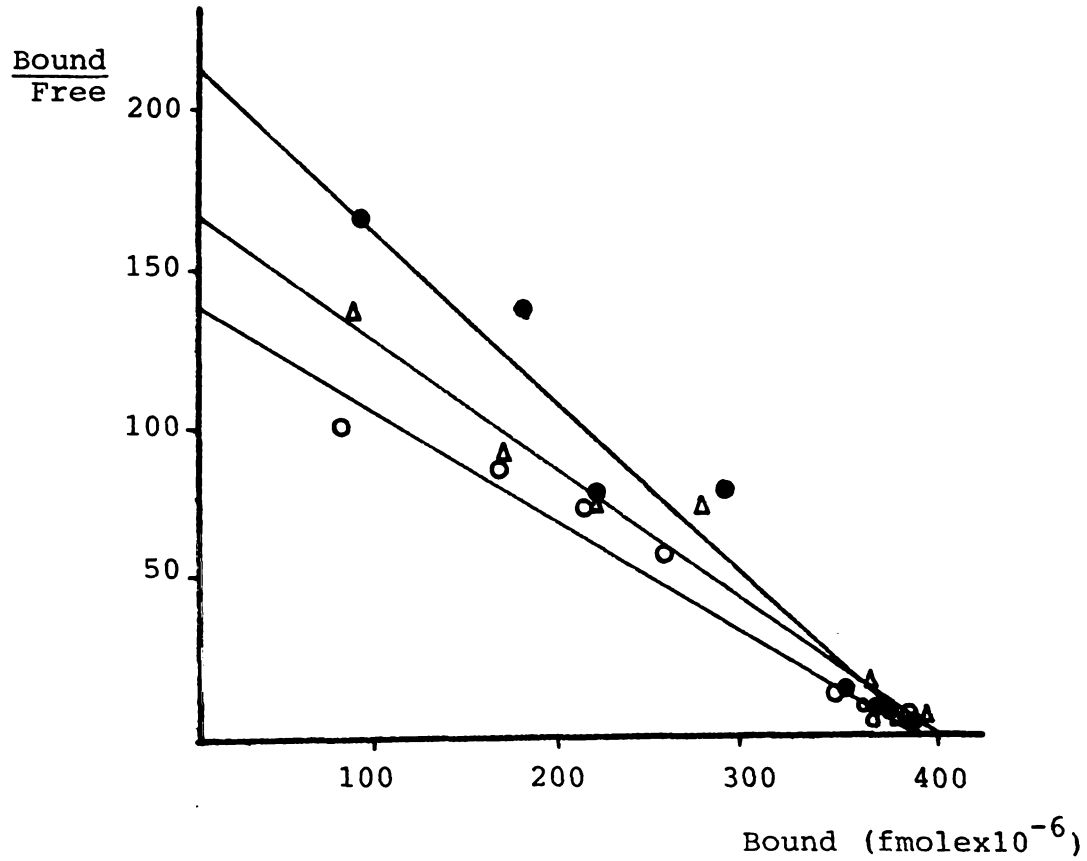


Figure14 : Scatchard plots of ^{3}H-dexamethasone interaction with HTC cytosol receptors at 10°C (●), 22°C (Δ) and 25°C (o). The total binding site concentration is 420x10^{-6}fmoles per mg of cytosol protein. The cytosol receptor protein is stabilized by 3 mM dithiothreitol and 10 mM sodium molybdate.

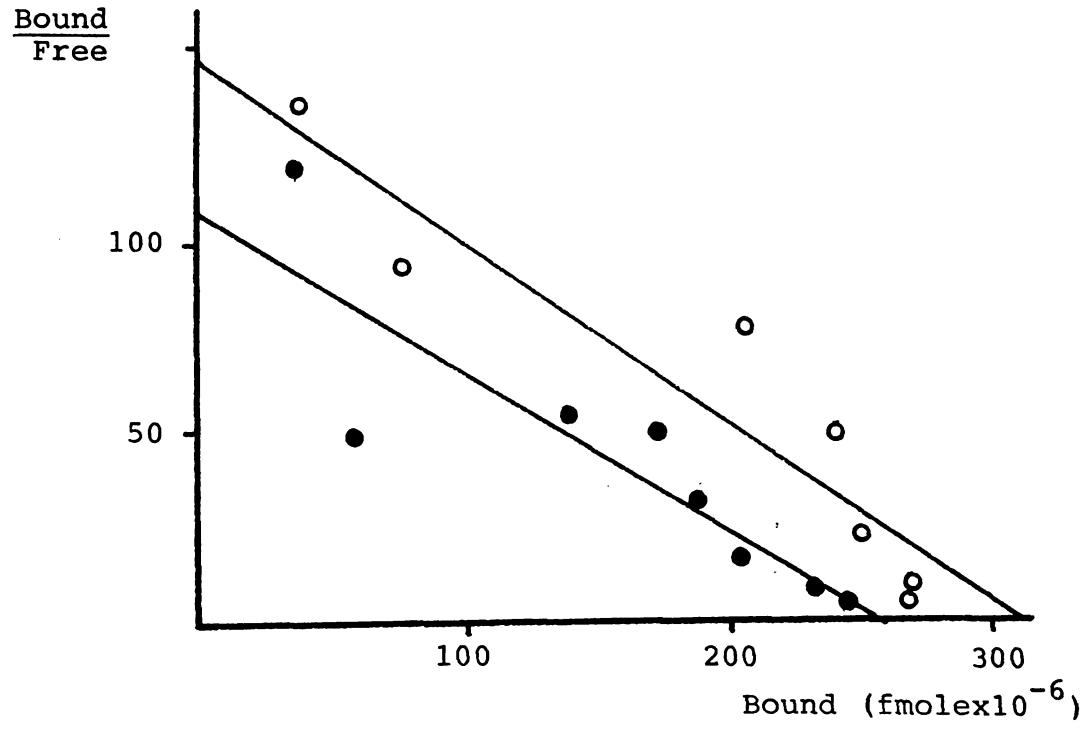


Figure 15: Scatchard plots of ^{3}H-dexamethasone interaction with HTC cytosol receptors at 4°C (o) and 18°C (●). The total binding site is 280x10^{-6}fmoles per mg of cytosol protein. The cytosol receptor protein is stabilized by 3mM dithiothreitol and 10 mM sodium molybdate.

receptor at higher temperatures; these include levamisole, fluoride, phosphate, arsenate, tungstate, vanadat and molybdate (64). We have attempted to study the kinetics of the glucocorticoid-receptor interaction under conditions in which there is minimal activation of the receptor-steroid complex. Sodium molybdate (Na_2MoO_4) was useful for this study since it both stabilizes the receptor (Neilson et al. 1977), is a phosphatase inhibitor and blocks receptor activation (64).

We also considered a number of other chemicals to add to the incubation system to prevent oxidation of sulfhydryl groups that also are responsible for receptor degradation (110-112); these include dithiothreitol (DTT), mercaptoethanol, thioglycerol and glutathiol. DTT was chosen in our experiments because it has a low oxidation reduction potential and thus can be effective at a low concentration (65). A total of 10 mM Na_2MoO_4 and 3 mM DTT was added to our incubation system to study the receptor stabilization. The results showed that we could extend the range of temperatures from -2° to 25°C (Figures 9-12). However, at 30° the receptor degraded quickly after 10 min of incubation (Figure 12).

3.3.2 Thermodynamic analysis of radiolabeled dexamethasone association with HTC cell cytosol receptors: Van't Hoff plot

From a series of Scatchard plots generated at eight temperatures, we obtained the affinities of radiolabeled dexamethasone for the HTC cell cytosol receptors. Figures 13, 14, and 15 show Scatchard plots

generated at different temperatures, and Table 4 gives values of affinities corresponding to the temperatures studied.

A Van't Hoff plot was generated by the PROPHET computer from the data. We obtained a curvilinear relationship of lnKa versus $\frac{1}{T}$ having a maximum value at 12°C (Figure 16). The second degree polynomial fit to the data by PROPHET expresses the relationship of affinity versus temperature as follows:

$$\ln K_A = -142.1 + 92.2\,X - 13.11\,X^2 \quad (X = \frac{1}{T} \times 10^3)$$

The correlation coefficient (R) for the fit is R = 0.88

3.3.2.1 Free energy change (ΔG) of the association

The free energy change (ΔG) for the steroid-receptor interactions can be directly calculated from the equilibrium association constants (K_a) by the following equation:

$$\Delta G = -RT \ln K_A$$

where R: gas constant (R = 1.987 cal $K^{-1}mol^{-1}$)

T: temperature in degrees Kelvin

K_a: apparent equilibrium association constant of the hormone for binding to the receptor expressed as $L^{-1}mol-1$

Temp. (^{o}C)	Time (min.)	K_D (nM)	LnK_A	$\frac{1}{T}x10^3$
-2	260	2.86	19.67	3.69
4	120	2.36	19.86	3.61
7	110	2.32	19.87	3.57
10	100	1.91	20.07	3.53
14	90	2.06	20.00	3.48
18	80	2.09	19.98	3.43
22	30	2.52	19.80	3.39
25	20	2.97	19.63	3.35

Table 4: Variation of the affinities of ^{3}H-dexamethasone for HTC cell cytosol receptors with temperature as determined from the data of figures 13-15.

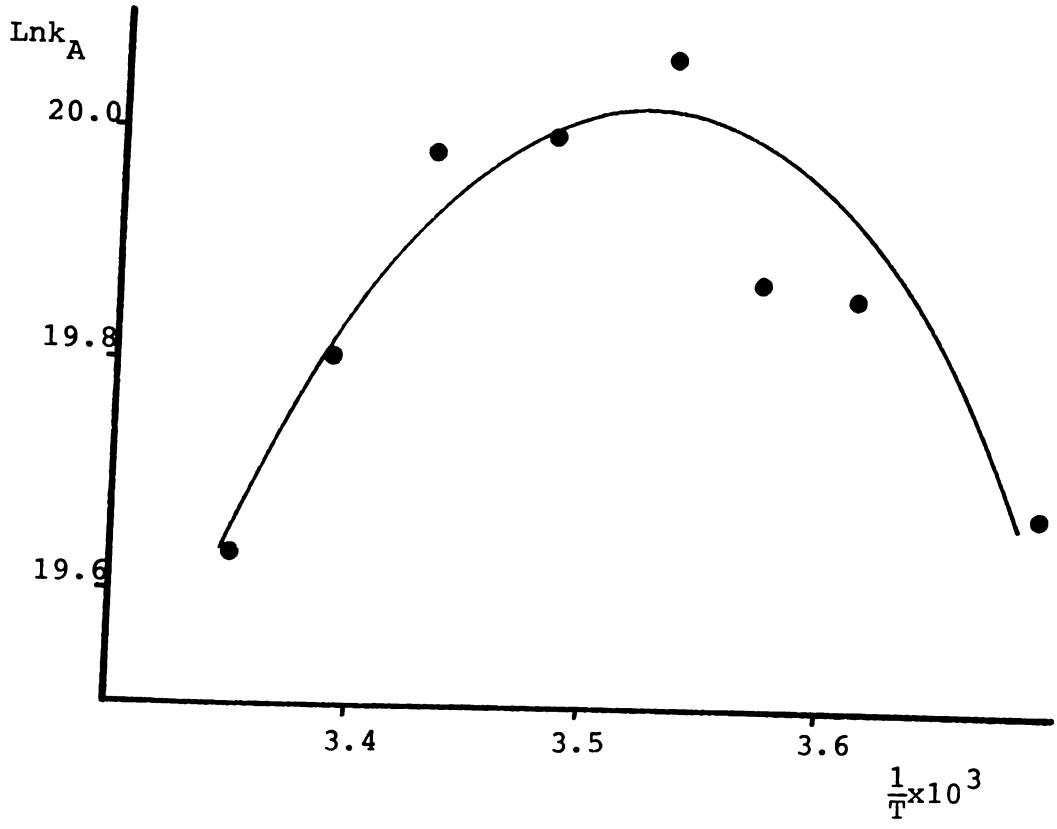


Figure 16 : Van't Hoff plot of ^{3}H-dexamethasone binding HTC cell cytosol receptors.

The free energy change (ΔG) is directly proportional to the affinity K_a; thus, a plot of ΔG against the temperature also shows a curvilinear relationship with a maximum ΔG at 25°C (as shown by Figure 17).

3.3.2.2 Enthalpy change (ΔH) of the association

A determination of the enthalpy change (ΔH) of the reaction requires knowledge of the temperature influence on the binding constant K_a. Unlike the result of using simple association reactions (Moore 1972) where the enthalpy change is either endothermic (reaction absorbing heat) or exothermic (reaction releasing heat), in the glucocorticoid-receptor interaction the enthalpy change has a marked temperature dependency and a nonlinear relationship over the range examined, shown as the Van't Hoff plot of $\ln K_a$ as a function of . The enthalpy could be determined from the slope of the second-degree polynomical least-squares fitted to the data points, since:

$$\Delta G = -RT \ln K_A;$$

$$\frac{\Delta G}{T} = -R \ln K_A;$$

$$\frac{\partial \frac{(\Delta G)}{T}}{\partial \frac{1}{T}} = \frac{-\partial R \ln K_A}{\partial \frac{1}{T}}$$

by definition $\frac{\partial \frac{(\Delta G)}{T}}{\partial \frac{1}{T}}$ is the heat change of the system or ΔH.

Thus $$-\frac{\Delta H}{R} = \frac{\partial \ln K_A}{\partial \frac{1}{T}}$$

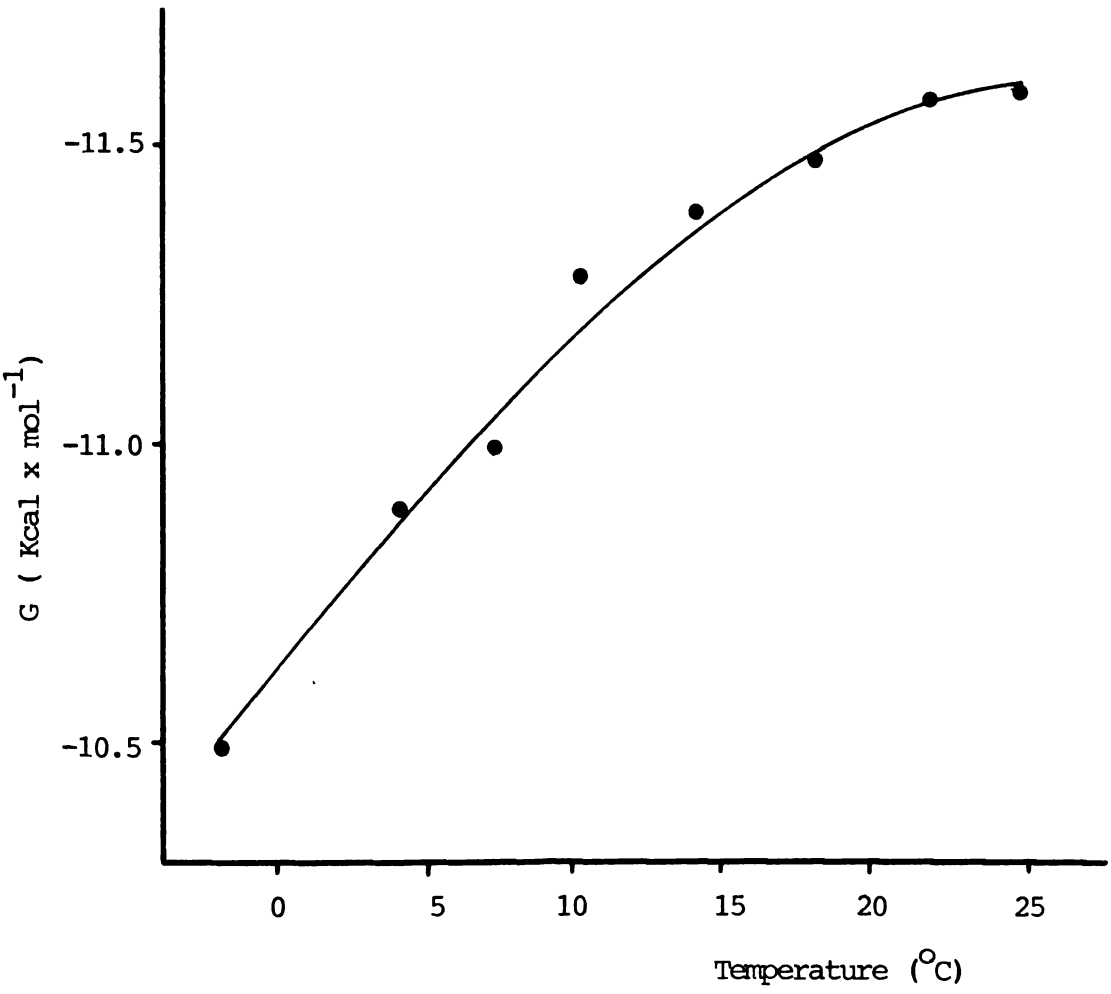


Figure 17 : Variation of the free energy change (ΔG^{o}) of glucocorticoid receptor binding with temperature in HTC cell cytosol

From the above equation, the decreasing slope (or $-\frac{\Delta H}{R}$) becomes smaller with increasing $\frac{1}{T}$ (or lower temperature T), indicating that the enthalpy decreases as the temperature increases, and the negative enthalpy change ($\Delta H < 0$) favors the formation of product at high temperatures (14°-25°C). By contrast, at low temperatures (-2° to 14°C) the positive enthalpy change ($\Delta H > 0$) works against the reaction. Table 5 and Figure 18 show the magnitude of the enthalpy change of the interaction of glucocorticoid with receptor from -2° to 25°C.

3.2.2.3 Entropy (ΔS) change of the association

The entropy change (ΔS) was obtained from the relationship of the change in free energy and enthalpy as follows:

$$\Delta G = \Delta H - T\Delta S$$

$$\Delta S = \frac{\Delta H - \Delta G}{T}$$

and the entropy unit (e.u.) is expressed as calories deg^{-1} mol^{-1}. Like the enthalpy change (ΔH), the entropy change (ΔS) increases as temperature decreases in the glucocorticoid receptor interaction. The entropy changes ranged from 72.5 e.u. to 9.9 e.u. at temperatures of -2 to 25°C, as shown in Table 5 and Figure 18. The relationship of entropy change with temperature was linear over this temperature range.

Temp.^{o}C	-2	4	7	10	14	18	22	25	Unit
ΔG	-10.5	-10.9	-11.0	-11.3	-11.4	-11.5	-11.6	-11.62	$Kcal.Mol^{-1}$
ΔH	9.0	4.8	2.8	0.7	-1.9	-4.5	-6.6	-8.7	$Kcal.Mol^{-1}$
ΔS	72.4	57.0	49.4	42.4	33.0	24.2	17.0	9.9	e.u
ΔC_p	-709	-679	-664	-650	-632	-615	-598	-587	$Cal.Deg^{-1}.Mol^{-1}$

Table 5: Thermodynamic analysis of dexamethasone interaction with HTC cell cytosol receptors.

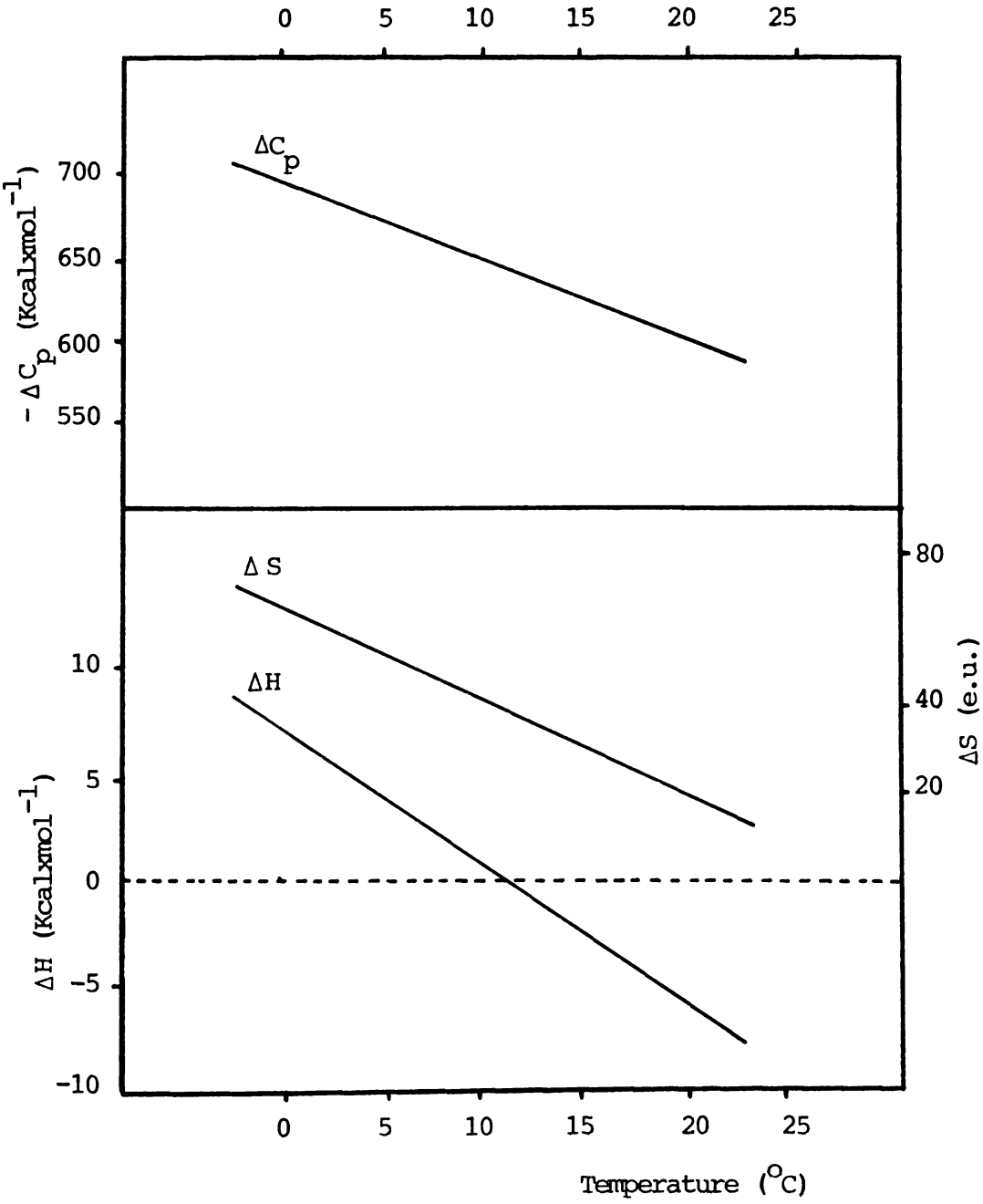


Figure 18: Variation of the enthalpy (ΔH), entropy (Δ S), and heat capacity change (ΔC_p) with temperature for ^{3}H-dexamethasone and HTC cell cytosol receptors interaction.

3.3.2.4 Heat capacity change (ΔC_p) of the association

There were large heat capacity changes (ΔC_p) in the process of glucocorticoid association with receptor as a result of the large temperature dependence in the enthalpy change (ΔH) of the interaction (Table 5 and Figure 18). The magnitude of ΔC_p was -700 to -600 cal $deg^{-1}mol^{-1}$ at temperatures from -2° to 25°C.

The heat capacity change (ΔC_p) is the change of heat (enthalpy change ΔH) with temperature as defined by the equation:

$$\Delta C_p = \frac{\partial \Delta H}{\Delta T}$$

Since the enthalpy change (ΔH) is also a function of temperature (T), as previously shown in Section 3.3.2.2:

$$-\frac{\Delta H}{R} = \frac{\partial \ln k_a}{\partial \frac{1}{T}}$$

then $$\Delta C_p = \frac{a \times R}{T^2}$$

where R is the gas constant

a x R is a constant number from the intergral of (ΔH) with $(\frac{1}{T})$

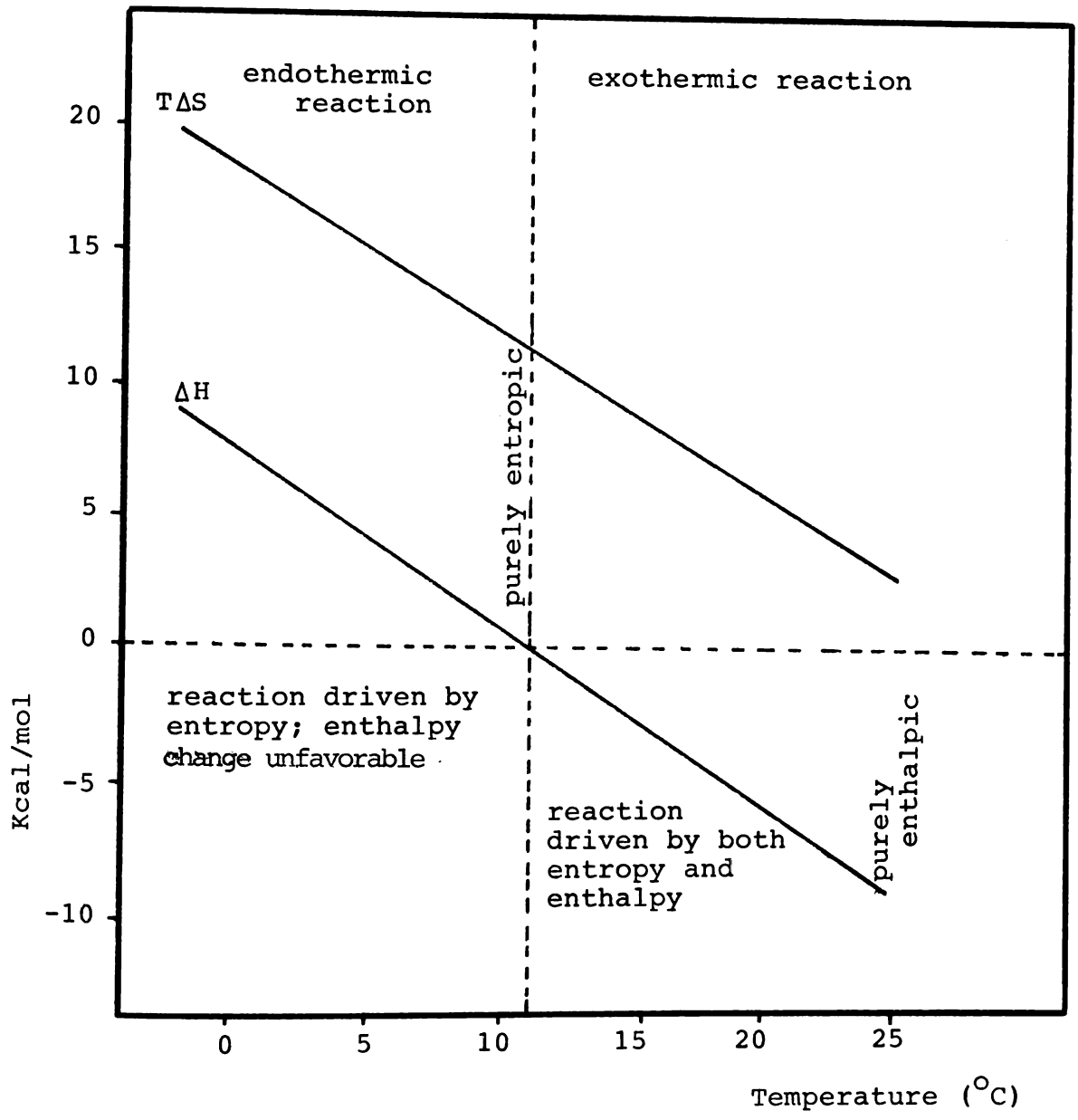


Figure 19 : Driving force in the interaction of ^{3}H-dexamethasone with HTC cytosol receptors.

Figure 19 depicts the force that drives the glucocorticoid hormone and receptor to form the hormone-receptor complex. The entropy contribution to the free energy change of the hormone-receptor interaction is negative throughout the range of temperature studied from -2°C to 25°C, whereas the contribution of enthalpy (ΔH) is biphasic. At low temperature, from -2°C to 11°C, the enthalpy change is positive ($\Delta H > 0$) and thus works against the reaction; at higher temperature from 11°C to 25°C the enthalpy is negative ($\Delta H < 0$) and contributes to the driving force of the reaction.

4.0 Interaction of radiolabelled dexamethasone with intact HTC cells

Data from previous work have provided a general description of the mechanism of glucocorticoid hormone action as follows. In the target cell, the steroid diffuses into the cytosol compartment to bind specificially to the receptors. Although the receptors have been termed "cytosol", it has not been established where inside the cell this initial binding occurs. In any event, the resulting receptor-steroid complex then undergoes an "activation" step that transforms it into a form that can bind with high affinity to the nucleus (87, 88, 90, 91, 92, 95, 105). This latter interaction then in some way stimulates the transcription of specific genes (12, 113, 114).

Although it has generally been assumed that the major driving force for the glucocorticoid-receptor interaction is due to the initial receptor-steroid binding, the role of membrane penetration or of conformational change subsequent to the initial binding has never

been addressed directly. These phenomena could affect the relationship between free and bound steroid and thus the thermodynamics of the system. There is precedent to consider these influences. For example, with thyroid hormones, the relationship between free and bound hormone is markedly different in intact cells and in cell-free preparations (personal communication) and with surface active hormones, internalization and other processes tend to result in the hormone-receptor complex having a higher affinity state than is reflected by the initial hormone-receptor interaction (115). It is thus of interest to determine whether binding of the glucocorticoid at the cytosol receptor is the major process in the overall kinetic events of steroid hormone action or whether some of these other considerations are important. To test these possibilities, a comparison was performed of the binding of dexamethasone by intact HTC cells in which the role of cell penetration and any conformational changes in the receptor-glucocorticoid complex associated with activation and nuclear binding in the overall binding thermodynamics can be studied and compared to the free energy changes associated with the receptor steroid interaction in cell-free cytosol.

4.1 Materials

The radiolabeled dexamethasone, non-labeled dexamethasone and growth medium are described in Section 3.1.

The hepatoma cells were obtained from the UCSF Cell Culture Facility at a concentration of 5×10^5 cells per ml in growth medium.

The cells were fed every 24 hr with an equal volume of growth medium; glutamine to a final concentration of 5% was also added.

The general characteristics of these cells were described by Thompson (61) as follows: "The rat hepatoma cell line came from an ascites tumor which in turn had been derived from a solid hepatoma (#7288c) originally induced by feeding male Buffalo rats a diet containing 0.04% N,N-2,7-fluoremylen-bis-2,2,2-trifluoroacetamide for 12.4 months. Primary culture was carried out by sterile peritoneal puncture and withdrawal of 0.1 ml ascitic fluid which was placed in T30 culture flasks to which 5 ml of growth medium was at once added. After an initial lag of a few days, a layer of epitheloid cells grew out. For the first eight months, growth was maintained in tightly stopped bottles in a standard laboratory incubator, but since then a humidified CO_2 incubator running with 3% CO_2-97% air has been used with the bottle stoppered loosely at 37°C.

The cells could be frozen in 5% glycerol-95% growth medium by standard techniques and stored in liquid nitrogen. Upon thawing after as much as a year of such storage, HTC cells exhibited the same growth and enzyme induction characteristics as the original line. The rat hepatoma cell line was originally cultured in October 1964, and then carried in an unbroken series of 59 transfers over 12 months. Since then, cells frozen at passage 33 have been used and carried another 35 transfers. Like other tumor lines in culture, this cell line formed multilayered confluent sheets on glass surfaces and exhibits logarith-

mic growth with a double line time of approximately 24 hours. Histologically they have the characteristics of "epitheloid" cells showing irregular cytoplasmic projections when growing in contact with glass and isolated from other cells, but becoming more rounded as intercellular contact is established. No blood elements or fibroblasts were seen. Compared to normal rats, which have a chromosome number of 42, a count of 100 mitotic figure HTC cells at the 46th transfer revealed a hypotetraploid number with a mean around 66, and 5% 2s and 1% 4s. Of special interest, since they are not seen in normal rat cells, were the several metacentric chromosomes observed in all mitotic figures studied from HTC cells."

From previous reports (25, 48) the HTC cell line was shown to have between 50,000 to 100,000 glucocorticoid receptors per cell that bind dexamethasone with high affinity (Kd = 10^{-9}M). In addition, the cytosol preparation does not metabolize the hormone during the course of incubation (48).

4.2 Methods

4.2.1 Culture of HTC cells

HTC cells were grown at 37°C in spinner culture by constant stirring with a magnetic bar suspended in the medium without contact with the bottom of the contaminent. The cells were given one volume of growth medium every 24 hr to keep the cell concentration at 7×10^5 - 1×10^6 cells/ml.

4.2.2 Harvesting the cells

The cells were harvested at a concentration of 7 x 10^5 to 1 x 10^6 cells/ml by centrifugation at 7,000 x g for 10 min in a Beckman TJ-6 centrifuge. The pellet was washed twice by resuspending it in an equal volume of Swim's 77 medium with vortexing and centrifuging. The final pellet was suspended in a volume of Swim's 77 medium 10% CO_2 (pH = 7.4-7.6) to obtain the final cell concentration of 5 x 10^6 to 7 x 10^6 cells/ml and 0.45 ml of this concentration is used in each tube for the binding studies.

4.2.3 Separation of bound and free ^{3}H-dexamethasone after intact HTC cell binding

After reaching equilibrium (as indicated in the time course study section), the mixture of intact cells and media containing ^{3}H-dexamethasone was centrifuged at 20,000 g in a Sorval 5412 centrifuge for 3 min to remove the free ^{3}H-dexamethasone in the supernatant medium. Free steroid was measured directly in the supernatant. The cell pellet was washed three times with a volume of 2.5 ml of cold PBS by resuspension, vortexing and centrifuging as described above. The washed pellet was suspended in Aquasol solution to measure the bound ^{3}H-steroid

4.2.4 Affinities, determined by Scatchard analysis, of dexamethasone for binding to intact HTC cells

In the time course study of dexamethasone binding to HTC cells at 4°C a constant amount of cells (5 x 10^6/ml) was incubated with a near

saturation concentration of ^{3}H-dexamethasone (1×10^{-7} M) in the absence and presence of excess of non-radiolabeled dexamethasone (1×10^{-5}). At various times, samples of 500 µl were taken to measure specific binding of the hormone to the cells as described. A progressive increase of specific binding of the hormone by the cells occurred over a two-hour period after which this binding began to reach plateau levels (Fig. 9). Although this time could be reflected by the attainment of binding equilibrium, this may also be apparent rather than real if there is substantial degradation of the receptor under the conditions used. To determine whether the time used provides a reasonable indication of the binding constant, the apparent affinity of dexamethasone for binding to receptors in intact HTC cells was examined at several different incubation times.

At 4°C, 1 ml containing 5×10^{6} cells were incubated with various concentrations of ^{3}H-dexamethasone (10^{-7} to 10^{-9}M) in the presence and absence of excess non-radiolabeled dexamethasone (1×10^{-5} M) to determine specific binding. At 3, 7, 11, 17 and 22 hr, 500 µl of the incubation solution was taken from each tube to determine the specific binding and free steroid concentration as described in section 4.2.3 for Scatchard analyses.

4.2.5 Time course study of the interaction at different temperatures

As with ^{3}H-dexamethasone binding by HTC cell cytosol, two pools of cells at 3×10^{6} to 5×10^{6} cells/ml in Swim's 77 plain solution with 10% CO_2 were incubated with a near saturated concentration of

^{3}H-dexamethasone of 3 x 10^{-8}M, with or without an excess of cold dexamethasone at 1 x 10^{-5}M. The mixtures were shaken at time intervals that varied with the temperatures studied (every 5 min for 37°C and every 30 min at 0°C). At specific time intervals, 500 µl of the mixture of each pool was taken to determine the amount of ^{3}H-dexamethasone specifically bound by the cells.

4.2.6 Affinity of radiolabelled ^{3}H-dexamthasone for binding to intact HTC cells at various temperatures (Van't Hoff plot)

As with the study of cytosol, with the intact HTC cells we tried to minimize variance by generating a series of binding studies at varying temperatures in the same experiment. Two sets of experiments were done for temperatures at 4°, 18°, 22°, 30° and 37°, and at 0°, 12°, 25°, 32° and 35°C. Cells at 3 x 10^{6} and 5 x 10^{6} cells/ml were incubated with 7 to 9 different concentrations of ^{3}H-dexamethasone ranging from 10^{-10}M to 10^{-7}M in the presence and absence of excess non-radiolabelled dexamethasone (1 x 10^{-5}M). The incubation volume was 500 µl in each 12 x 75 mm glass tube (Scientific Products). At equilibrium, as determined from the time course studied at different temperatures, the tubes were transferred to ice water and centrifuged at 20,000 rpm to separate bound and free steroid as described in section (4.2.3). The ^{3}H-dexamethasone bound by the cells was expressed as fmol x 10^{-6} per cell and the free radiolabelled ^{3}H-dexamethasone as 10^{-9} M or nM units.

4.3 Results

4.3.1 A theoretical calculation of dexamethasone interaction with intact HTC cells

A Scatchard plot was obtained from data by incubating ^{3}H-dexamethasone at different concentrations with a fixed concentration of cells allowing the reaction to reach maximum binding, as indicated by the time course studies in 4.2.5. Least squares analysis of the Scatchard plots gave a straight line with a correlation coefficient of R = 0.7 - 0.9 within ten experiments at different temperatures (Figures 20 and 27).

Equation 20 of the following analysis would give a linear Scatchard plot, provided that the amount of free nuclear acceptor (N) remains unchanged during the study course of the binding of the hormone-receptor complex with the nucleus. Since this is, in fact, the case, there must be a very large number of acceptors for the receptor-steroid complexes within the nucleus.

The early events in glucocorticoid hormone action described in Section 1.7 could be expressed by the following steps:

$$S \rightleftharpoons S' + R \overset{K_1}{\rightleftharpoons} RS \overset{K_2}{\rightleftharpoons} RS^{*} \overset{K_3}{\rightleftharpoons} RS^{**} + N \overset{K_4}{\rightleftharpoons} RNS$$

Symbols are defined as follows (all are equlibrium concentrations in one cell):

$[S]$: concentration of free steroid in solution. The free steroid (S') inside the cell is assumed to be equal to the free steroid in the solution; thus it is ignored in our calculations.

$[RS]$: concentration of steroid-receptor complex.

$[R]$: concentration of free receptor.

$[RS^*]$: concentration of activatable receptor-steroid complex.

$[RS^{**}]$: concentration of activated steroid-receptor complex.

$[N]$: concentration of free acceptor in the cell nucleus that binds receptor.

$[RNS]$: concentration of steroid-receptor-nuclear complex.

$[R_t]$: total amount of cytoplasmic receptor.

$[N_t]$: total amount of nuclear receptor.

$[V_c]$: volume of each cell.

K_1, K_2, K_3, and K_4 are equilibrium constants for each step shown above.

Derivation of specific bound over free (B/S) vs. specific bound (B) relationship for the steroid binding to the cell is as follows:

$$[R_t] = V_c\,([R] + [RS] + [RS^*] + [Rs^{**}] + [RNS]) \quad (1)$$

$$[N_t] = V_c\,([N] + [RNS]) \quad (2)$$

Mass action implies:

$$K_1 = \frac{[RS]}{[S]\,[R]} \quad (3) \qquad K_3 = \frac{[RS^{**}]}{[RS^*]} \quad (5)$$

$$K_3 = \frac{[RS^*]}{[RS]} \quad (4) \qquad K_4 = \frac{[RNS]}{[N].[RS^{**}]} \quad (6)$$

The concentration of all the species containing steroids can be expressed as a function of (RS) as following, using equations (3) to (6):

$$[S] = \frac{[RS]}{K_1.[R]} \quad (7); \qquad [RS^*] = K_2(RS) \quad (8);$$

$$RS^{**} = K_2K_3(RS) \quad (9); \qquad [RNS] = K_2K_3K_4 \cdot N(RS) \quad (10)$$

The bound is the total steroid specifically bound by the cell. The free (F) is the (S) concentration or solution outside the cells as mentioned. The total specifically-bound hormone is:

$$B = V_c ([RS] + [RS^*] + [RS^{**}] + [RNS]) \qquad (11)$$

$$= V_c ([RS] + K_2[RS] + K_2K_3[RS] + K_2K_3K_4[N].[RS])$$

$$= V_cK_2[RS] \left(\frac{1}{K_2} + 1 + K_3 + K_3K_4[N]\right)$$

let

$$\delta(N) = K_2\left(\frac{1}{K_2} + 1 + K_3 + K_3K_4[N]\right); \text{ this is a constant number}$$

provided that the number of free nuclear acceptors [N] is very large compared to the number of nuclear receptor occupied (RNS) thus:

$$B = V_c(RS)\cdot\delta(N) \qquad (12)$$

$$[R_t] = V_c[R] + V_c ([RS] + [RS^*] + [RS^{**}] + [RNS])$$

$$[R] = \frac{[R_t]}{V_c} - ([RS] + [RS^*] + [RS^{**}] + [RNS])$$

$$= \frac{[R_t]}{V_c} - ([RS] + K_2[RS] + K_2K_3[RS] + K_2K_3K_4[N][RS])$$

$$= \frac{[R_t]}{V_c} - K_2\left[\frac{1}{K_2} + 1 + K_3 + K_3K_4.[N]\right].(RS)$$

$$= \frac{[R_t]}{V_c} - \delta(N)(RS) \qquad (13)$$

From equation (3):

$$\frac{[RS]}{[S]} = K_1[R] = K_1\left(\frac{[R_t]}{V_c} - \delta(N)[RS]\right) \quad (14)$$

Similarly

$$\frac{[RS^*]}{[S]} = K_2\frac{[RS]}{[S]} = K_2K_1\left(\frac{[R_t]}{V_c} - \delta(N)[RS]\right) \quad (15)$$

$$\frac{[RS^{**}]}{[S]} = K_2K_3K_1\left(\frac{R_t}{V_c} - \delta(N)[RS]\right) \quad (16)$$

$$\frac{[RNS]}{[S]} = K_2K_3K_4K_1[N]\left(\frac{[R_t]}{V_c} - \delta(N)[RS]\right) \quad (17)$$

From (11):

$$\frac{B}{F} = V_c\left(\frac{[RS]}{[S]} + \frac{[RS^*]}{[S]} + \frac{[RS^{**}]}{[S]} + \frac{[RNS]}{[S]}\right) \quad (18)$$

substituted by (14), (15), (16) and (17), equation (18) becomes:

$$\frac{B}{S} = V_cK_1\left(\frac{[R_t]}{V_c} - \delta(N)[RS]\right)(1 + K_2 + K_2K_3 + K_2K_3K_4[N])$$

$$= V_cK_1\left(\frac{[R_t]}{V_c} - \delta(N)[RS]\right)\underbrace{K_2\left(\frac{1}{K_2} + 1 + K_3 + K_3K_4[N]\right)}_{[N]}$$

$$= V_cK_1\left(\frac{[R_t]}{V_c} - \delta(N)[RS]\right)\delta(N)$$

$$= \delta(N).K_1[R_t] - \delta(N)^2.K_1V_c[RS] \quad (19)$$

From equation (12):

$$[RS] = \frac{B}{V_c.\delta(N)}$$

Thus equation (19) becomes:

$$\frac{B}{S} = \delta(N).K_1[R_t] - \delta(N)^2.K_1V_c.\frac{B}{V_c\,\delta(N)}$$

or

$$\frac{B}{S} = -\delta(N).K_1B + \delta(N).K_1[R_t] \quad (20)$$

Scatchard plot of the above equation will have a negative slope crrespondent to $-\delta(N).K_1$ and the abscissa intercept of the line is $\delta(N).K_1R_t$. Since K_1, R_t are constant, thus the straight line of Scatchard plots obtained in the experiments of ^{3}H-dexamethasone binding to intact HTC cells has confirmed the fact that $\delta(N)$ is a constant value, i.e., the concentration of the nuclear acceptor is excessive large compared to that of other components involved in the hormone action such as the hormone, receptor, the hormone receptor complex, its activated form.

4.3.2 Properties of the interaction

Changes of apparent affinity of the glucocorticoid for receptors in intact HTC cells versus time at 4°C are shown in Fig. 20 by five Scatchard plots generated at 3, 7, 11, 17 and 22 hr. The changes in measured affinity and total binding site concentration with time are also shown in Fig. 21 and are listed in Table 6. The K_a increases with progressive incubation time, although it begins to plateau after about 1 hr. During this time, there is a progressive decrease in the binding site concentration, which is presumably due to progressive degradation of the receptors. Thus at this temperature in the intact cell system, it is not possible to obtain "absolute" equilibrium. Nevertheless, there exists a range of affinity and total site changes that allow us to reach a "relative" value of equilibration. The data of Fig. 21 and Table 6 show that the changes in apparent affinity and total receptor sites are minimized between about 11 to 22 hr. It is thus possible to choose the optimum conditions for which the values of affinity and total binding sites could be used to interpret the situation closest to the absolute equilibration of the system. Observations similar to this were also reported by Koblinsky et al (69). Thus, for these studies at 4°C the incubation time was 11 hr. The decreases in the total number of receptors observed could be due to degradation of receptor protein or viability of the cells or both.

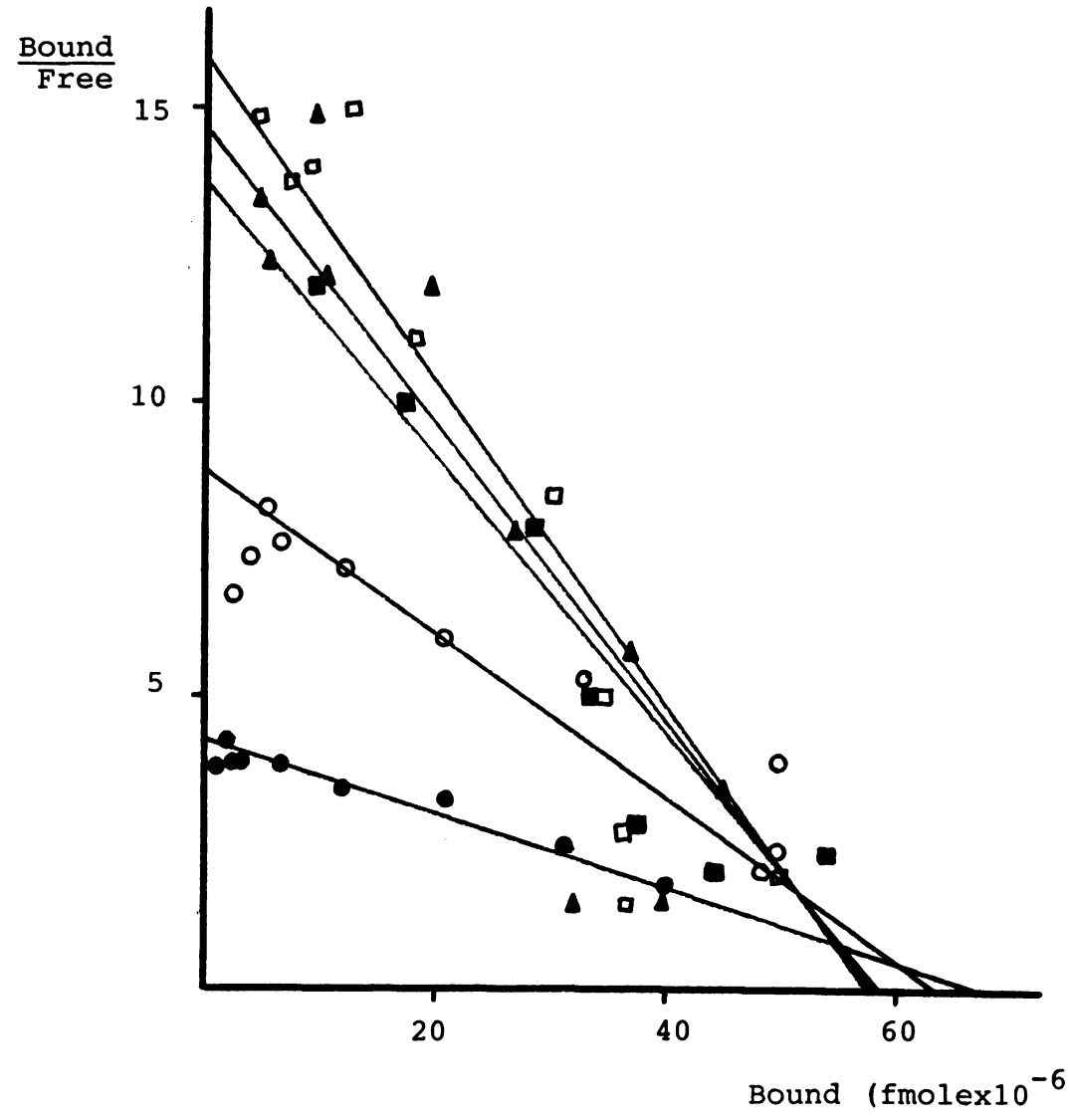


Figure 20: Changes of the apparent affinities and binding sites with incubation time in ^{3}H-dexamethasone binding to intact HTC cells. 3hrs (●), 7 hrs (○), 11 hrs (Δ), 17 hrs (■) and 22 hrs (□).

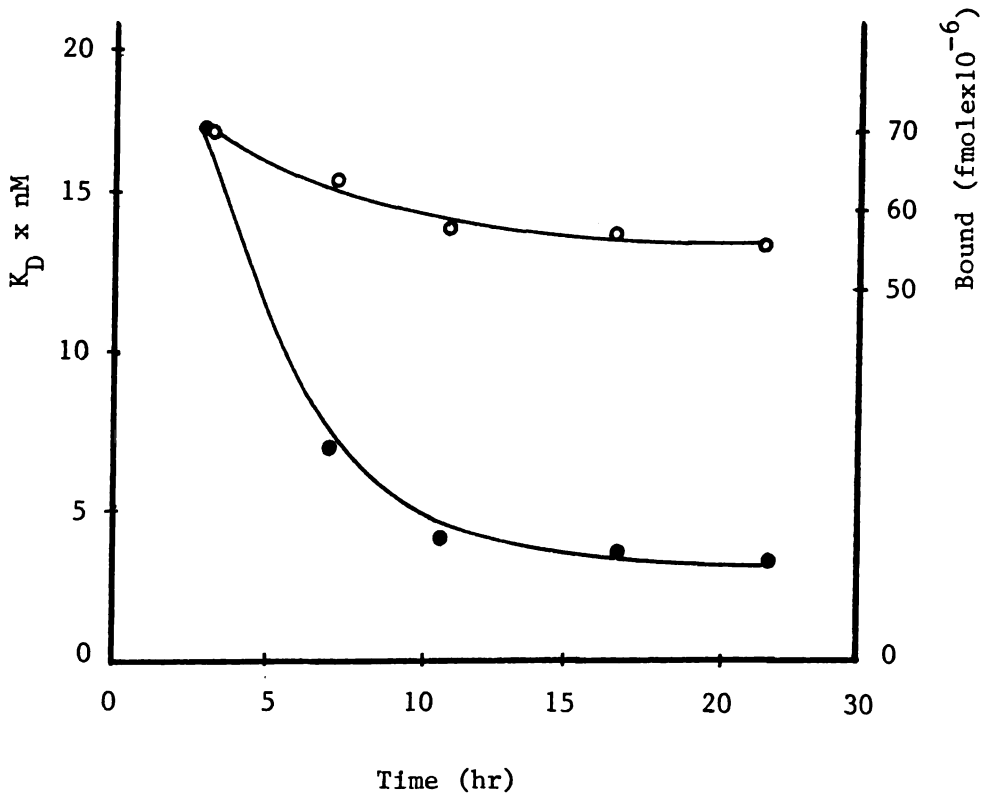
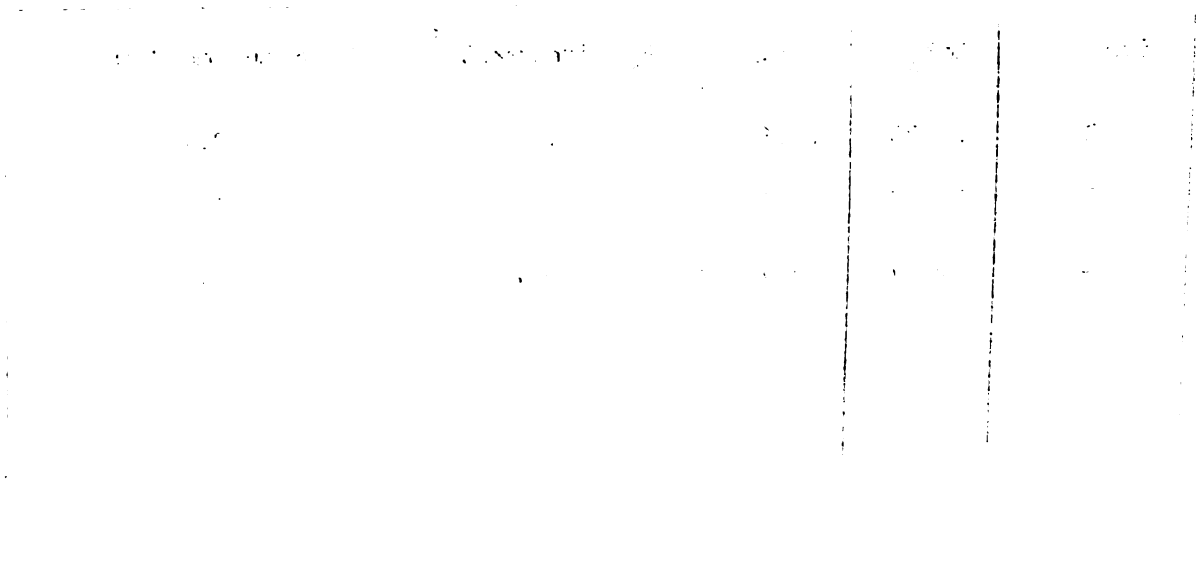


Figure 21: Effect of incubation time on the appearent equilibrium dissociation constant (K_D) (●) and the concentration of binding site (o).

Time (hr)	LnK_A	K_D (nM)	R_t (fmolex10^{-6})	Site per cell x 10^3
3	17.85	17.6	70.0	42.1
7	18.76	7.1	64.0	38.5
11	19.31	4.1	57.5	34.6
17	19.38	3.8	57.0	34.3
22	19.5	3.4	55.0	33.1

Table 6: Variation of the measured apparent equilibrium constants (K_A) and binding sites with different incubation times at 4°C.



4.3.3 Time course of the interaction as a function of temperature

Duplicate cultures of HTC cells at 5 x 10^6 cells/ml were incubated with 3 x 10^{-8}M ^{3}H-dexamethasone in the absence and presence of excess radionert 3 x 10^{-5}M dexamethasone to assess specific binding as described in section 2.1.2. Specific binding was determined at different time intervals and temperatures. Specific binding increased with increasing incubation time, but reached a plateau as the system equilibrated. The time required for this equilibrium state varied with temperature and decreased with increasing temperature. We obtained equilibrium times of 11 hr for 4°, 8 hr for 12°, 6 hr for 8°, 3.75 hr for 22° and 45 min for 37°, Figures 22-26. Based on these results, we selected incubation times for temperatures, 0°, 25°, 30°, 33° and 35°C of 17 hr, 3 hr 10 min, 2 hr 20 min, 1 hr 20 min and 50 min, respectively, to obtain maximum binding.

4.3.4 Themodynamic analysis of the interaction

We calculated apparent affinity constants for the ^{3}H-dexamethasone-receptor interaction in HTC cells from Scatchard plots generated at the various temperatures studied (Figures 27, 27a and 27b). These equilibrium association constants ($\ln K_a$) were plotted versus x 10^3 (T_{kelvin} = 273° C + t°) to obtain a Van't Hoff plot for the thermodynamic analysis of the interaction. Using the PROPHET computer to fit the second-degree polynomial to the Van't Hoff plot, as in the cytosol study, the relationship of temperatures x 10^3 versus $\ln k_A$ is

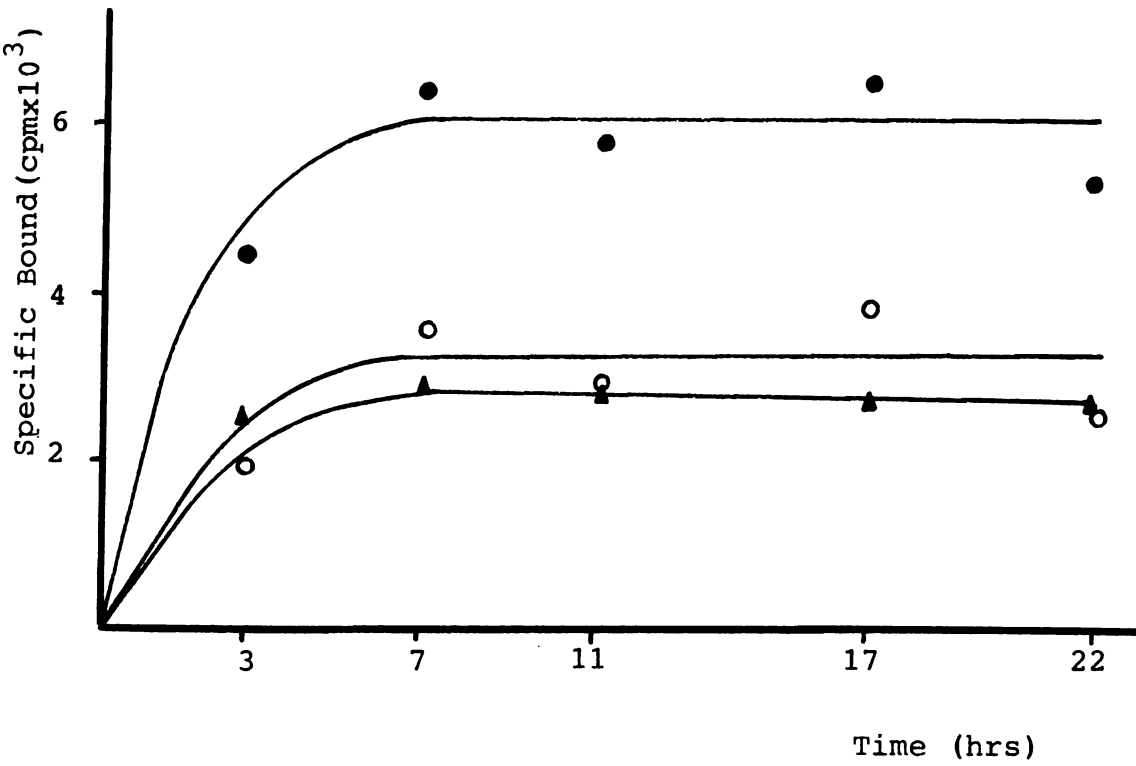


Figure 22: Time course of dexamethasone binding by intact HTC cells at 4°C (●). Also shown are the amounts of specific binding by cytosol (○) and nuclei (▲).

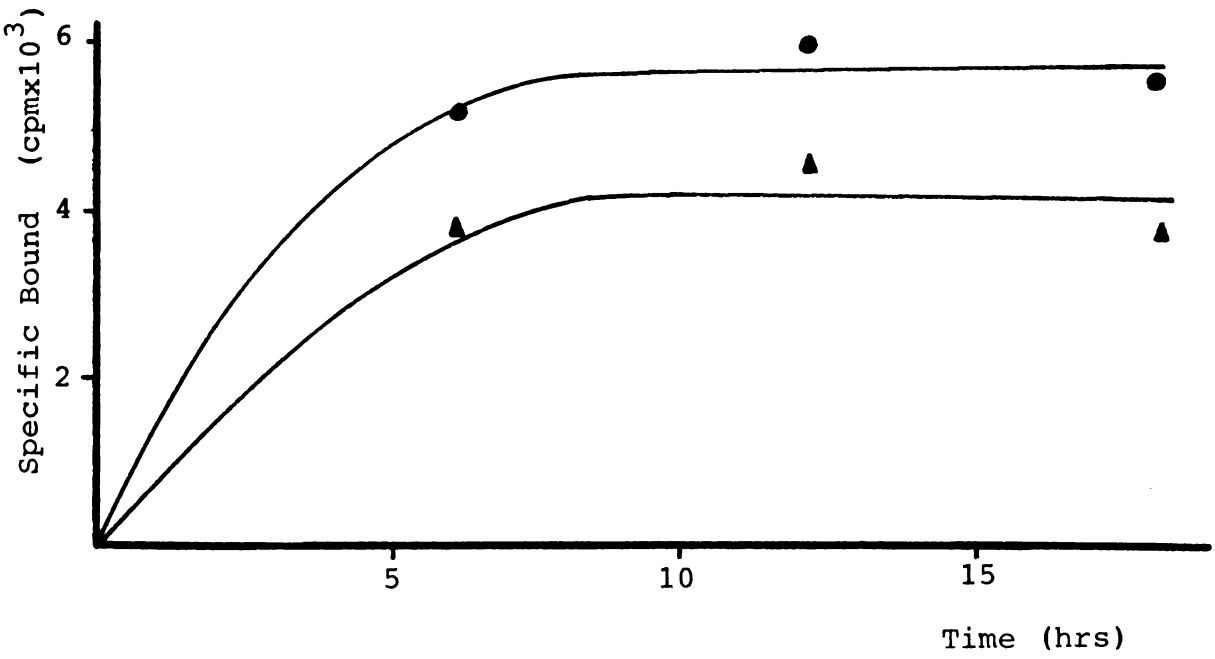


Figure 23 : Time course of specific ^{3}H-dexamethasone binding by HTC cells at 12°C (●). Also shown is the specific nuclear binding (▲).

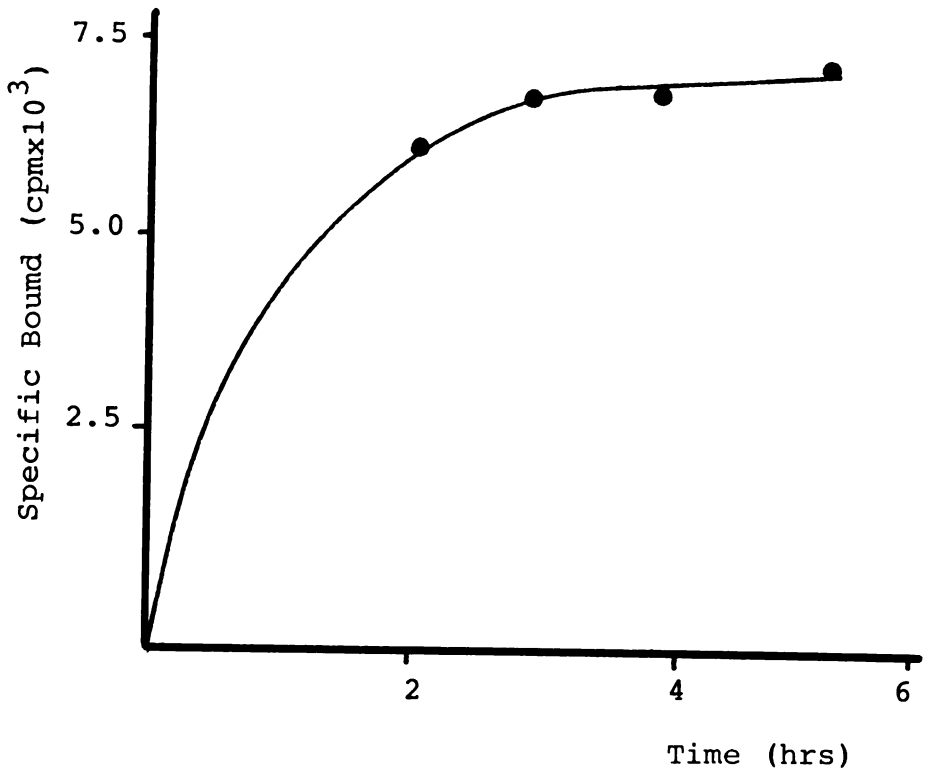


Figure 24 : Time course of ^{3}H-dexamethasone binding to HTC cells at 18^{o}C.

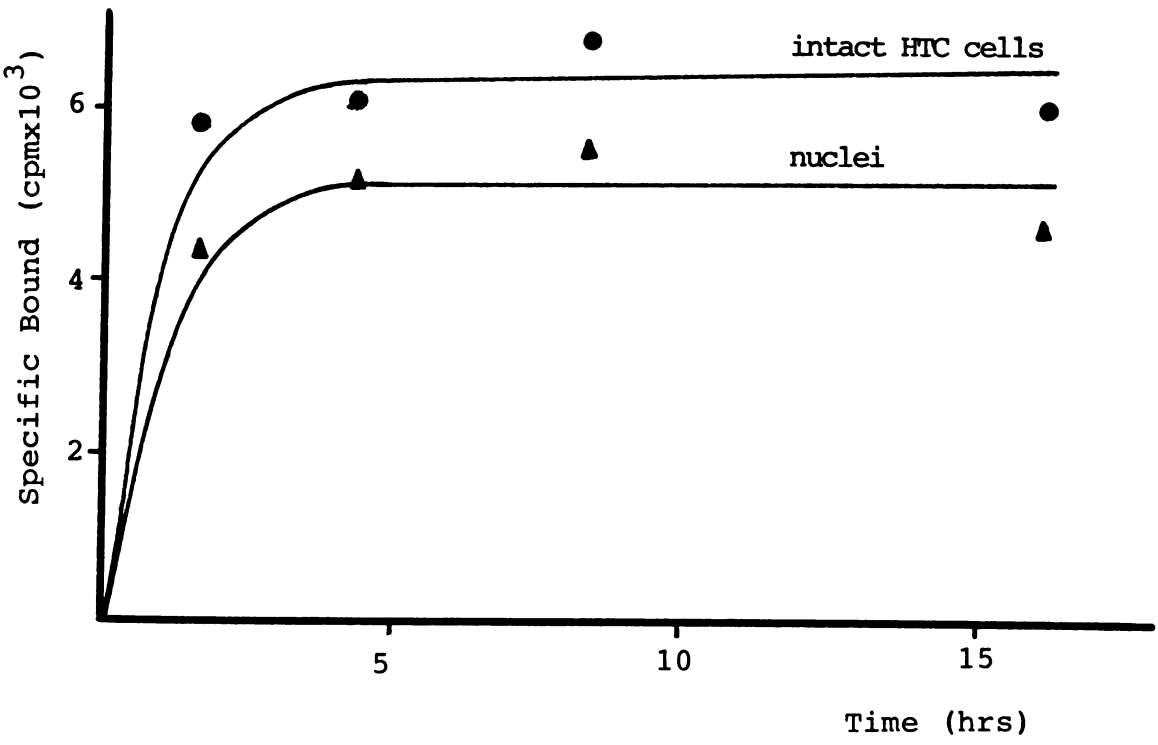


Figure 25 : Time course of ^{3}H-dexamethasone binding by intact HTC cells at 22°C. Also shown are the amounts of specific nuclear binding.

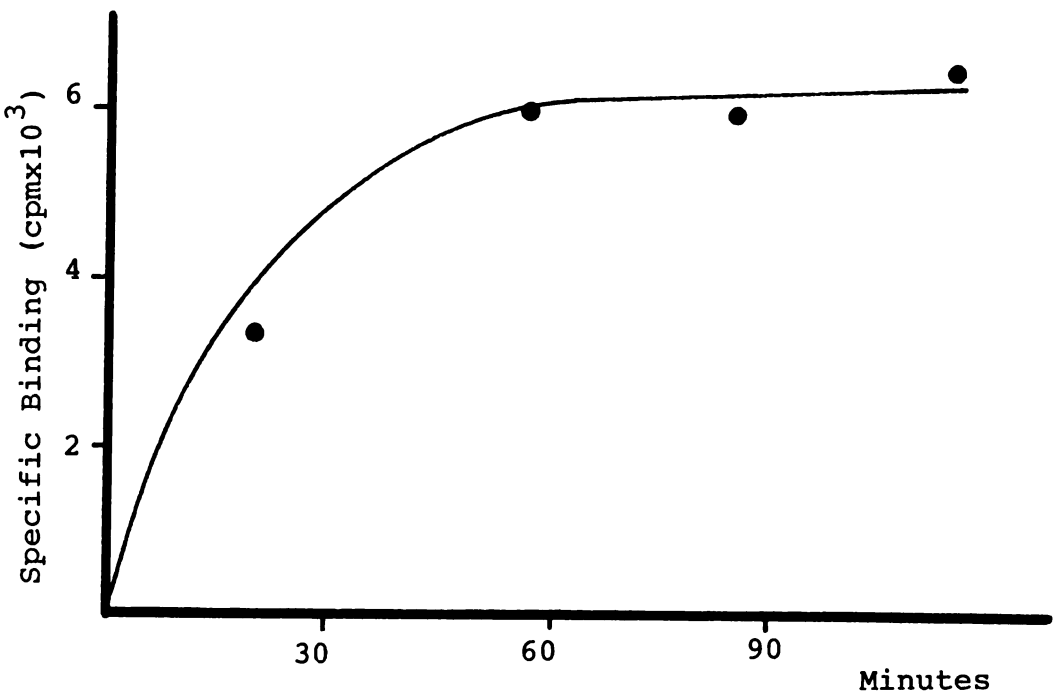


Figure 26: Time course of ^{3}H-dexamethasone binding by intact HTC cells at 37^{o}C.

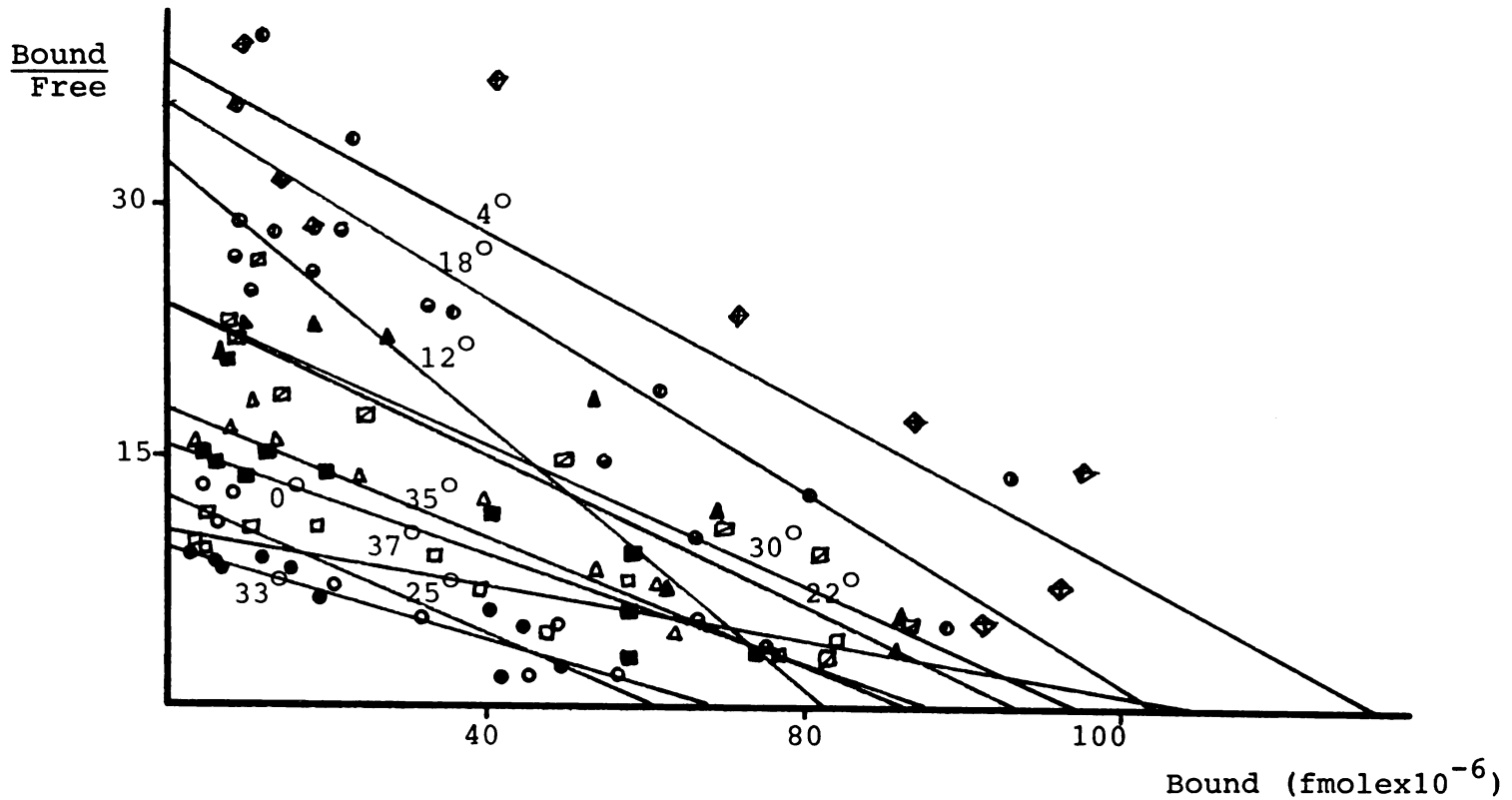


Figure 27: Scatchard plots of specific ^{3}H-dexamethsone binding by intact HTC cells at 0°, 4°, 12°, 18°, 22°, 25°, 30°, 33°, 35° and 37°C as indicated on the graph. The total binding site varied from 60 to 90x10^{-6} fmole for the first set of experiments (fig. 27a) and from 90 to 140x10^{-6} fmole for the second set of experiments (fig. 27b).

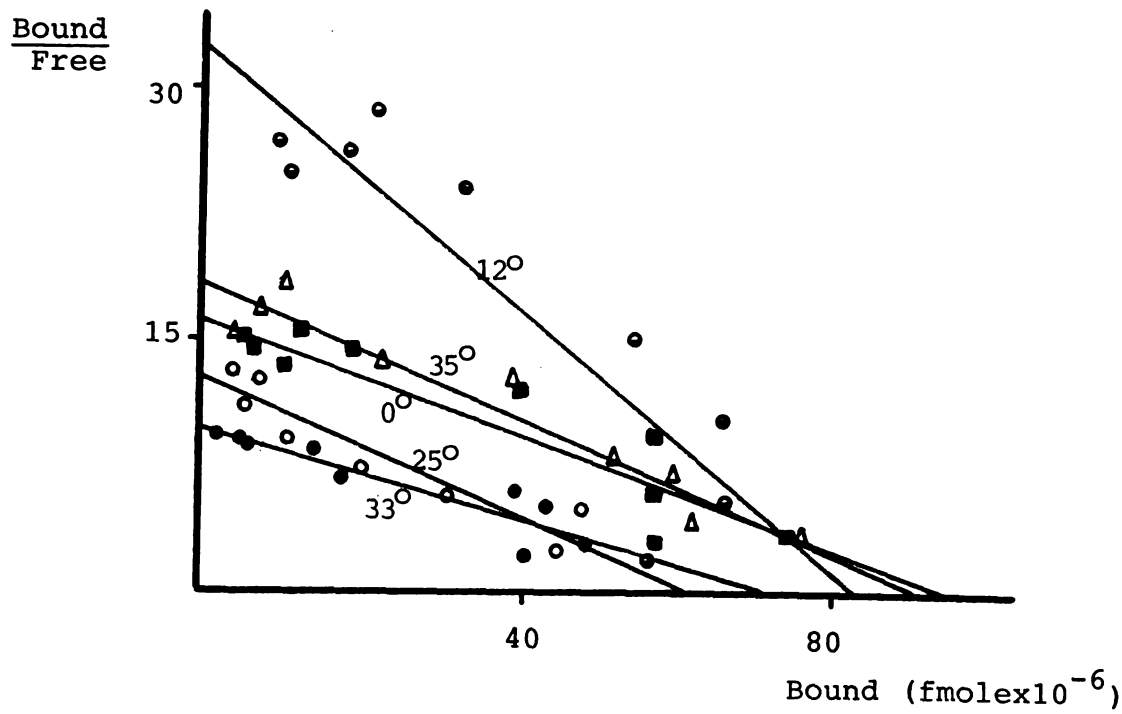


Figure 27a: Scatchard plots of specific ^{3}H-dexamethasone binding by intact HTC cells at 0°, 12°, 25°, 33° and 35°C.

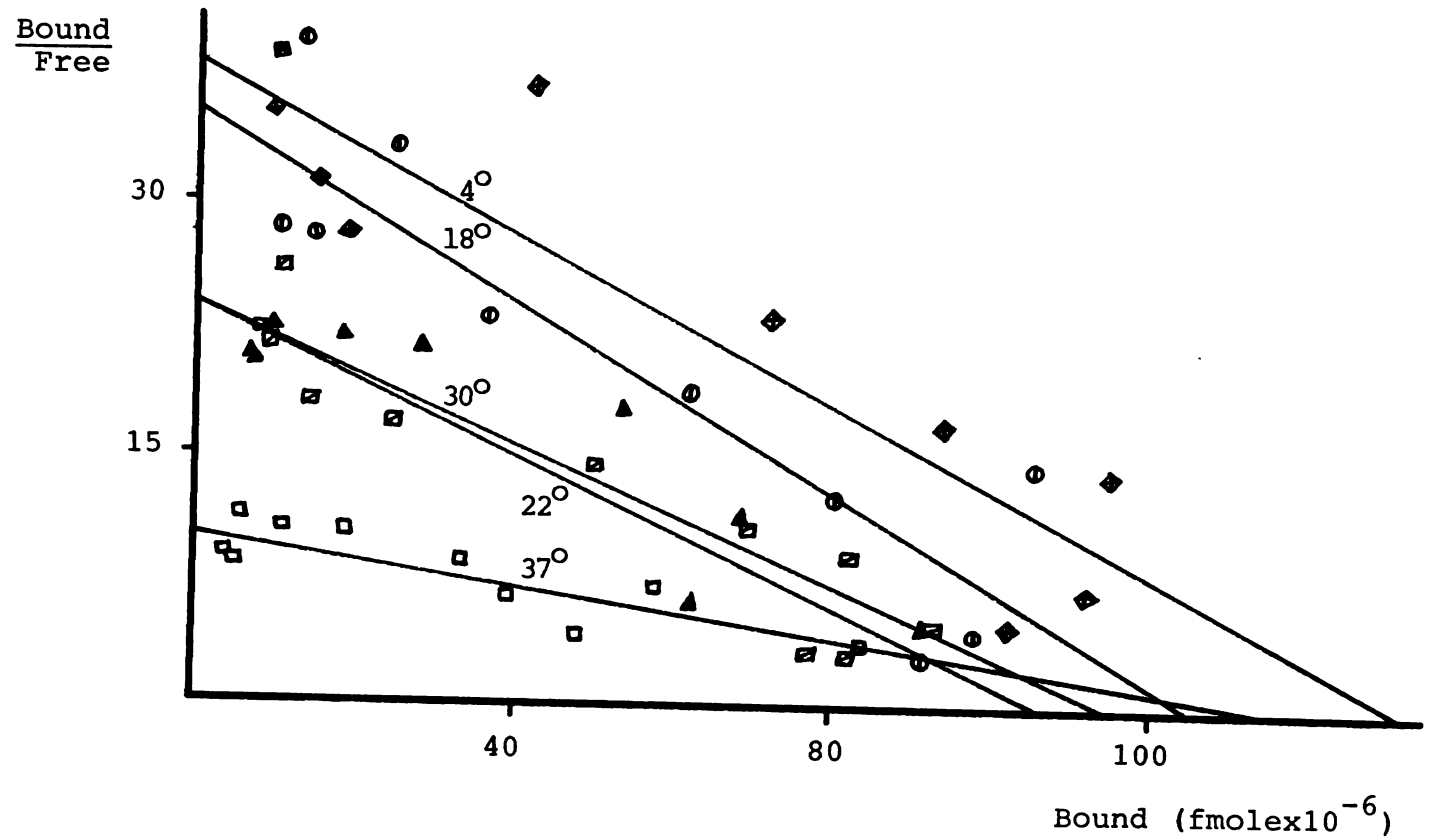


Figure 27b: Scatchard plots of specific ^{3}H-dexamethasone binding by intact HTC cells at 4°, 18°, 22°, 30° and 37°C.

a curvilinear one with an optimal affinity value at 12°C, as with isolated cytosol. The fit curve can be expressed as the following equation:

$$\ln K_A = -212.2 + 133.6\ X - 19.24\ X^2 \quad \text{where } X = \frac{1}{T} \times 10^3$$

The correlation coefficient of the fit is R = 0.7 - 0.9

Thermodynamic values of the steroid-receptor interaction in the intact cells, calculated from the Van't Hoff plot of Figure 28 (Sections 3.3.2.1, 3.3.2.2, 3.3.2.3 and 3.3.2.4 for the cytosol study), are summarized in Table 7.

The free energy change (ΔG) of the system is also a curvilinear relationship with temperature (shown in Figure 29) and the optimum free energy change occurs at 33°C. All of the free energy changes favor the binding of ^{3}H-dexamethasone with the cell ($\Delta G < 0$). The enthalpy change (ΔH) is linearly related to temperature (Figure 30) and decreases as temperatures increase. At low temperatures for 0° to 15°C the enthalpy changes of the system are positive ($\Delta H > 0$), whereas at higher temperatures for 15° to 37°C the enthalpy is negative ($\Delta H < 0$) (shown in Table 7). Similarly, the entropy change (ΔS) is positive ($\Delta S > 0$) at lower temperatures from 0° to 22°C and negative ($\Delta S < 0$) at higher temperatures (from 30° to 37°C), and the entropy decreases linearily as temperatures increase (shown in Table 7 and

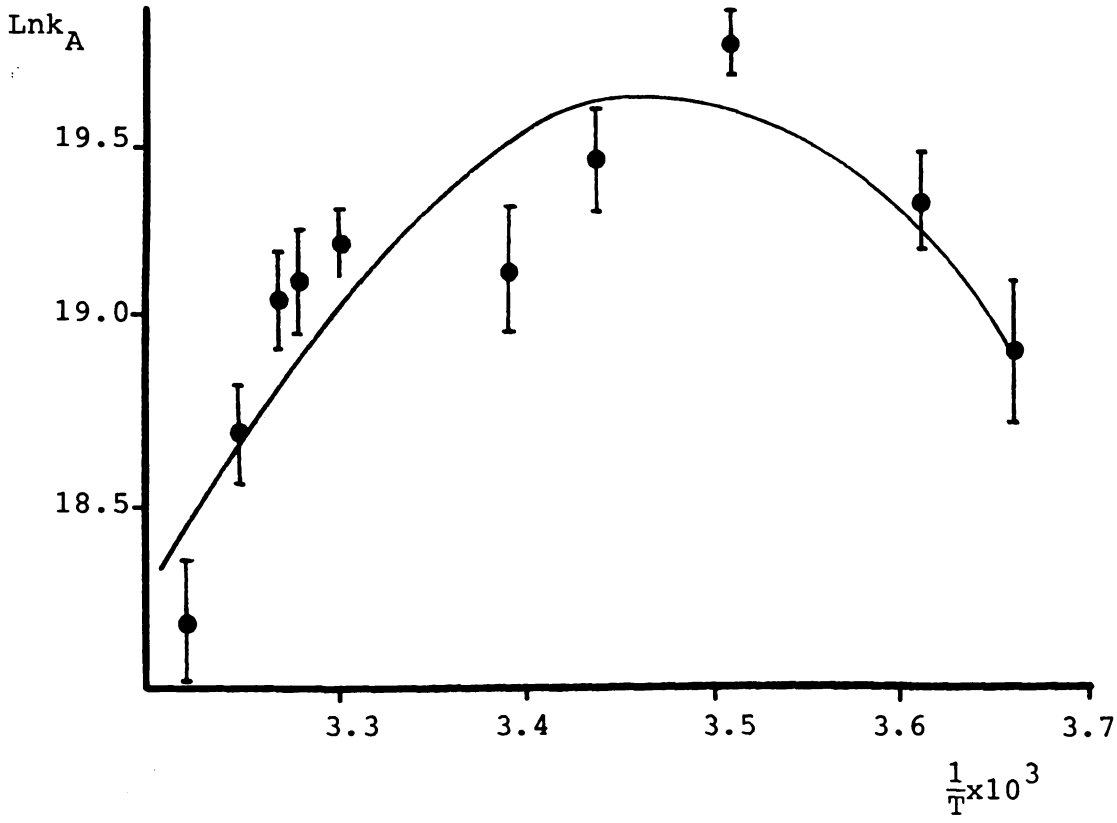


Figure 28 : Van't Hoff plot of ^{3}H-dexamethasone binding to intact HTC cells

Temp. ^{o}C	0	4	12	18	22	30	32	33	35	37	Unit
ΔG	-10.30	-10.65	-11.20	-11.26	-11.28	-11.58	-11.59	-11.65	-11.45	-11.20	$Kcal.Mol^{-1}$
ΔH	14.40	10.50	2.70	-2.70	-6.30	-13.10	-14.8	-15.60	-17.20	-19.20	$Kcal.Mol^{-1}$
ΔS	90.30	76.50	49.00	29.00	16.80	-6.30	-10.60	-13.00	-18.00	-26.00	e.u.
ΔC_p	-1000	-990	-940	-900	-870	-830	-820	-810	-800	-790	$Cal.Deg^{-1}$ Mol^{-1}.

Table 7: Thermodynamic analysis of the interaction of dexamethasone with intact HTC cells.

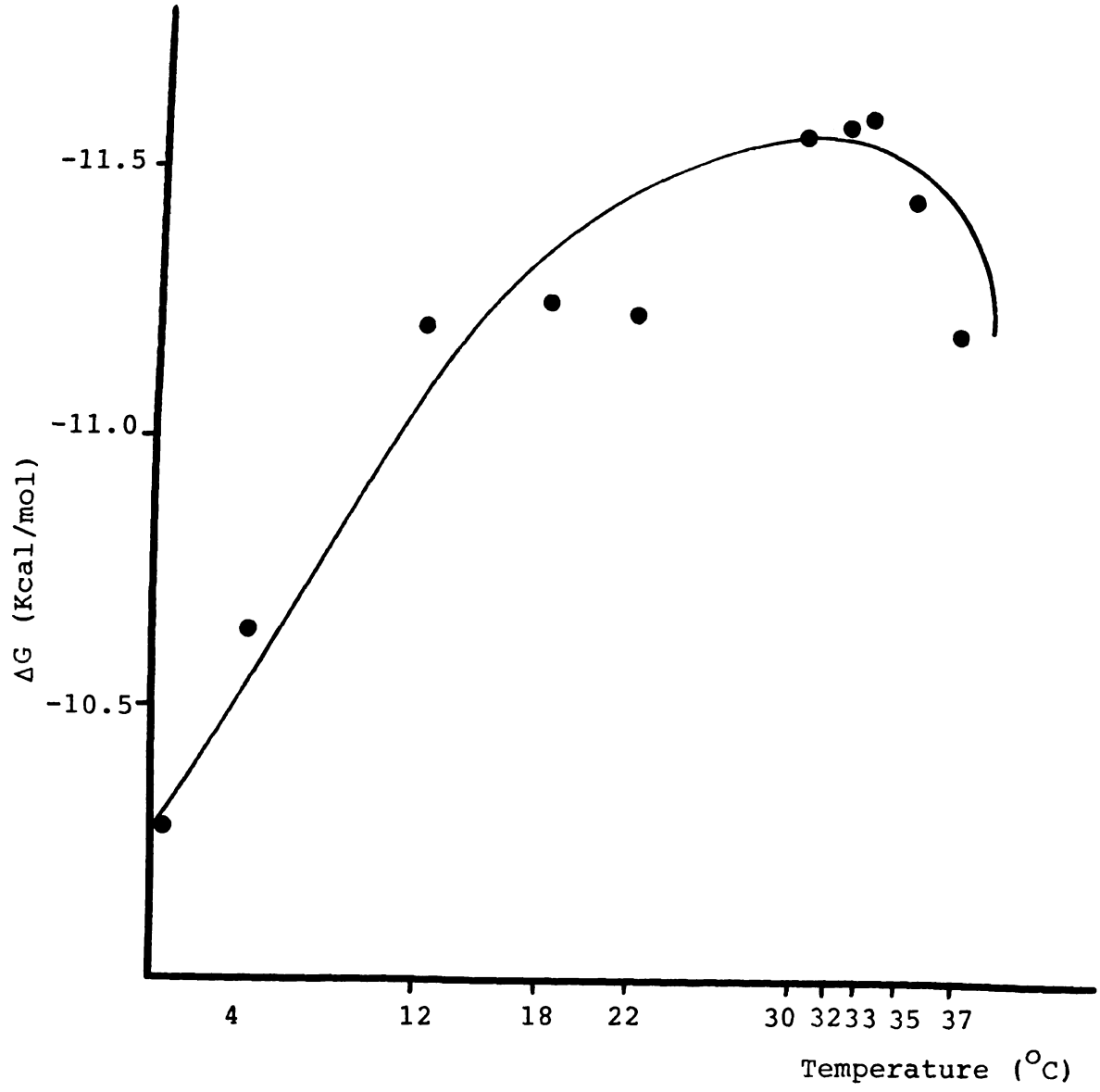


Figure 29 : Variation of the apparent free energy changes (ΔG) of glucocorticoid receptor binding with temperatures in intact HTC cells.

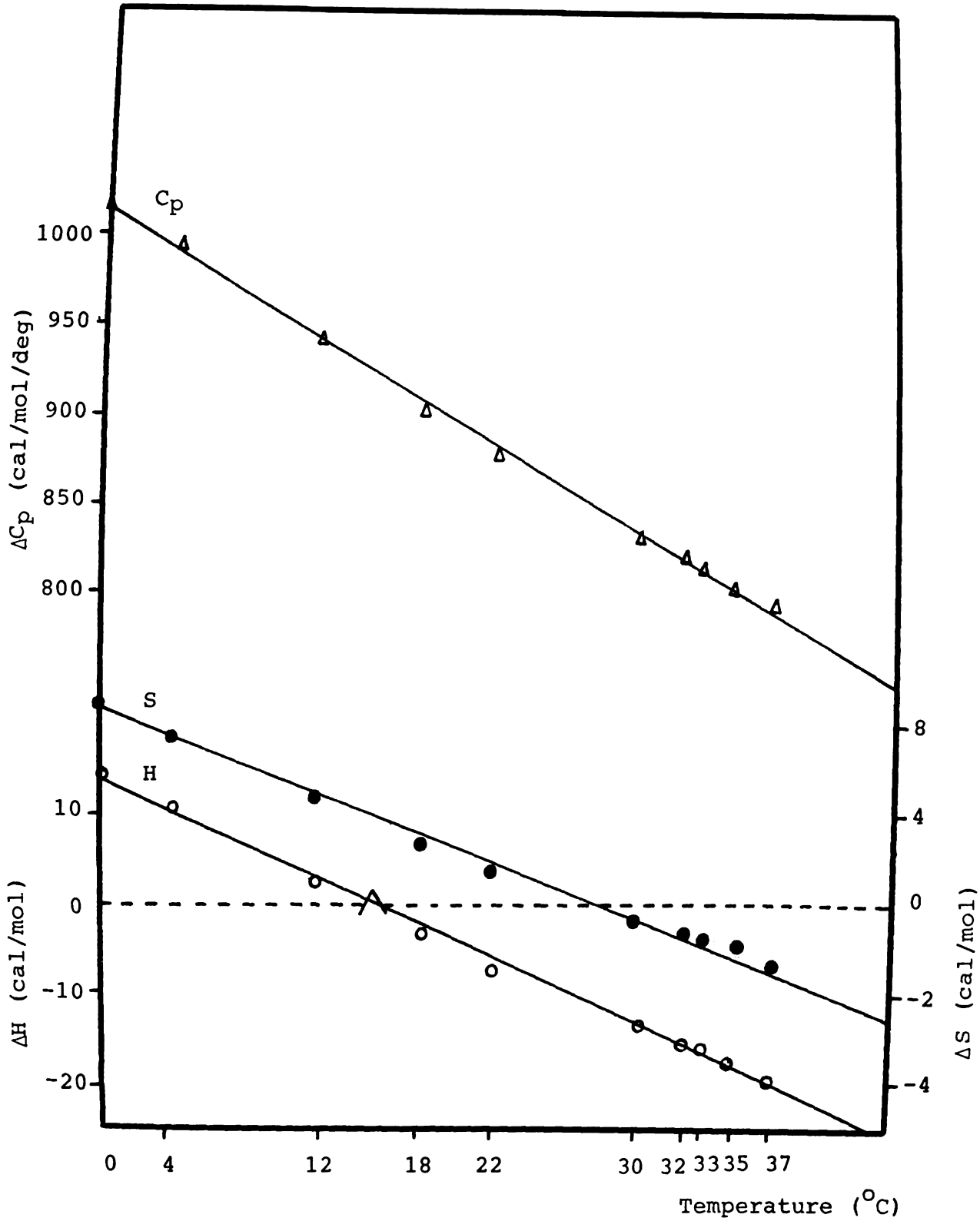


Figure 30 : Variation of the enthalpy change (ΔH), entropy change (ΔS) and heat capacity change (ΔC_p) with temperatures in ^{3}H-dexamethasone interaction with intact HTC cells.

Figure 30). There also exists a large heat capacity change (ΔC_p) from the interaction. The heat capacity change decreases slightly (and linearily) as temperature increases (Table 7). Finally, the above thermodynamic parameters should be considered as "apparent", because they reflect the overall binding process and could be due to influences of several of the steps (Section 1-7) in the hormone receptor interaction in the cell.

5.0 Effect of temperature on nuclear binding of the hormone-receptor complex measured in intact cells

Nuclear binding of the activated glucocorticoid hormone-receptor complex is the final step in the early events of glucocorticoid hormone action. With the present methodology, it is not possible to obtain an affinity of the complex for the nuclear acceptor measured with the use of crude cytosol in a cell-free system, because, as pointed out by Simons et. al. (70), there exist inhibitor proteins in a reconstitutional experiment that will not allow a correct interpretation of the affinity.

These studies were performed to ask whether factors operative in the cell have a major effect on the nuclear binding. First, comparing the relationship of temperature to nuclear binding as related to the same effect on isolated cytosol binding, it will be possible to assess whether forces are operative to increase or decrease the overall process. Secondly, the relationship between cytosol and nuclear binding

might differ at various temperatures and this might provide a better indication of the total content of acceptor sites. Thus, in the present study, the hormone was incubated with whole cells at various temperatures for a time to allow for maximum binding to occur, then the cells were fractionated and the amount of hormone-receptor complex accumulated in various fractions was measured.

5.1 Materials

These are described in Section 4.2.

5.2 Methods

Cells at 5 x 10^6 to 8 x 10^6/ml were incubated in S77 medium with 10% CO_2 (pH 7.4 - 7.6) and radiolabeled glucocorticoid concentrations from 2 x 10^{-8}M to 3 x 10^{-8}M in the presence and absence of non-radiolabeled dexamethasone (Section 2.1.2). At various temperatures (4°, 12°, 22°, 25° and 37°C), 300 µl of the incubated mixture were taken at various times to measure the specific binding of radiolabeled dexamethasone by intact cells as described in Section 4.2.3. At the same time equal amounts of cells were fractured by quickly freezing the cell suspensions at -70°C in dry ice and ethanol for 3 min followed by quickly thawing the frozen cells in flowing water at room temperature (20°C). The process was repeated one more time and then the lysed cells were spun at 20,000 rpm for five min in an Eppendorf centrifuge. The supernant medium was aspirated, and the pellets were washed four times with 300 µl of PBS and then recollected by centrifugation at 20,000 rpm for 2 min each time. The final pellet

was collected in a volume of 300 µl of PBS and suspended in 3 ml of Aquasol solution for counting the amount of specific binding of radiolabeled dexamethasone in the nucleus. The incubation times were varied to ascertain the extent of equilibrium of the interactions. Temperatures and extension times of the study are shown in Table 8.

5.3 Results

5.3.1 Effect of temperature on nuclear binding

The amount of specific binding of ^{3}H-dexamethasone with intact cells represents the total hormone binding to the cytoplasmic receptor and the nucleus. The time courses of nuclear binding studied at 4°, 12° and 22°C (Figures 22, 23, 25) show that, at various temperatures, the amount of glucocorticoid-receptor complex activated and bound to the nucleus varies. For example, 45% and 80% of the total complexes are nuclear-bound at 4° and 22°, respectively (Table 8).

5.3.2 Temperature-dependence of the nuclear binding process

Table 8 shows the average amount of nuclear binding of the complex at each temperature, and Figure 31 shows the influence of temperature on nuclear binding. There appears to be an optimum range of temperatures from 25° to 37°C where maximum binding of hormone-receptor complexes to the nucleus is around 80%. These values do not change in a major way even with more prolonged incubation times.

Temp. (^{o}C)	$\frac{\text{Specific nuclear binding}}{\text{Total binding in whole cell}} \times 100$
4	41 (17 hr), 43 (7 hr), 45 (11 hr), 49 (22 hr), 55 (3 hr).
12	67 (18 hr), 72 (6 hr), 78 (12 hr).
22	75 (1 hr 20 min), 77 (12 hr), 82 (6 hr), 84 (3 hr).
25	83 (1 hr 15 min).
37	84 (45 min).

Table 8: Variation of the amount of ^{3}H-dexamethasone -receptor complex binding to the nucleus with temperature. Hours in the parenthesis indicate the time of incubation.

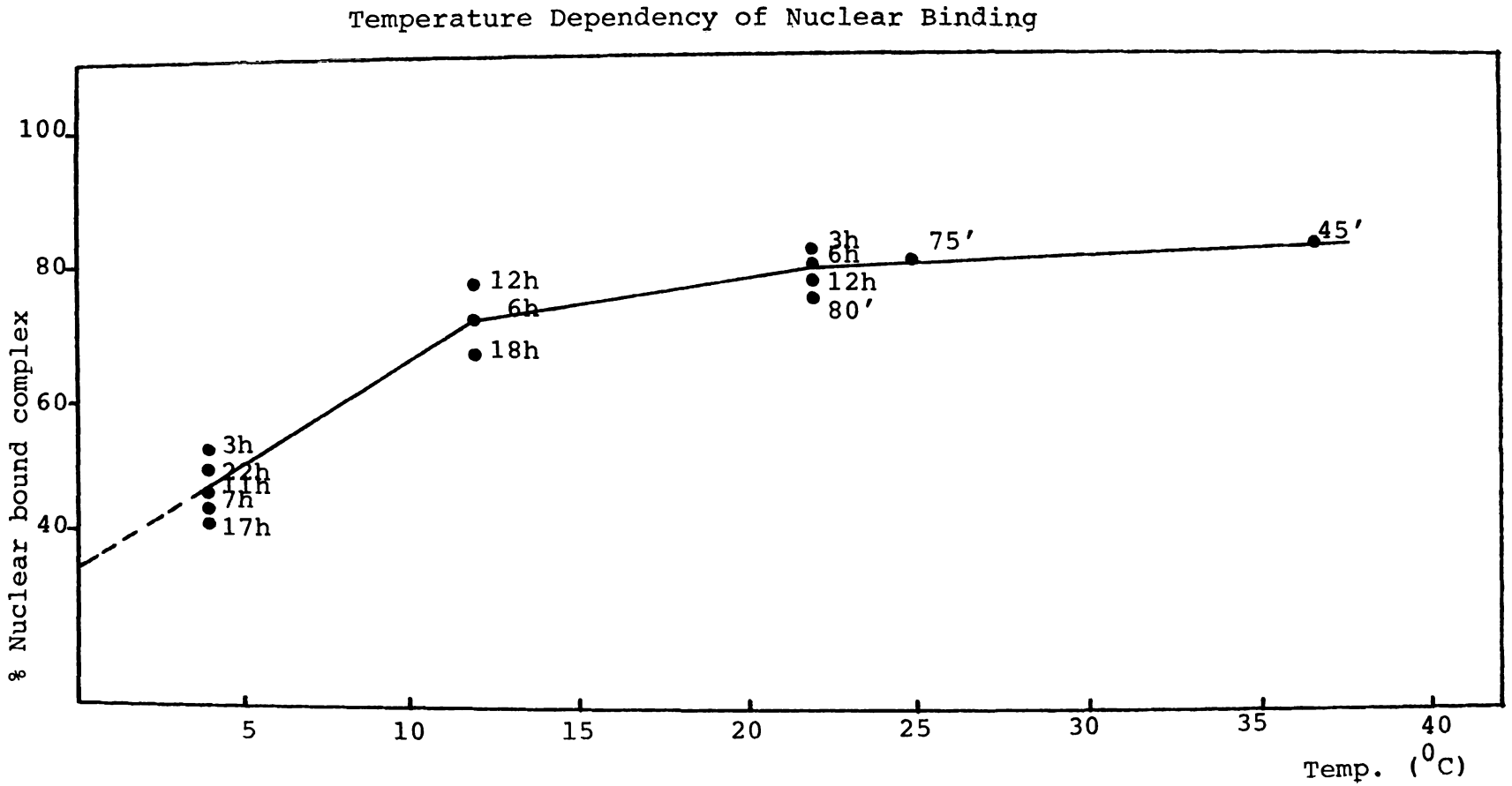


Figure 31: Temperature dependence of nuclear binding of radiolabelled dexamethsone by intact HTC cells.

6.0 Study of radiolabeled dexamethasone association with cytosol and nuclear receptors after incubation of the hormone with intact HTC cells

The "apparent affinity" of the ^{3}H-dexamethasone interaction with receptors in intact cells reflects the combined influences of steroid uptake, hormone binding to cytoplasmic receptors, activation of the complexes and nuclear binding of the complexes. It is therefore of interest to be able to fractionate the cell into cytosol and nuclei after incubation of the hormone with cells and determine the apparent affinity of ^{3}H-dexamethasone association with receptors in these cell components by Scatchard analysis.

6.1 Materials

The cells, buffer solutions, ^{3}H-dexamethasone and non-radiolabeled dexamethasone are the same as described in Sections 3.1 and 4.1.

6.2 Methods

Cells at 5 x 10^6/ml were incubated with varies radiolabeled dexamethasone concentrations from 10^{-7} to 10^{-9} M that were proportionally divided into duplicates of ten tubes (0.5 ml of incubation mixture in each tube) one in the presence and the other in the absence of 4 x 10^{-5} M non-radiolabeled dexamethasone. At 112°C, there were two identical sets of incubations at described by the conditions above. The binding was allowed to reach maximal levels (after 8 hr of incubation). The first set was collected by centrifuging the mixtures

at 20,000 rpm in Eppendorf centrifuge tubes for 2 min; the supernatant was taken to measure the free radioactive hormone and the cell pellet was washed three times, each time with 1 ml of PBS; the last pellet was suspended in 0.5 ml PBS, and 0.3 ml were collected in 3 ml of Aquasol, a scintillation counting solution, to measure the specifically bound ^{3}H-dexamethasone. The second set of incubation tubes was taken to measure the specific binding of ^{3}H-dexamethasone in the cytoplasm and nuclei. The cells were pelleted as above in Eppendorf centrifuge tubes and the supernatant was taken for free hormone measurement. The pellet was then washed with PBS three times, and the final pellet was suspended in 500 ml of PBS buffer. The cells were fractionated by quickly freezing the above cell suspension at -70°C (in dry ice and ethanol solution) for 3 min, followed by a quick thawing of the frozen cells in flowing water at room temperature (20°C). The process was repeated one more time, and then fractionated cells were spun at 20,000 rpm for 5 min in an Eppendorf centrifuge. The supernatant was taken to measure the specific binding of ^{3}H-dexamethasone by the cytoplasmic receptors, as described in Section 3.2.1, and the pellet was washed four times with a volume of 300 µl of PBS buffer, collected by centrifuging at 20,000 rpm in an Eppendorf centrifuge for 2 min each time. The final pellet was collected in a volume of 300 µl of PBS and suspended in 3 ml of Aquosol for counting the specific binding of ^{3}H-dexamethasone in the nucleus.

6.3 Results

6.3.1 Saturation binding of radiolabeled dexamethasone to HTC cell nuclear acceptors compared to cytosol receptors

The specific binding of ^{3}H-dexamethasone-cytosol receptor complex (RS) changes with the concentration of ^{3}H-dexamethasone (S), but the cytosol receptors approach saturation at 1.5×10^{-9}M ^{3}H-dexamethasone (Fig. 32). However, a plot of the specific binding of the hormone-receptor nuclear complex (RNS) as a function of the hormone-receptor complex (RS) concentrations is a straight line (Fig. 33).

6.3.2 Comparative Scatchard analysis of the affinities of radiolabeled dexamethasone binding to intact HTC cells, cytosol and nucleus

Plotting of the ratio of specifically bound labeled ^{3}H-dexamethasone in the whole cells, cytosol, and nuclei to the free ^{3}H-dexamethasone concentration versus the correspondent specific binding gave three parallel Scatchard plots, indicating similar apparent affinities of the steroid for the cells, cytosol, or nuclei. This also shows that the extent of relative saturation of cytosol nuclear binding is similar over wide ranges of steroid concentrations. The results are shown in Figure 34 for an experiment in which the incubations with steroid were conducted at 12° and the affinities derived from the slopes of the plots for all three cases are around K_d = 4.5 nM. Results similar to this for experiments conducted at 37°C were also reported by Bloom et al. (68).

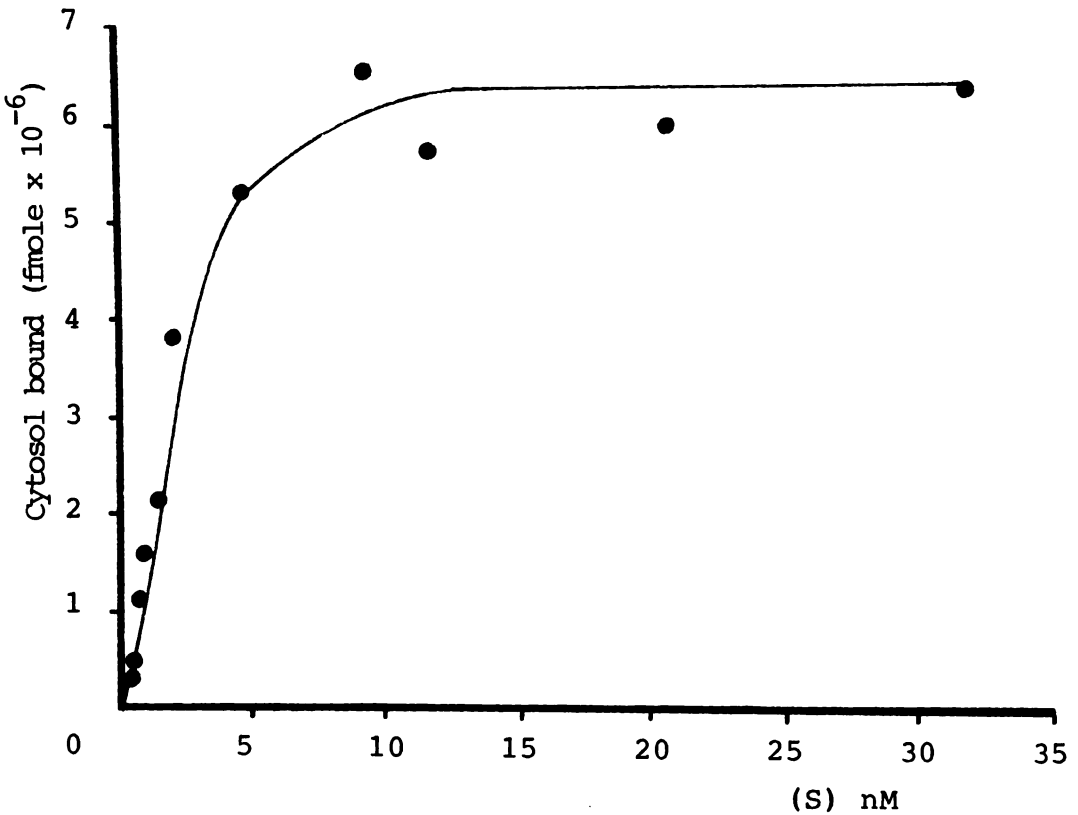


Figure 32 : The cytosol receptors are saturated at ^{3}H-dexamethasone concentration as low as 15 to 35 nM

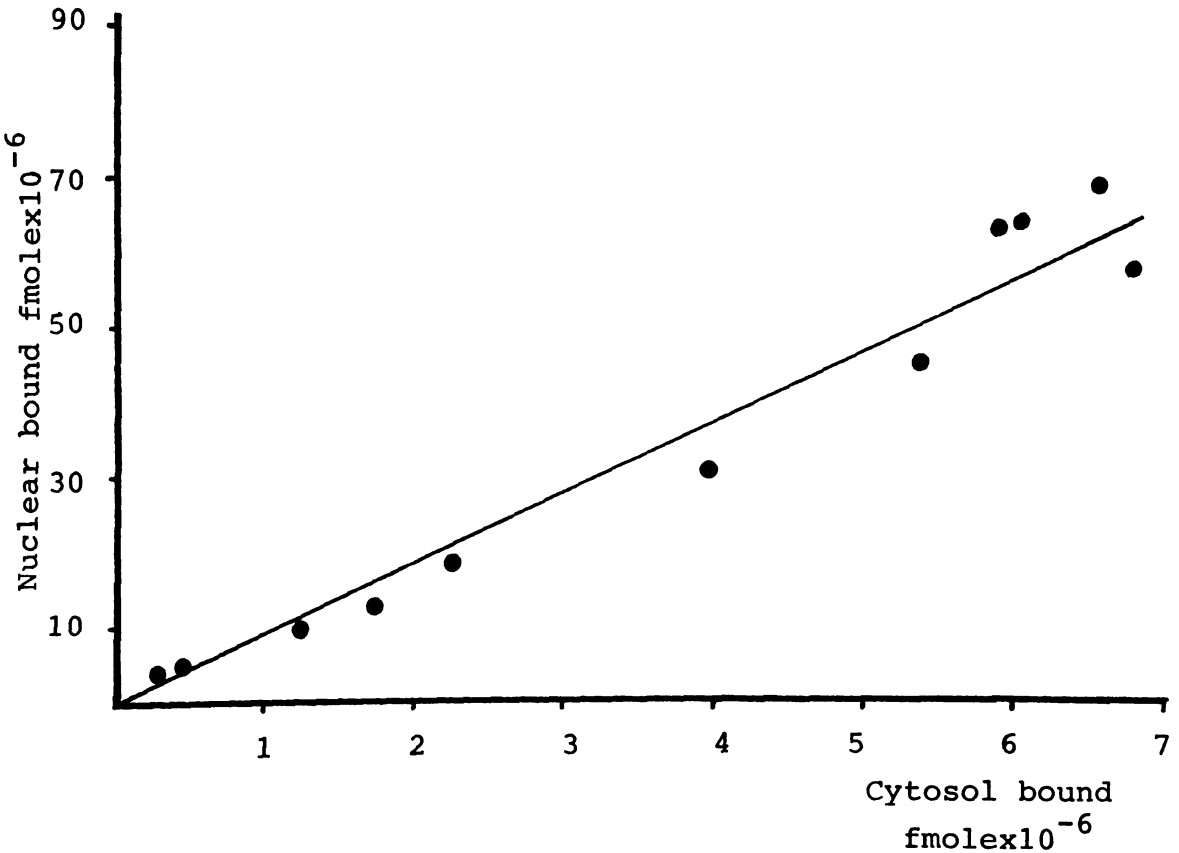


Figure 33 : Nuclear acceptor sites are far from being saturated with receptor -dexamethasone complex in HTC cells

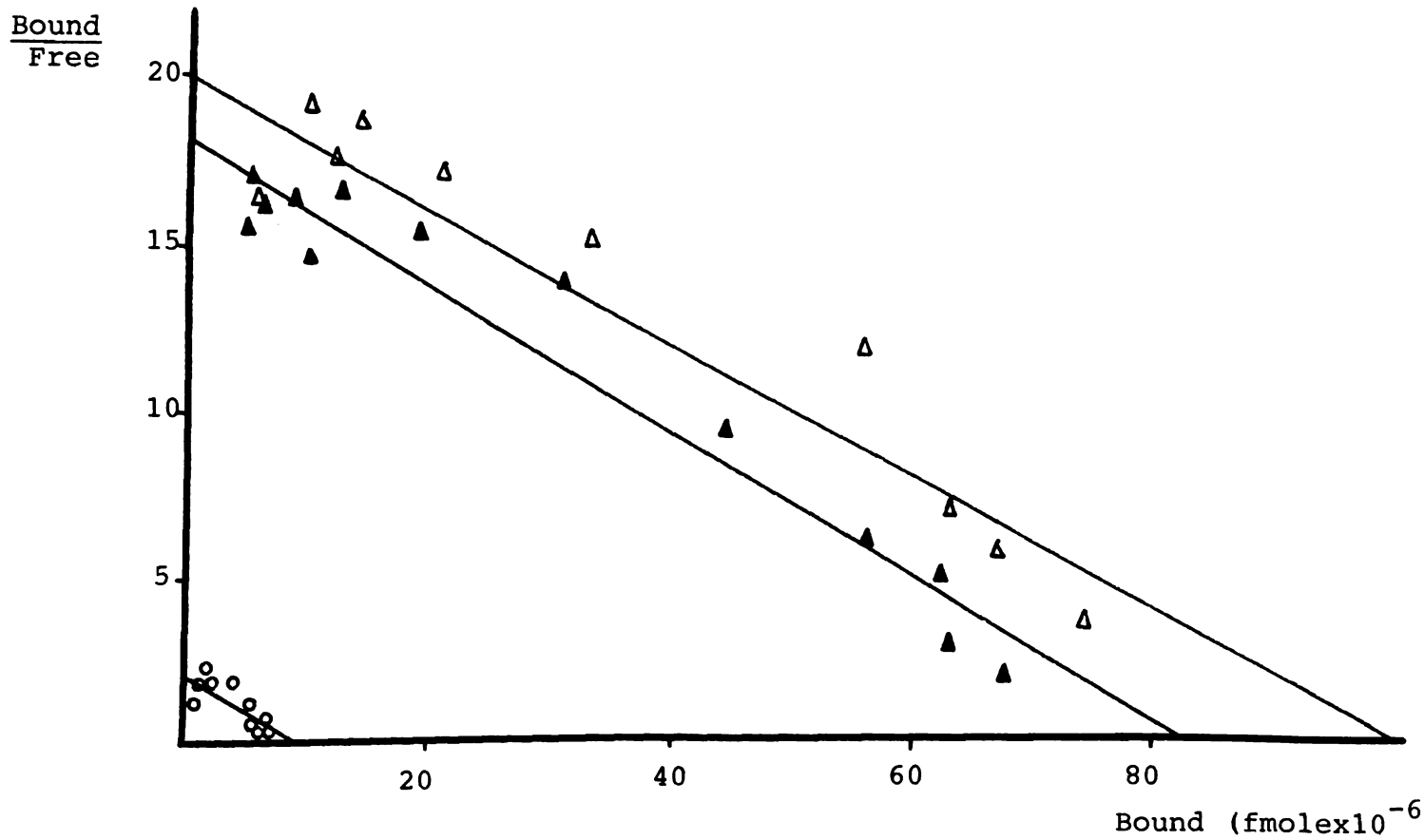


Figure 34 : Scatchard plots of ^{3}H-dexamethasone binding to intact HTC cells (Δ), the nucleus (▲) and cytosol (o) at 12°C .

6.3.3 Relationship between cytosol and nuclear binding: Assessment of the content of nuclear acceptor sites

Plotting the ratio of nuclear to cytoplasmic binding versus the nuclear binding allowed us to obtain a Scatchard plot of the nuclear binding of the ^{3}H-dexamethasone complex (Fig. 35). Within the range of cytosol complex concentration obtainable, the line appeared to be parallel to the abscissa; there is no tendency of the curve to approach the abscissa; the proportion of total receptors bound by the nucleus (70 to 80% of the total receptors) is constant at all relative saturations of the receptor by the steroid. These data suggest that the nuclear acceptor concentration exceeds by several-fold the number of the cytosol receptors. Findings of E. Bloom et. al. (68) who measured binding at 37°C agree with this result.

7.0 Discussion

7.1 Thermodynamic analysis of glucocorticoid hormone interactions with HTC cell cytosol receptors - The hydrophobic effect

One of the basic and interesting problems of glucocorticoid and receptor interactions is the nature of the forces that bring about the interaction of the hormone and receptor. As mentioned in the Introduction there has been increasing interest in using thermodynamic analyses to study the nature of hormone-receptor interactions in glucocorticoid-responsive systems. Koblinsky et al. (1972) (69) and Schaumberg and Jogensen (1968) (84) found a linear relationship between the corticosterone affinities for rat thymocyte receptors and temperature.

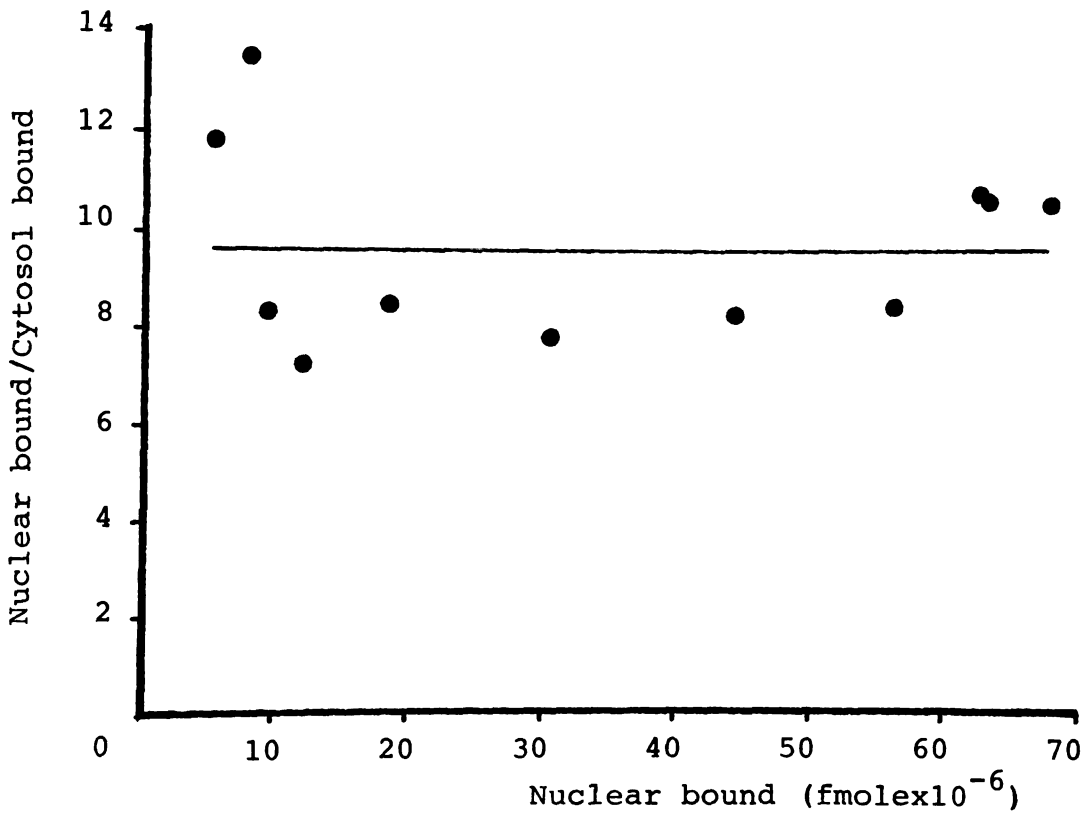


Figure 35 : Scatchard analysis of ^{3}H-dexamethasone receptor complex binding to nuclei performed in intact HTC cells.

The consistently negative entropy change of the interaction (ΔS = -18 e.u.) they obtained led them to the conclusion that the binding is not primarily driven by hydrophobic interactions and that it involves mainly changes in the conformation of the receptor molecule. However, the latter group's study was limited by analyses at only four temperatures (from 4° to 37°) (84). A hydrogen-binding model was also proposed by Mornon et. al. (1977) (71a). A hydrophobic effect as the major force of glucocorticoid receptor interaction was obtained by Rousseau and Schmidt (1977) and by Wolff et. al. (72) who studied the interaction with HTC cell cytosol receptors. However, this work was done before the finding of the receptor stabilization effect of dithiothreitol (DTT) and sodium molybdate and, as a consequence, did not exclude the possibility of slight receptor degradation, which would result in overestimation of hormone affinity. In the present report we have studied these interactions under more stabilized conditions by using sodium molybdate, DTT, and a more potent glucocorticoid.

Most recently Mickelson and Westphal have studied the influence of temperature on the binding of cortisol to CBG and obtained findings similar to ours, although they drew different conclusions (73). By their analysis, there are two phases in the temperature dependence of cortisol binding to guinea pig CBG, described as the Van't Hoff plot. The enthalpy change (ΔH) is negative ($\Delta H < 0$) for all temperatures exa-

mined from 4°C to 41°C, and the entropy change is positive ($\Delta S > 0$) at low temperature (4°C) and negative ($\Delta S < 0$) at high temperature (37°C). They conclude that the binding process is not driven by hydrophobic force. However, their conclusion is based on a notion by Kauzman (1959) on hydrophobic interaction, by which the enthalpy change of the process would be positive ($\Delta H > 0$) or nearly athermal and the entropy change ΔS would also be positive ($\Delta S > 0$). However, this is no longer an appropriate definition for hydrophobic interaction (discussed in following sections).

We undertook to examine the Van't Hoff plot of the Westphal data with the PROPHET computer. There are three possible ways to fit their binding data: the single least-squares fit, the biphasic least-squares fit, and the polynomial fit. Our analysis shows that the data are best fitted by a second-degree polynomial, with a correlation coefficient of $R^2 = 0.988$, whereas the least-squares fit for the biphasic plot gives $R^2 = 0.966$. From the polynomial fit of their data, we obtained thermodynamic results similar to those of our present study of the dexamethasone interaction with glucocorticoid receptors in HTC cells, by which both the enthalpy and entropy changes of cortisol binding to CBG are temperature-dependent and a large negative heat capacity change (ΔC_p) in the binding from -800 to -1,000 cal $deg^{-1}mol^{-1}$ is obtained. Figure 36 and Table 9 describe the above results. These are thermodynamic characteristics of a hydrophobic interaction, as will be discussed more extensively in the following sections.

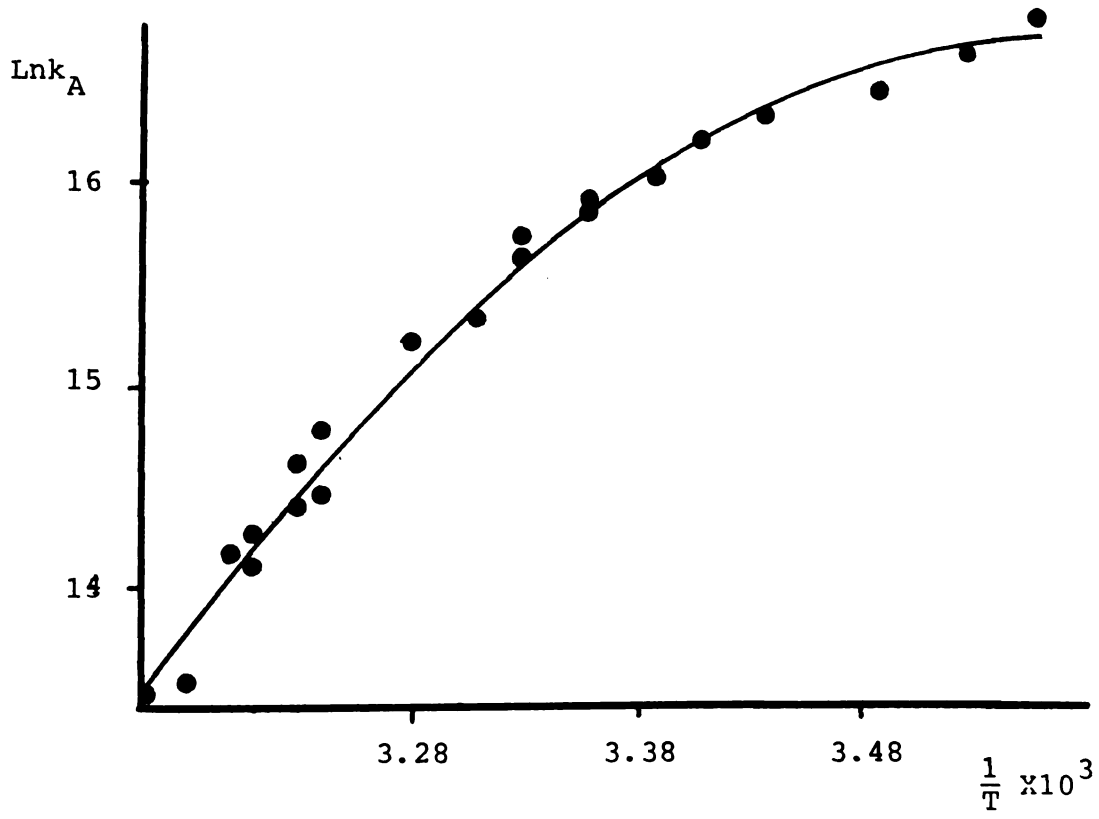


Figure 36: Van't Hoff plot of the Westphal data of cortisol binding to CBG but using polynomial fitting. The correlation coefficient of the fit is R^2 = 0.988 obtained by PROPHET computor analysis.

No.	$\ln K_A$	$\frac{1}{T} \times 10^3$	Temp. T/°C	$-\Delta G$ (Kcal.mol^{-1})	$-\Delta H$ (Kcal.mol^{-1})	$-\Delta S$ (e.u.)	$-\Delta C_p$ (cal.deg^{-1}.mol^{-1})
1	13.47	3.18	314/41	8.40	31.21	72.64	779
2	13.53	3.20	312.5/39.5	8.40	29.90	68.80	786
3	14.11	3.23	309.5/36.5	8.67	27.60	61.15	801
4	14.12	3.22	310.5/37.5	8.74	28.37	63.22	796
5	14.22	3.23	309.5/36.5	8.77	27.60	60.80	801
6	14.40	3.25	307.7/34.7	8.80	26.07	56.10	811
7	14.46	3.26	306.7/33.7	8.81	25.30	53.76	816
8	14.62	3.25	307.7/34.7	8.94	26.27	56.30	811
9	14.78	3.26	306.7/33.7	9.01	25.30	53.10	816
10	15.22	3.30	303/30	9.16	22.30	43.35	836
11	15.33	3.33	300/27	9.14	19.92	36.00	853
12	15.63	3.35	298.5/25.5	9.27	18.39	30.33	862
13	15.76	3.35	298.5/25.5	9.34	18.39	30.33	862
14	15.86	3.38	295/23	9.30	16.09	23.00	882
15	15.92	3.38	296/23	9.36	16.09	22.70	876
16	16.03	3.41	293/20	9.33	13.78	15.20	894
17	16.21	3.43	291.5/18.5	9.39	12.25	9.80	903
18	16.33	3.46	289/16	9.38	9.95	-1.97	919
19	16.44	3.51	285/12	9.31	6.11	-11.24	945
20	16.62	3.55	282/9	9.31	3.04	-22.25	965
21	16.80	3.60	277/4	9.25	0.80	-30.50	1,000

Table 9: Thermodynamic analysis of cortisol binding by CBG from the data of Westphal (73).

7.1.1 Free-energy changes of the interactions

As the hormone interacts with the receptor to form a complex (RS): $R + S \rightleftarrows RS$; in thermodynamic terms, we are looking at the change of free energy (ΔG) of the system from reactants (R + S) to product (RS).

As in the case of any polyatomic molecule, steroid and receptor possess their energy ($G^{\bullet}$) from four degrees of freedom: translation, rotation, stretching, and bending. Upon accommodation to form steroid-receptor complex (RS), the molecules have to constrain their free movements, resulting in a loss of free energy (ΔG) to form a more stable product RS:

$$\Delta G \text{ system} = \Delta G_{(RS)} - \Delta G_{(R + S)}$$

The free energy change of the system was obtained from the relationship of affinity of steroid and receptor at a certain temperature, as expressed by the following equation:

$$\Delta G = -RT \ln K_A \qquad (1)$$

Where R: gas constant (1.987 cal $K^{-1}mol^{-1}$)

T: temperature degrees Kelvin

K_A: equilibrium constant of the association of steroid and receptor.

We obtained K_A values at temperatures of -2°, 4°, 8°, 10°, 12°, 14°, 18°, 22° and 25°C by plotting the binding data using the Scatchard technique; the data were obtained by measuring dexamethasone-receptor binding by HTC cell cytosol at concentrations of radiolabeled dexamethasone from 5×10^{-8} M to 5×10^{-10}M. For these experiments, 3 mM DTT and 10 mM Na_2MoO_4 were added at 0°C before the incubation to block the activation of the receptor-steroid complex and to minimize receptor protein degradation as mentioned previously (3.3.1). Knowledge of the free energy changes cannot by itself reveal any information regarding the nature of the interactions between the steroids and the receptors. To obtain such information, we studied the changes of enthalpy (ΔH), entropy (ΔS), and heat capacity (ΔC_p) of the system.

7.1.2 The enthalpy change (ΔH) of the interaction

In a chemical reaction, the net change from rearrangement of bonds in reactants to form a new product is associated with a change of heat in the system under a constant pressure, as expressed by:

$$\Delta H = \int_{Ti}^{T\delta} \Delta C_{p\delta T} \qquad (2)$$

The enthalpy change (ΔH) of the system is the product (expressed as cal/mol of the heat capacity with the change of temperature (ΔT). A negative enthalpy change ($\Delta H < 0$) implies an exothermic reaction releasing heat into the surroundings. By contrast, a reaction associated with a positive enthalpy change ($\Delta H > 0$) absorbs heat from the surrounding (endothermic reaction). Although the enthalpy change of a reaction, in principle, could be measured directly by a calorimeter, in our system, it was necessary to calculate the heat change (enthalpy) of the steroid-receptor interaction from a Van' Hoff plot, in which the $\ln K_A$ is plotted versus $\frac{1}{T}$. Unlike many simple association reactions, the enthalpy change (ΔH) in our study exhibited a marked temperature dependence (Figure 18), as shown by the curvilinear slope of the Van't Hoff plot that could be expressed as:

$$\ln k_A = -142.1 + 92.2 \left(\frac{1}{T} \times 10^3 \right) - 13.11 \left(\frac{1}{T} \times 10^3 \right)^2 \quad (3).$$

From this relationship, the enthalpy change (ΔH) is derived as follows:

$$\frac{\partial \ln K_A}{\partial \frac{1}{T}} = 92.2 \times 10^3 - 26.22 \left(\frac{1}{T} \times 10^6 \right) \quad (4)$$

since $\Delta G = -RT \ln K_A$

$$\frac{\partial \ln K_A}{\partial \frac{1}{T}} = \frac{\partial \frac{\Delta G}{T}}{\partial \frac{1}{T}} * \frac{1}{T} \qquad (5)$$

$$\frac{\partial \ln K_a}{\partial \frac{1}{T}} = -\frac{\Delta H}{R} \qquad (6)$$

According to equation (6), the enthalpy change (ΔH) of the system could be obtained by multiplying (-R) by the slope of the second-degree polynomial least-squares fitted to the data points of the Van't Hoff plot expressed by equation (3). The curve (Figure 16) provides a good fit to the data points with a correlation coefficient R = 88, and the decreasing slope with increasing $\frac{1}{T}$ indicates that the enthalpy change decreases as the temperature increases. Thus, the enthalpy change is not characteristic of either an endothermic or exothermic reaction. This implies that there exists interference of a third component in addition to the hormones and receptors themselves. At high temperatures (14°-25°C) our studies showed a negative enthalpy change ($\Delta H < 0$) from -1.9 Kcal to -8.7 Kcal per mole, which drives the reaction to favor the formation of product (RS). At low temperature (-2° to 10°C), the enthalpy change is positive, ($\Delta H > 0$) which is not favorable for the product formation of hormone receptor complex.

7.1.3 Entropy changes (ΔS) of the interaction

Entropy is a measurement of the disorder of a system. It is a state function which means the change depends only on the final and

initial states of the system, the more disorder the higher the entropy. The entropy change in our system was obtained from the energy change (ΔG) and enthalpy change (ΔH).

$$\Delta S = \frac{\Delta H - \Delta G}{T}$$

Our results show an increasing entrophy change (ΔS) as temperature decreases. This confirmed the fact that there were more than two components participating in the system because the binding of steroid to receptor would decrease freedom in the system thus decreasing entropy. At high temperatures ranges (18°-25°C), the entropy changes (ΔS) were from 24.2-9.9 e.u., at lower temperatures (18° to -2°C) the contribution of the entropy change (ΔS) was larger than that at higher temperatures (24 to 72 e.u.). This phenomenon can be explained by the role of water molecules in the system. X-ray crystallography shows that in ice, water molecules hydrogen bond to one another such that the lone electron pair of the oxygen atom interacts with the hydrogen atom of a neighbor water molecule and the two hydrogen atoms interact with the two other oxygen atoms of neighbor water molecules in a tetrahedral structure (Figure 37). The hydrogen bond enthalpy is small, 1-2 Kcal/mole compared to 100 Kcal/mole in a covalent bond. In the aqueous state, water molecules form a mobile network through hydrogen bonds of each molecule with four

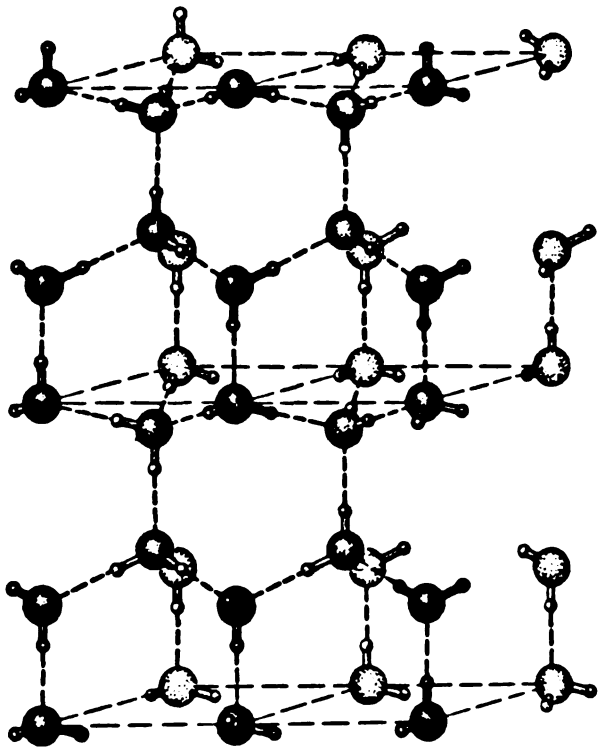


Figure 37: The structure of ordinary ice (115).

tetrahedrally converted neighbor molecules. The network is not a rigid one, and changes of neighbor molecules occur rapidly. When a steroid is introduced into this network, a hole is created, and some hydrogen bonds in the original network are broken. The hydrophobic steroid backbone does not allow it to interact strongly with the surrounding water molecules. As a result these water molecules orient themselves in some way to reform hydrogen bonds that are disrupted by the introduction of the hydrophobic molecule. The result is that the water molecules around the steroid actually become more ordered with a lower entropy or constrained in higher free energy. Since there is little change in the number of hydrogen bonds, the enthalpy change (ΔH) is small (the maximum change is 9 Kcal/mole), but the rearrangement of water molecules around the hydrocarbon molecule, however, is associated with a negative entropy change ($\Delta S < 0$).

Let us now consider the case where a receptor protein is introduced to the system. The hydrophobic domains of the receptor would have effects on the water in the system similar to those of the steroid, and the net result is that the system will be at higher free energy and lower entropy. This important clue was observed by Frank and Evans (1945) and reinstated by Charles Tanford (74). As the steroid and receptor associate, the number of water molecules at the accessible area between the two molecules will be released, favoring the increasing entropy and decrease of free energy of the system as an

overall result (Figure 38). Thus, the association of the steroid and receptor is not due to attraction but to repulsion of both by water. Such hydrophobic interactions are important in many biological systems. Other examples are the self-association of glucagon (75), the formation of the protein-protein interface between insulin and its receptor (76), the formation of the trypsin PFI complex and the association of α and β oxyhemoglobin (77).

It should be noted that the term "hydrophobic interaction" is used to describe the combined effects of London, Van der Waals, and hydrogen binding interactions of processes in aqueous solutions. It is not a "force" different from those effects. Hydrophobic interactions in our case, are characterized by a low enthalpy change and are entropy driven at low temperatures. Water is a network whose mobility depends on temperature. At low temperatures, there is less mobility and more hydrated water surrounds the surface area of steroid and receptor. Upon asosociation, these constrained hydrated water molecules are released, resulting in large increases in entropy. At high temperatures, there is thermal displacement of the hydrated water resulting in less hydration at the surface area (Figure 39). The entropy change observed at high temperature is thus less than that at low temperature.

There is a difference between the observed value and net entropy change. The net entropy change is larger than the observed value,

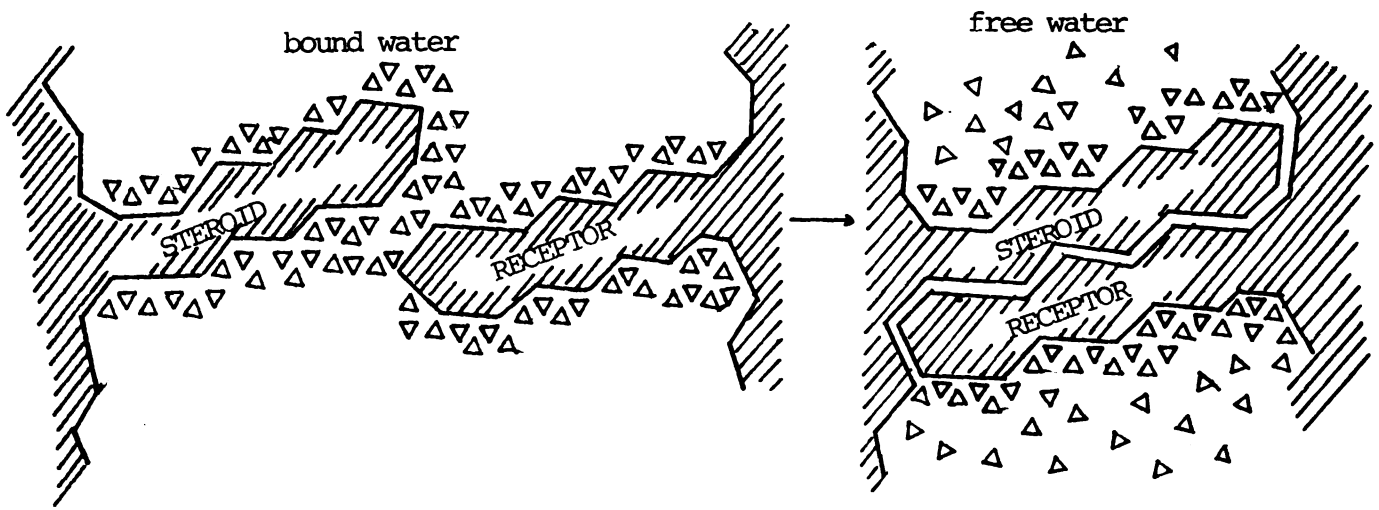
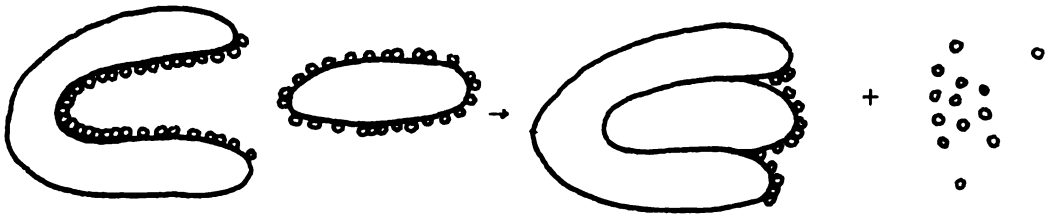


Figure 38: Schematic representation of hydrophobic interaction. Releasing of water molecules increases entropy of the system.

$$R_{H_2O} + S_{H_2O} \rightleftharpoons RS_{H_2O} + nH_2O$$

$$\Delta G = \Delta H - T\Delta S$$

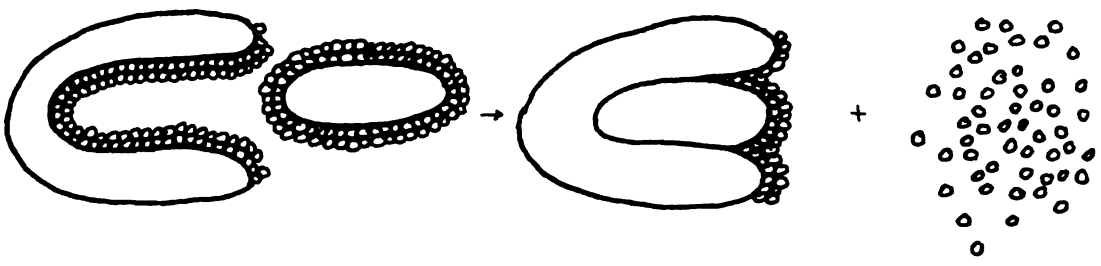
At high temperature



ΔG: negative
ΔH: negative
ΔS: increases
ΔC_p: negative

Reaction is both enthalpy and entropy driven

At low temperature



ΔG: negative
ΔH: positive
ΔS: increases
ΔC_p: negative

Reaction is entropy driven

Figure 39: Model of hydrophobic interaction of glucocorticoid hormone and receptor at high and low temperatures.

because there is a standard entropy change of -45 e.u. at 25°C due to the loss of translational and rotational freedom as free steroid and receptor combine (ΔS_{RS}) (77).

$$\Delta S_{observed} = \Delta S_{RS} + \Delta S_{H2O}$$

For example, at 25°C, the observed entropy change of the glucocorticoid hormone-receptor interaction ($\Delta S_{observed}$) is 10 e.u.

$$10 \text{ e.u.} = -45 \text{ e.u.} + \Delta S_{H2O}$$

$$\Delta SH_2O = 55 \text{ e.u.}$$

Thus, the entropy change contributed to the system by water as high as 55 e.u. even at 25°C. Similarly this value is more than 120 e.u. at -2°C.

The change of enthalpy is relatively small (-8.7 Kcal to 9 Kcal/mole) compared to the change of entropy upon steroid-receptor association, since the change in the number of hydrogen bonds is small and the enthalpy of hydrogen bonding is weak (1-2 Kcal/mole).

The enthalpy and entropy changes of the steroid and receptor association are temperature dependent and decrease with increasing temperature; this phenomenon argues against the hypothesis that hydrogen bonding plays a major role in driving the association as suggested by Mormon et al. (1977).

The influence of enthalpy change (ΔH) and entropy change (ΔS) on the formation of (RS) is expressed as:

$$\Delta G = \Delta H - T\Delta S$$

Because both ΔH and ΔS are temperature-dependent, it is of interest to compare their contributions at various temperatures at which there are different affinities (K_a) as shown by the curvature of the Van't Hoff plot. As temperature decreases from 25° to 14°C, the enthalpy change is negative and the entropy change (ΔS) increases from 10 e.u. to 33 e.u. (Table 6). Thus, both enthalpy change ($\Delta H < 0$) and entropy ($\Delta S > 0$) change contribute to the free energy change ($\Delta G < 0$) of steroid receptor complex formation. The increasing affinity of steroid receptors at decreasing temperature is due to the increasing role of entropy at lower temperatures. Entropy dominates the driving force as enthalpy becomes more positive at temperatures below 16°C (Figure 16). The contributions of enthalpy and entropy as driving forces for the interaction of hormone and receptor are summarized in Figure 19.

The magnitudes of enthalpy and entropy do not provide any information regarding the hydrophobicity of the binding. Formisano (80) analyzed the effects of nonpolar residues on the thermodynamics of coil-to-helix transition of the polypeptide glucagon. The enthalpy and entropy changes of helix propagation are always positive for valine and negative for alanine, but they are positive at low temperature

and negative at high temperature for leucine. Hydrophobic interactions are therefore characterized by a large temperature dependence of their thermodynamic contents (i.e. enthalpy and entropy) and not necessarily by a large entropy change.

7.1.4 Heat capacity (ΔC_p) of the interaction

The heat capacity change (ΔC_p) is the change of enthalpy (ΔH) with temperature.

$$\Delta Cp = \frac{\delta \Delta H}{\delta T}$$

Because enthalpy change (ΔH) is also a temperature dependent term, as a result, ΔCp calculated from equation (5) of Section 7.1.2 is:

$$\Delta Cp = - \frac{26.22 \times 10^6 \times R}{T^2}$$

Within the range of temperatures in our study we obtained a negative heat capacity change ($\Delta C_p < 0$) from -587 cal. $mol^{-1}deg^{-1}$ to -709 cal. $mol^{-1}deg^{-1}$ as temperatures varied from 25° to -2°C. As indicated by a number of authors (75, 78-80), the heat capacity change (ΔC_p) appears to be the most useful parameter in interpreting the hydrophobic effect. Water molecules surrounding the hydrophobic steroid and receptor have lower enthalpy than free water due to tighter bonding of their reorganized hydrogen bonds. These lower enthalpy water molecu-

les behave like a "heat sink" and acquires a higher heat capacity compared to free water molecules. Consequently, upon binding, it is the variation of heat capacity (ΔC_p) between these hydrated and free water molecules that results in a negative heat capacity change ($\Delta C_p < 0$).

$$\Delta C_{p(system)} = \Delta C_{p(free\ water)} - \Delta C_{p(hydrated\ water)}$$

Lower temperatures favor hydration and therefore increase heat capacity; thus upon binding there will be larger changes in heat capacity at lower temperature than at higher temperatures. The magnitude of the heat capacity change from -600 to -800 cal $deg^{-1}mol^{-1}$ also correlates reasonably with the figure of -20 cal $deg^{-1}mol^{-1}$ given by Edelhock and Osborne (1976) (79) for each methylene group ($-CH_2-$) transferred from water to a nonpolar medium.

The number of hydrated water molecules determines the size of the "heat sink" or the heat capacity (ΔC_p) of the system. Due to the thermal mobility of the network of water, the hydration is "rich" at low temperatures and "poor" at high temperatures. The rich hydration affords a higher heat capacity (ΔC_p) whereas lower heat capacity exists in the case of poor hydration. However, the change of heat capacity with temperature is small compared to entropy and enthalpy changes.

As stated previously, the enthalpy (ΔH) and entropy (ΔS) changes of the system are temperature dependent; in fact, they are heat capacity dependent as expressed by their mathematical definitions:

$$\Delta H = \int_{T_i}^{T_f} C_p \Delta T$$

$$\Delta S = \int_{T_i}^{T_f} \frac{C_p}{T} \times \Delta T$$

The changes of enthalpy (ΔH) and entropy (ΔS) of a system are products of its heat capacity (ΔC_p) and temperatures (T). The negative enthalpy change ($\Delta H < 0$) at high temperatures is due to low hydration or lower heat capacity (ΔC_p). The reverse is true in the case of low temperatures. Similarly, the heat capacity is also responsible for the large and small change of entropy at low and high temperatures due to rich and poor hydration respectively (Figure 39).

In conclusion, the heat capacity (ΔC_p) of the aqueous biological system in which glucocorticoid hormone and receptor interacts, determines the magnitude and direction of the thermodynamic changes (enthalpy, entropy) of the system that describe the hydrophobic property of the glucocorticoid and receptor interactions.

7.2.0 Two face interaction of glucocorticoid hormone with the receptor

The change in hydration of the surface area of the hormone and the receptor account for the free energy change of the binding hormone to receptor. Thus, it is appropriate to search for a relationship between free energy change and the surface area involved in the binding. A knowledge of this relationship will be helpful in understanding the structural interaction of steroid with receptor. Chothia (81) has reported the relationship between the surface area of proteins and the strength of protein-protein interaction as:

$$\Delta G = \Delta G_S + \Delta G_t$$

Where ΔG is the observed free energy of association, ΔG_S is the free energy of association which is proportional to the surface area involved in hydrophobic bonding and is equal to ΔG_S (steroid) + ΔG_r (receptor). ΔG_t is the free energy associated eith translational and rotational entropy loss where two molecules are brought together. It is estimated that for each A^{o2} surface removed from contact with water (water released from steroid receptor association in our case) where is a free energy gain of 24 calories (81). The surface area was calculated by the method of Bondi (82) by which the steroid molecules is divided into different surface areas according to the hydration degree in this area. The surface area is measured as A^{o2}. An example is the surface areas of dexamethasone is shown as Figure 40 following:

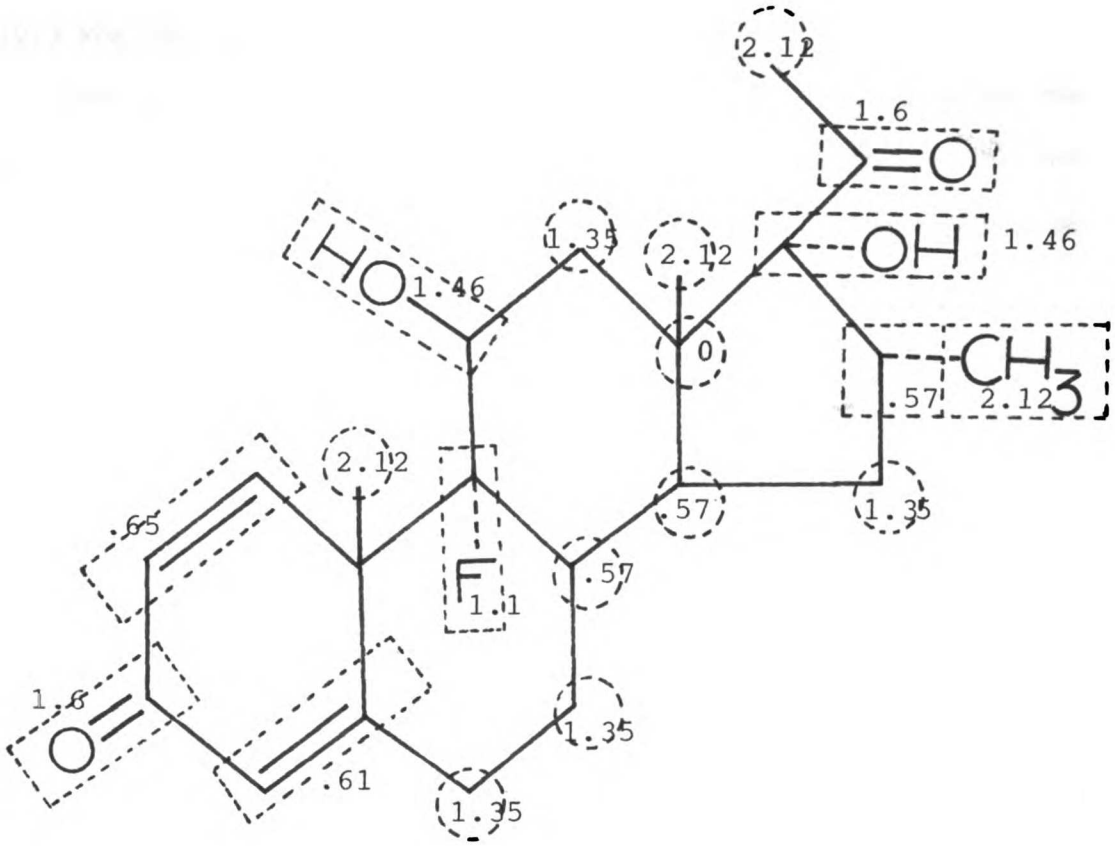


Figure 40 : Bondi surface area of dexamethasone. The numbers are in Angstoms square ($\AA^2$) of hydrated areas corresponding to the degree of hydrophobicity.

According to Page and Jencks (1971) (78), as two molecules associate they reduce their translational and rotational motions that result in loss of entropy. The average loss was calculated as ΔG_t = 12.3 kcal/mol at 0°C.

The hydrophobic energy changes derived from surface area was based on 24 cal/A^{o2} at 25°C (Jain-Chothia, 1976) corrected to 22.5 cal/A^{o2} for processes occurring at 0°C. The surface area of the receptor and steroid required for the calculation of ΔG_R (receptor) and ΔG_S (steroid) were taken as one half (for a one face binding analysis) or equal to (for a two face binding analysis) the Bondi surface area of the steroid. The method of calculation and the results are shown in Figure 40 and Table 10 and its footnote (Wolff et al. (72)). Positive values for the net change in ΔG are calculated for one face steroid receptor interaction; the correlation between $\Delta G_{obs}/\Delta G_{calc}$ is 38% for cortisone, 41% for cortisol and 37% for dexamethasone. In contrast, by assuming that both faces of the steroid are inserted into the receptor, the correlation is 78% for progesterone, 76% for corticosterone, 82% for cortisol and 87% for dexamethasone (data of progesterone, corticosterone and cortisol are from the calculations of Wolff et al. (72)). Some of the pitfalls of the calculation were discussed reporting the hydrophobic interaction of glucocorticoid hormone and the receptor (72) as follows: "Most of the shortfalls in the calculated energy of the two face models could be due to neglecting van der Waals forces. Assuming the protein density at the receptor

Free energies (Kcal/mol)	one face of steroid involved				two faces of steroid involved			
	prog.	cortico.	cort.	dex.	prog.	cortico.	cort.	dex.
ΔG (observed)[a]	-8.6	-10.2	-9.6	-10.7	-8.6	-10.2	-9.6	-10.7
ΔG (translation and rotation)[b]	12.3	12.3	12.3	12.3	12.3	12.3	12.3	12.3
ΔG (steroid)[c]	-4.0	-4.3	-4.5	-4.8	-8.1	-8.6	-9.0	-9.6
ΔG (receptor)[d]	-4.0	-4.3	-4.5	-4.8	-8.1	-8.6	-9.0	-9.6
ΔG (calculated)[e]	4.3	3.7	3.3	2.7	-3.9	-4.9	-5.7	-6.9
$\frac{\Delta G \text{ (ster.)} + \Delta G \text{(recept.)}}{\Delta G \text{ (obsd.)} - \Delta G \text{(trans.\& rot.)}}$	38%	38%	41%	37%	78%	76%	82%	87%

Table 10: Estimation of the extent of hydrophobic binding of various steroids to the glucocorticoid receptors. The calculations show a better correlation with a two face than a one face model for the binding (table 10 cont.).

TABLE 10 FOOTNOTE:

a. ΔG (observed) = $-RT\ln k_a$ at 0°C.

b. Determined by Page and Jencks (1971) to be the average translational and rotational energy loss for a bimolecular reaction at 0°C. Calculation of the translational and rotational entropy loss through statistical thermodynamic of the steroid molecule in a bimolecular reaction finds the entropy loss to be approximately 76 e.u. (76 Kcal). The translational and rotational energy loss of the receptor in a bimolecular reaction would be expected to be minimal as association of the acceptor with the relative small steroid molecule would have very little affect on the translational and rotational entropy for the receptor as a whole. Moreover, Page and Jencks have noted that in equilibrium complex, some of the rotational and translational entropy loss in forming the complex is converted into a low frequency stretching, vibration, into an internal rotation and into four low frequency bending modes. These may contribute up to 30 e.u. (9Kcal) of residue entropy to the loose complex. Thus, the estimate of 45 e.u. for the translational and rotational entropy loss for steroid complex formation appears reasonable.

c. Determined by multiplying one-half or one times the Bondi surface area of the steroids times 22.5 cal/A^2

d. ΔG (calcd) = $\Delta G_t + \Delta G_s + \Delta G_r$

site to be around 1.4 g/cm^3 (Lon Richards, 1954) and the free energy contribution for each van der Waals contact as -0.2 Kcal/mol (Ramachandram and Saniseleharam, 1968), a free energy of binding of -4 Kcal/mol could result from van der Waals forces in the two face models. Any remaining deficit may be caused by overestimating ΔG_t through too low a value for entropy associated with low frequency vibrations of the steroid receptor complex. Even though there is uncertainty in these estimates, the results are consistent only with the view that those cases of high affinity steroid-protein binding can be generated through hydrophobic interactions and that most of the steroid is enveloped by the receptor."

Another parameter that has been mentioned as a prominent measurement of hydrophobic effect, the heat capacity (ΔC_p), could also be used to estimate the mode of steroid interaction; a loss of -20 cal x deg^{-1}mol^{-1} is obtained upon removal of hydrated water from both faces of the $-CH_2-$ groups. Thus the approximately 21-methylene groups of the steroid molecule account for a 21 x (-20) = -420 cal deg^{-1}mol^{-1} change in heat capacity for the steroid and an equal amount for the receptor as it envelopes both sides of the steroid; the change in heat capacity of the system is estimated at -840 cal x deg^{-1}mol^{-1} for the two face model; we found values in our experiment that are from -600 cal deg^{-1}mol^{-1} to -700 cal deg^{-1}mol^{-1}. The change in observed heat capacity (ΔC_p) accounted for 71% to 83% of the calculated heat capa-

city value. The lack of a complete accounting in this is perhaps due to the difference in heat-capacity change for polar substituents, heteroatoms, alkylsubstituents, etc. or else to the presence of van der Waals' forces. A recent report on crystallographic work on the uteroglobin receptor and progesterone interaction (83) also revealed that a structural change in the receptor and a folding model engulfing both faces of the steroid.

7.3.0 Thermodynamic analysis of the interaction of dexamethasone with the whole cell

7.3.1. Background

Dexamethasone and the receptor interact with high affinity by hydrophobic forces, forming the hormone-receptor complex. This is followed by an activation process that allows the complex to bind to the chromosome (Section 1.7). The process of glucocorticoid hormone action is thus a continuous flow of events, and the hydrophobic binding of the steroid to the receptor is the point that initiates the total kinetic sequences and thermodynamic properties of this initial step with respect to the overall kinetic steps of events of glucocorticoid hormone action.

Study of the temperature effect on glucocorticoid binding to an intact cell would provide information regarding the binding of glucocorticoid with cytoplasmic receptors as well as other kinetic sequences of the hormone-receptor intractions. The binding of steroids with

intact cells has not been previously studied extensively because of the system's complex nature. As briefly mentioned, there have been a few reports on the thermodynamic effects of glucocorticoid and intact cell interactions within the last decade, but the studies were limited to only two or three temperatures. For instance, Schaumberg and Bojensen reported in a three temperature experiment that the binding of corticosterone to thymocyte cells varies linearly; the equilibrium dissociation constant (K_d) increases as temperature increases (84). On the contrary, MacDonald reported that the affinity (K_d) of ^{3}H-triamcinolone to glucocorticoid receptor in spleonic lymphocyte cells at 0°C is 5.6 nM, and at 37°C is 0.85 nM (85). Jones, Sherman and Bell also reached the same conclusions as MacDonald when they studied a series of glucocorticoids within two temperatures (0° and 37°C) in rat thymus cells (86).

To obtain a less ambiguous analysis of the glucocorticoid and receptor interactions in cells we studied the temperature effect on the equilibrium dissociation constant of the glucocorticoid with HTC cells at a number of temperatures. Technically, we treated the system as two separate reactants in a second order kinetic model. Intact cells were directly incubated with ^{3}H-dexamethasone at concentrations ranging from 1.5×10^{-9}M to 1.5×10^{-7}M. The incubation was allowed to reach equilibrium as we obtained maximum binding.

7.3.2. Properties of radiolabeled dexamethasone binding to intact HTC cells

The binding of glucocorticoids to an intact cell entails a complicated kinetic process that includes penetration of the cell membrane, binding to cytosol receptors to form a hormone-receptor complex, activation of this complex, and its translocation to the nucleus of the cell. We first investigated the time course of ^{3}H-dexamethasone binding to intact HTC cells at different temperatures to study the equilibrium of the binding. The time required for maximum binding decreases with increasing temperature (Figures 22-26). This measurement is affected by receptor degradation and whether the equilibrium of the binding has been achieved. At 4°C, we examined five Scatchard plots in a period of 22 hours and found increasing affinities and decreasing of total receptor with time as shown in Figures 20 and 21. This observation was also reported by Koblinsky et al. in cytosol which was not stabilized by DTT and molybdate (69). We thus interpreted the equilibrium of the system for the optimum value at which the binding affinity and the reactivity of the cell are both significant when combined at the expense of an average of 14% cell degradation during the incubation time of 22 hours. We chose to use equilibrium times for the temperatures studied at 17 hr for 0°C, 11 hr for 4°C, 8 hr for 12°C, 6 hr for 18°C, 3 hr 45 min for 22°C, 3 hr 5 min for 25°C, 2 hr 20 min for 30°C, 1 hr 30 min for 33°C, 50 min for

35°C, and 45 min for 37°C. We used these temperatures to generate Scatchard plots of ^{3}H-dexamethasone binding after incubation of the steroid with intact HTC cells. Ten Scatchard plots were generated. The "apparent" equilibrium constants obtained provided an integented assessment of the overall sequences of events in the hormone-receptor interactions as shown in Section 4.2.6.

7.3.3. Free energy (ΔG), enthalpy (ΔH), entropy (ΔS) and heat capacity (ΔC_p) change of the interaction

Similar to the thermodynamic analysis of ^{3}H-dexamethasone binding by HTC cell cytosol, a Van't Hoff plot was generated to analyze thermodynamic parameters of the interactions of ^{3}H-dexamethasone with intact cells. This reflects "apparent thermodynamic" values of the events in glucocorticoid-receptor interactions. We obtained a curvilinear relationship of affinity versus temperature in the Van't Hoff plot (Figure 28). The calculation from the plot showed that the apparent free energy change favored the interaction of dexamethasone with the whole cell; contribution of enthalpy change of the interaction is also temperature dependent and the enthalpy drives the interactions at high temperatures from 18° to 37°C whereas the entropy change drives the interaction at low temperatures from 0° to 22°C and the entropy change is also a temperature dependent parameter. There exists also a large heat capacity change (ΔC_p) from the interactions.

7.3.4. Comparison of the thermodynamic effects of the interaction of ^{3}H-dexamethasone with HTC cell cytosol receptors and HTC intact cells: An implication that the rate determinant step is at the level of the cytosol receptor-steroid interaction

There may not be a single explanation for the changes in the magnitudes of affinity (K_a) free energy (ΔG), enthalpy (ΔH), entropy (ΔS) and heat capacity (ΔC_p) in the interaction of dexamethasone with an intact cell compared to its cytosol receptor, because as shown by the calculation in section 4.3.1, the interaction in an intact cell is a complicated cascade of kinetic processes.

In our experiments, the thermodynamic curve (Van't Hoff plot) of the dexamethasone and intact cell interaction is similar in shape to the curve of ^{3}H-dexamethasone binding by cytosol (Figure 41), the curves differ only in the magnitude of the values of affinity (K_d). Consequently, free energy change (ΔG), enthalpy change (ΔH), entropy change (ΔS) and heat capacity change (ΔC_p) also differ. In fact, the slopes of both Van't Hoff curves are nearly parallel to each other, and they share the same optimum affinities at temperatures for 12°-14°C. This implies that the interaction of glucocorticoid and receptor at the cytoplasmic level is the major contributor to the kinetic and thermodynamic changes as compared to any transport process, or conformational changes associated with other steps such as the activation and nuclear binding processes.

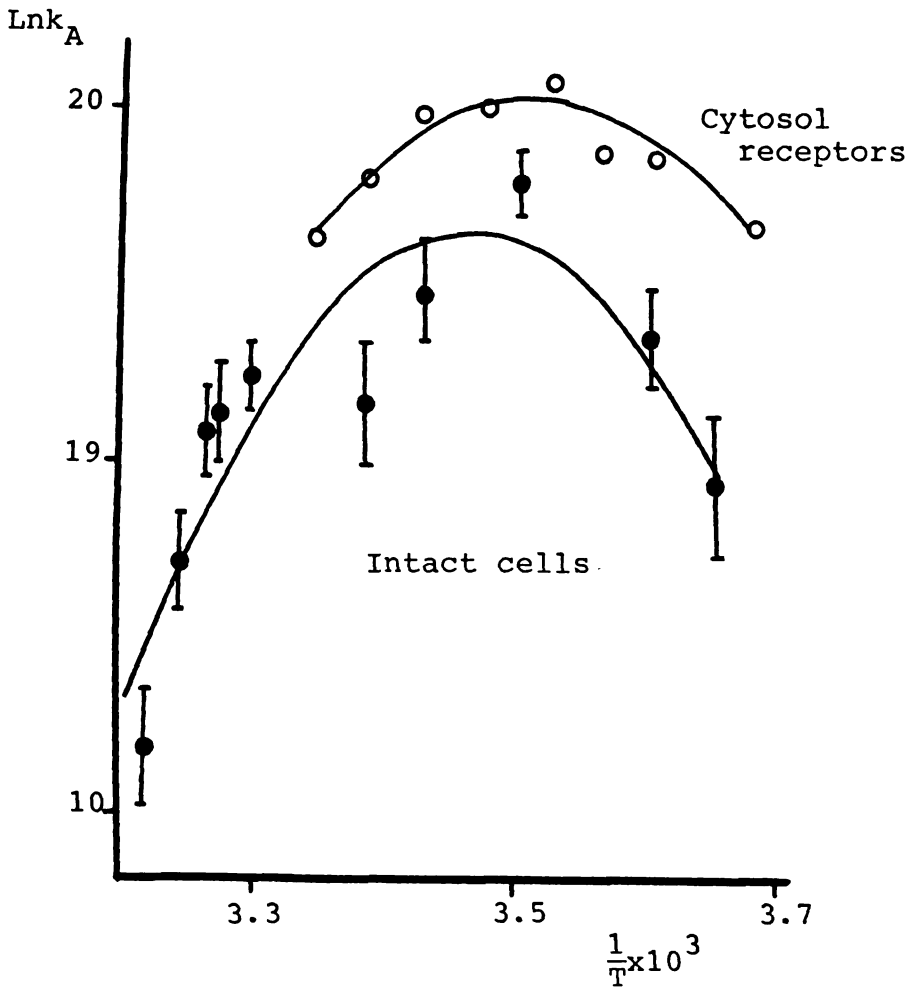


Figure 41 : Van't Hoff plot of ^{3}H-dexamethasone binding to intact HTC cells and its cytosol receptors. The cytosol receptor protein is stabilized by 3 mM dithiothreotol and 10 mM sodium molybdate.

We can, thus, conclude that the interaction of glucocorticoid hormone with the cytoplasmic receptor is the rate determining step in the mechanisms of glucocorticoid hormone action.

7.4.0 Temperature dependency of nuclear binding

The last steps in the early events of glucocorticoid receptor interaction are the activation of the complex followed by its binding to the nucleus. Previous studies have shown that, at low temperatures, glucocorticoids form a non-activated complex with receptors in either intact target cells or cytosol and that this complex does not bind nuclei in cell-free systems; warming will lead to the formation of activated complexes that can bind in the nucleus of nuclear-bound complexes (87, 90, 95). This temperature effect is not known to be reversible: numerous studies have shown that heating of cytosol that has been equilibrated with steroid followed by cooling it promotes binding of the complex to nuclei (87, 90, 92, 95-97). Using DEAE-cellulose chromatography to study the time course of ^{3}H-dexamethasone-cytosol complex formation at 37°C, Munck later demonstrated that the non-activated form is the first to appear and gives rise subsequently to activated complexes (49).

Although a high salt concentration has also been shown to affect the activation process (93, 94, 98-100), throughout the time course studied, the salt concentration within the cell and in the incubation medium was constant at varying temperatures. Thus, the observed action is an effect solely of temperature change.

In the present experiment, we examined the possibility that glucocorticoid receptors could be activated in intact cells and the effect of temperature on the binding of the activated complex to nuclei and the changes of this effect at different temperatures.

The total nuclear and cytosol binding as a function of temperature was measured. Nuclear binding increased progressively with increasing temperature, varying from about 45% of the total receptor-glucocorticoid complexes at 4°C to 80% at 37°C. By contrast, total cellular binding was as great at 0°C as at 37°. The studies of Bloom et. al. (68) indicated that the limitation of nuclear binding at 0°C is due to the activation process rather than nuclear binding, since a transient eluation of the temperature resulted in progressive nuclear binding at 0°C. Munck et. al. (49) have also concluded that activation occurs more rapidly than nuclear binding. These results, plus the finding of a progressive increase in the relative proportion of the complexes bound to the nucleus with increasing temperature implies that nuclear binding itself is not impaired by nuclear membrane penetration of the receptors, assuming that they really are cytoplasmic in the absence of the steroid (an assumption that has never been proven).

As noted in Fig. 31, increases in the time of incubation beyond those required for maximal formation of the cytoplasmic receptor-dexamethasone complex do not result in an appreciable increase in the proportion of complexes bound by the nucleus. Thus, activation and

subsequent nuclear binding appear to follow cytosolic receptor-dexamethasone complex formation quickly, and there appears to be no further progression of these processes. These data may imply some rate-limiting component of the activation rather than just slow kinetics at lower temperatures. Whereas conclusions cannot be drawn with certainty, the data may point to some modification step such as dephosphorylation, rather than other processes such as reversible aggregation of receptor units, as being responsible for the activation.

7.5 Relation between nuclear acceptor occupancy compared with the cytosol receptor-dexamethasone complex concentration; evidence for a large excess of acceptor sites in the nucleus

After binding to the specific receptor in the cytosol, the activated hormone-receptor complex binds to the nuclear acceptor sites. Attempts to access the number of nuclear acceptor sites in vitro experiments have been reported by a number of investigators (87-92) but their results has been challenged mostly due to technical problems of the presence of an inhibitor protein reported by Simon (70) in such in vitro experiments. However, there is evidence showing that there are a limited number of nuclear acceptor sites that exceeds the quantity of cytosol receptors (66, 67, 68). The major study in the intact cells was by Bloom et. al. (68) in which a plot of the quantity of nuclear-bound receptor-dexamethasone complexes as a function of the receptor-dexamethasone complexes was linear and a Scatchard plot of

the data was parallel or nearly parallel to the abscissa. In the current studies, this type of analysis was performed after incubation of cells with the steroid at lower temperature (12°) with the hope that the affinity of the complex for the nucleus might be higher and that this might result in a Scatchard plot with enough slope to obtain a better indication of the site concentration. However, again the results showed a linear relationship between the nuclear and cytosolic complexes and a Scatchard plot was nearly parallel to the abscissa (Fig. 34). Thus, these data support the previous conclusion that the quantity of cytosolic receptors is far less than the number of nuclear acceptor sites. The latter are at least five-fold in excess of the quantity of receptors in the cell and are probably present in even greater excess. Although it has been argued that these "acceptors" are nonspecific DNA binding sites, it is noteworthy that the association of receptors with them paralleles precisely the induction of tyrosine aminotransferase in HTC cells and an "excess acceptor" hypothesis could also explain the lack of "spare receptors" in these cells (68).

8.0 Thermodynamic mechanism of glucocorticoid hormone action

In summary, the present studies have provided additional information regarding the early events in glucocorticoid hormone action. Of major importance was the obtaining of data that provides additional support for the notion that the hormone receptor interaction is driven dominantly by hydrophobic effects. The data in this case come from studies with isolated cytosol in which receptor activation is blocked

by sodium molybdate that along with the incubation with DTT also enhanced receptor stability. This allowed us to examine binding over a wider temperature range (from -2°C to 25°C) than before and to exclude the potential criticism of the previous work of Wolff et. al. (72) that activation or denaturation at the high temperature may have contributed to the shape of the Van't Hoff plot ($\ln K_a$ vs $1/T$). In addition, the data were extended by the use of another agonist, dexamethasone, in addition to corticosterone that was used previously (72).

Dehydration of both the receptor binding sites and the steroid, which is regarded as the main component of the hydrophobic effect associated with hormone-receptor binding, correlated well with the observed changes of entropy, enthalpy and heat capacity. In aqueous solution, both the steroid and the receptor binding site, although predominantly hydrophobic, are nonetheless hydrated by water. These steroid or receptor-associated water molecules must rearrange their hydrogen bondings and become more constrained than their neighboring free water molecules.

It is this restriction that decreases their entropy and enthalpy. Upon steroid receptor binding, the hydrated water at the surface where the steroid and receptor interact, are released and the difference in entropy, enthalpy and heat capacity between the free steroid and receptor associated water molecules accounts for the major hydrophobic changes of the system as reflected by the Van't Hoff analysis. For

example, at low temperature both hormone and receptor are strongly hydrated and we observed a large entropy change which is correspondent to the release of a large amount of hydrated water. Due to the thermal motion of water molecules, the degree of hydration decreases as temperature increases; corresondingly, we observed a decrease in entropy of the system as temperature increases. The enthalpy change of the system is small because only hydrogen bond rearrangement is involved, but the temperature dependence of enthalpy changes is similar to that of the entropy changes. Releasing of the hydrated water increases the enthalpy of the system. However, this enthalpy change increases the free energy of the system, and thus works against the entropy and does not favor the reaction. Nevertheless, the hydration cannot explain the negative enthalpy change at higher temperatures from 12°C to 25°C; this is explained by the enthalpy change contribution of the hormone and receptor. Free hormone and receptor have more mobility than does the complex; thus the complex acquires a lower enthalpy. Upon forming the complex, the hormones and receptors produce a negative enthalpy change, this change is regarded as intrinsic enthalpy change of the hormone and receptor and remains relatively constant with temperature changes compared to that of the system. However, this decrease is not observed at lower temperature, because the positive changes of enthalpy due to the large amount of hydrated water is greater than the negative change in enthalpy of hormone and

receptor. At high temperature, as mentioned, the system is poorly hydrated and the contribution of enthalpy from hormone and receptor becomes more prominant and observed as a negative enthalpy.

Although we did not observe a negative entropy change in the cytosol system, in the intact cell experiments at temperatures higher than 25°C, negative entropy changes do occur. At or above this temperature, the amount of water associated with the hormone and receptor is less and the decrease in entropy resulting from hormone-receptor binding is a major entropy contribution to the system compared to that from increasing entropy from dehydration of water. The net result was observed to be negative.

How does the heat capacity fit into this hydration and dehydration model? Water has a higher heat capacity than, for example, iron because it has a higher capacity to retain heat. In our system, the differences in heat capacity following hormone-receptor binding are more subtle, because we are comparing the change of heat capacity of the same substance (water) but at different environments. As discussed, the nature of bound water is different from that of free water due to the constraints of hydrogen bonding; these constraints give the receptor- and steroid-associated water a higher heat capacity. The bound water thus behaves as a heat container or heat sink. The association of hormone and receptor releases the bound water which results in a decrease in its heat. We observed a large change in heat

capacity of the system associated with steroid-receptor binding. The reason that heat capacity is the most favorable parameter in explaining the hydrophobicity of the interaction is due to the unique role of the hydrated water in the heat capacity change of the system. Being mainly hydrophobic, the heat capacities of the hormone, receptor and the complex are very similar. Thus, unlike the case with entropy, or enthalpy changes, the hormone and receptor contribute little to the heat capacity change of the system. The change is accounted for mainly by the hydrated water released into free water upon hormone-receptor binding; this also is the definition of hydrophobic interaction.

The degree of hydration correlates with the surface area of the hormone and receptor. The associated areas of hormone and receptor determine the binding free energy of the hydrophobic association. The binding energy is thus calculated from the surface area of the steroid and the literature estimate from the protein-protein interaction. The correlation of the observed binding energy (from the affinity values of hormone-receptor binding with the calculated energy from surface area, supports the model that the receptor engulfs both sides of the hormone; the free energy obtained from dehydration of both sides of the hormone gives a negative free energy change, whereas a one face model results in positive free energy changes.

We also found that the shape of the Van't Hoff plots of steroid receptor binding when the steroid was incubated with intact cells was similar to that obtained with isolated cytosol. This observation suggests that the major driving forces of the intact cell binding are similar to those of the initial receptor-steroid interaction. This conclusion has several implications.

The first relates to the mechanism of steroid uptake. Whereas with thyroid (105), catecholamine (106) and polypeptide (107) hormones, specific uptake mechanisms appear to account for the hormones' entry, this has not been thought to be the case with the steroid hormones (25, 46, 47). Due to the hydrophobicity of steroids and that of the cell membrane, the steroids have been hypothesized to enter the cell by passive diffusion. Our findings of similar thermodynamic parameters for binding in both cytosol and intact cells supports strongly the above hypothesis. If, for example, there were a major element of transport associated with steroid uptake, it is likely that much more marked decreases than were observed in uptake would occur at lower temperatures where these processes are relatively inactive. Again, the data suggest that cellular uptake does not provide a significant barrier to steroid entry.

Since the Van't Hoff plots of intact cell and cytosol binding are similar, the data also imply that the major driving force for the overall binding is the initial steroid-receptor interaction and not other changes such as conformational ones associated with activation

or nuclear binding. Although this might seem to be trivial conclusion, there are several examples, e.g. with catecholamines, where following the initial hormone receptor binding the complex becomes transformed into a much higher affinity or lower affinity state. In fact, several lines of evidence suggest that the off-rate of the steroid from the nonactivated form of the receptor differs from that of the activated form (108). Thus, the transformation or changes in conformation associated with nuclear binding (that has not to my knowledge been addressed specifically) could in principle dominate the overall driving force of the reaction. However, this appears not to be the case.

It is noteworthy, however, that the overall affinity of the steroid for the receptor for intact cell binding as compared to cytosol binding was found to be somewhat lower at all temperatures examined. For instance at 4°C the K_d for dexamethasone binding by isolated cytosol was 2.3 nM whereas the intact cell value was 4.0 nM. This difference could be due to the different conflicts surrounding the receptors in intact cells and cytosol. Alternatively, the difference could reflect the participation of others in processes such as conformational changes, activation or nuclear binding that occur in the cell, but not in the cytosol, or that the membrane does provide a passive gradient to steroid entry that is constant with temperature.

However, even if this were the case, the conclusion would not overall be changed that the major driving forces are comprised by the initial receptor-steroid interaction.

Information regarding the nuclear binding process was also obtained. After maximum binding of various hormone concentrations to intact cells, by cell fractionation the amounts of the hormone binding to the nuclei and receptors was measured. The saturation binding study has shown that the cytosol receptors are nearly saturated at 1 x 10^{-7}M of the hormone, whereas the nuclear acceptor sites are still far away from being saturated. This observation is further supported by the nuclear Scatchard analysis by which the plot is almost parallel to the abscissa. This observation is not due to the limited amount of activated hormone-receptor complexes or their slow binding to the nuclei, because from 40% to 80% of the total hormone-receptor complexes are bound to the nuclei in the equilibrum time of 45 min to 22 hr measured at the affinity of the hormone receptor complex to the nuclei has not been measured. This answer will have to await for the success in purification of glucocorticoid receptors that will eliminate artifacts that influence the binding process a mentioned by S. Simons (70).

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PART II

MECHANISM OF AGONIST-ANTAGONIST GLUCOCORTICOID HORMONE ACTION

PART II

Mechanism of Agonist - Antagonist Glucocorticoid Hormone Action

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1.0 Introduction

Glucocorticoids act by binding to specific receptor proteins. It has been proposed that in the absence of glucocorticoid, the receptor is in an inactive state, and that the binding of an active steroid stabilizes the receptor in an active conformation. Glucocorticoid antagonist steroids also exist and block the ability of agonists to act. The aim of this study is two-fold: first, to provide more evidence regarding the existence of different conformational states of glucocorticoid receptors; and second, to investigate the mechanism of agonist/antagonist glucocorticoid interactions with those receptors.

The activities of steroids in relation to glucocorticoid-responsive systems have been categorized into four classes according to their inductive effects on tyrosine aminotransferase (TAT) activity in hepatoma tissue culture (HTC) cells by Samuels and Tomkins, 1970 (1):

a) Pure agonist or optimal inducers: These are glucocorticoids which, at high concentrations, can induce the enzyme TAT to maximal levels. Pure agonists may be "weak" or "strong", depending on their affinity for the glucocorticoid receptors.

b) Partial agonists or suboptimal inducers: This group includes compounds which can induce enzyme activity, but not to the maximal level, even at high concentrations. Furthermore they can inhibit the action of optimal inducers down to the levels elicited by the partial agonist alone.

c) Pure antagonists or anti-inducers: These steroids have no inductive ability, but they can inhibit the induction by agonists or partial agonists.

d) Inactive glucocorticoids or inactive inducers: These steroids neither induce nor inhibit the actions of agonists.

Since the discovery in the mid 1960's of the anti-glucocorticoid properties of progesterone (2, 3), many steroidal compounds have been reported to antagonize glucocorticoid activity in in vitro test systems (Table I). Although the application for these results to in vivo systems has encountered difficulties (4-6), these antagonists have proven to be extremely useful to probe the mechanisms of glucocorticoid hormone action. Table I lists a number of steroids classified into the above four groups by Rousseau and Schmidt (7). It also shows the affinities of these steroids for binding to the glucocorticoid receptors. Because not all glucocorticoids are available in radioactive form, the affinity determinations were based on measurements of the ability of non-radioactive glucocorticoids to compete with radiolabeled dexamethasone (an optimal inducer) for binding.

An accumulation of data regarding the interactions of agonist/antagonist glucocorticoids with cellular receptors has established a number of working hypothesis for their mechanism of action. In general, the hypothesis support two conformations of receptors that reflect those associated with pure agonist and antagonist steroids made prior to the identification of receptors (1).

No.	Steroids Systematic name	Trivial name	Affinity (nM)* Mean	 Range	Activity † Mean	 Range	Class
I	9α-Fluoro-16α-methyl-11β, 21-dihydroxy-1,4-pregnadiene-3,20-dione	Desoxymethasone	0.82	0.34 → 1.3	88	80 → 96	O
II	9α-Fluoro-16α-methyl-11β, 17α, 21-trihydroxy-1,4-pregnadiene-3, 20-dione	Dexamethasone	5.6	1.9 → 8.8	100	88 → 116	O
III	11β, 21-Dihydroxy-4-pregnene-3, 20-dione	Corticosterone	7.0	6.4 → 7.4	85	80 → 94	O
IV	6α-Methyl-11β, 17α, 21-trihydroxy-4-pregnene-3, 20-dione	6α-Methylcortisol	8.3	6.1 → 104	100	94 → 104	O
V	21-Hydroxy-4-pregnene-3, 20-dione	11-Deoxycorticosterone	9.1	4.1 → 14.1	31	28 → 33	S
VI	11β-Hydroxy-4-pregnene-3, 20-dione	11β-Hydroxyprogesterone	10.3	10.0 → 10.6	9	6 → 12	S
VII	11β, 17α, 21-Trihydroxy-4-pregnene-3, 20-dione	Cortisol	10.5	5.0 → 18.1	111	101 → 119	O
VIII	11β, 17α, 21-Trihydroxy-1, 4-pregnadiene-3, 20-dione	Prednisolone	12.4	9.5 → 15.3	112	109 → 114	O
IX	11β, 17α, 21-Trihydroxy-1, 4, 7-pregnatriene-3, 20-dione	7-Dehydroprednisolone	19.1	12.2 → 26.1	62	55 → 64	S
X	1,4-Pregnadiene-3, 20-dione	1-Dehydroprogesterone	20.7	12.2 → 29.2	0.2	−1.3 → 1.0	A
XI	11β, 16α, 17α, 21-Tetrahydroxy-4-pregnene-3, 20-dione	16α-Hydroxycortisol	30.5	17.2 → 43.8	61	52 → 71	S
XII	6α, 16α-Dimethyl-4-pregnene-3, 20-dione	6α, 16α-Dimethyl-Progesterone	32.3	26.6 → 37.9	15	14 → 16	S
XIII	11β, 21-Dihydroxy-18-al-4-pregnene-3, 20-dione	Aldosterone -	40.1	23.6 → 49.4	71	65 → 80	S
XIV	4-Pregnene-3, 20-dione	Progesterone ✓	51.6	31.2 → 72	2.5	2.3 → 2.7	A
XV	17α, 21-Dihydroxy-1, 4, 9(11)-pregnatriene-3, 20-dione (21-acetate)	1,9 Dehydrocortexolone (acetate)	53.7	31.3 → 76.2	8.1	7.0 → 10	S
XVI	11β, 17α, 21-Trihydroxy-4, 6-pregnadiene-3, 20-dione	6-Dehydrocortisol	54.8	23.6 → 86	52	48 → 56	S
XVII	16α-Methyl-17α, 21-dihydroxy-4-pregnene-3, 20-dione	16α-Methylcortexolone	61.3	43.8 → 78.8	14	12 → 17	S
XVIII	16α-Methyl-4-pregnene-3, 20-dione	16α-Methylprogesterone	80.1	60.3 → 119	0	−4 → 2	A
XIX	6α-Methyl-4, 16-pregnadiene-3, 20-dione	6α-Methyl-16-dehydroprogesterone	88.7	69.8 → 108	0.6	0.2 → 1	A
XX	17α, 21-Dihydroxy-4-pregnene-3, 20-dione	Cortexolone	128	74.5 → 181	14	13 → 15	S
XXI	16α, 17α-Dihydroxy-4-pregnene-3, 20-dione	16α, 17α-Dihydroxyprogesterone	180	130 → 230	1.2	0.6 → 1.5	A
XXII	17α-Hydroxy-4-pregnene-3, 20-dione	17α-Hydroxyprogesterone	192	138 → 245	1.4	0.0 → 1.8	A
XXIII	17α-Methyl-17β-hydroxy-1,4-androstadiene-3-one	17α-Methyl-1-dehydro-testosterone	304	283 → 326	0.6	0.2 → 0.9	A

(Table 1 cont.)

No.	Steroids: Systematic name	Trivial name	Affinity (nM)* Mean	Range	Activity† Mean	Range	Class
XXIV	16α, 21-Dihydroxy-4-pregnene-3, 20-dione (21-acetate)	16α-Hydroxydeoxycorticosterone (acetate)	318	267 → 368	0.2	−0.6 → 1.1	A
XXV	21-Hydroxy-5β-pregnane-3, 20-dione	5β-Dihydrodeoxycorticosterone	419	329 → 509	0.4	0.2 → 0.7	A
XXVI	17α-Methyl-17β-hydroxy-4-androstene-3-one	17α-Methyltestosterone	429	360 → 498	0.1	−1 → 1.3	A
XXVII	16α-Methyl-17α-hydroxy-4-pregnene-3, 20-dione	16α-Methyl-17α-hydroxyprogesterone	586	469 → 703	0	−1 → 1	A
XXVIII	17α, 21-Dihydroxy-4-pregnene-3, 11, 20-trione	Cortisone 7	631	521 → 740	61	55 → 66	(S)
XXIX	14α, 17α, 21-Trihydroxy-4-pregnene-3, 20-dione	14α-Hydroxycortexolone	692	552 → 831	0.4	0 → 0.6	A
XXX	17β-Hydroxy-4-androstene-3-one	Testosterone	695	631 → 759	0	0 → 0	A
XXXI	17β-Hydroxy-1,4-androstadiene-3-one	1-Dehydrotestosterone	869	579 → 1286	0.1	−2 → 0.7	A
XXXII	21-Hydroxy-4-pregnene-3, 11, 20-trione	11-Dehydrocorticosterone	919	—	31	26 → 37	(S)
XXXIII	11β, 17α, 21-Trihydroxy-5α-pregnane-3, 20-dione (21-acetate)	5α-Dihydrocortisol (acetate)	932	642 → 1216	11	7.8 → 14	S
XXXIV	17α, 21-Dihydroxy-1,4-pregnadiene-3, 11, 20-trione	Prednisone	1019		42	34 → 51	(S)
XXXV	17-Keto-4-androstene-3-one	Androstenedione	2060	—	1.6	0.7 → 2.6	i
XXXVI	11β-Hydroxy-17-keto-4-androstene-3-one	11β-Hydroxyandrostenedione	2200	—	1.3	0.9 → 1.7	i
XXXVII	11α-Hydroxy-4-pregnene-3, 20-dione	11α-Hydroxyprogesterone	2231	1628 → 2835	0.4	0 → 0.6	i
XXXVIII	16β-Methyl-4-pregnene-3, 20-dione	16β-Methylprogesterone	2401	2313 → 2489	1.2	0.8 → 2.3	i
XXXIX	11β, 17α, 21-Trihydroxy-5β-pregnane-3, 20-dione (21 acetate)	5β-Dihydrocortisol (acetate)	3035	1495 → 4574	2.1	0.5 → 3.5	(i)
XL	11β, 17β-Dihydroxy-4-androstene-3-one	11β-Hydroxytestosterone	3231	1630 → 4051	−0.2	−0.8 → −0.4	(i)
XLI	21-Hydroxy-4, 16-pregnadiene-3, 20-dione (21 acetate)	16-Dehydrodeoxycorticosterone (acetate)	7973	3893 → 12053	1.3	0.5 → 1.8	i
XLII	1-Methyl-11β, 17α, 21-trihydroxy-1,4-pregnadiene-3, 20-dione	1-Methylprednisolone	8144		1.4	0.7 → 1.7	i
XLIII	20α-Hydroxy-4-pregnene-3-one	20α-Hydroxyprogesterone	> 10000	—	1.1	−1.2 → 2.5	i
XLIV	3α, 11β, 21-Trihydroxy-5β-pregnane-20-one	Tetrahydrocorticosterone	> 10000	—	2	1.5 → 2.1	i

* Equilibrium dissociation constant (0.) of steroid-receptor interaction determined in HTC cell cytosol by competition with [^{3}H]-dexamethasone, as described in the text.

† Based on four determinations of the effect of 10^{-5} M steroid on tyrosine aminotransferase activity in HTC cells at 37°. Data are in percent of steady-state induction (61.84 mU/mg protein) over basal level (3.0–3.8 mU/mg protein) obtained with dexamethasone. Activity classes are symbolized as follows: O: optimal inducers; S: suboptimal inducers; A: anti-inducers; i: inactive glucocorticoids. Classifications in parentheses are discussed in the text.

Table 1: Classification of glucocortiocoids (7).

Based on this classification, Samuels and Tomkins proposed an allosteric receptor model for the interaction of agonist/antagonist glucocorticoids with the receptor (1). The model later received support when the receptors were detected (8) and those occupied by the agonist dexamethasone were found to have properties different from those of unoccupied or antagonist-associated (progesterone) receptors.

This observation was followed by extensive study of the kinetic behavior of the agonists dexamethasone and cortisol and the pure antagonist progesterone with respect to HTC cell cytosol receptors (9). First, the results showed that progesterone has a lower affinity (K_d = 2.0 - 2.5 x 10^{-8} M) than that of dexamethasone (K_d = 2 x 10^{-9} M) and that it binds to a single class of receptor sites. In competition experiments, progesterone was then observed to inhibit the specific binding of dexamethasone to the same cytoplasmic receptors. Dexamethasone also prevented the specific binding of progesterone to the extent predicted from the relative affinities of the two steroids for the specific receptors. Thus, it was concluded that dexamethasone and progesterone bind to the same receptor site.

The allosteric model proposed two conformational states of the receptors, one active and the other inactive. In the absence of steroids, the receptor predominantly exists in the inactive state. Agonist binding promotes a shift toward an active conformation; anti-inducers, on the other hand, bind to the inactive form and do not cause an increase in the concentration of active receptors. This

model assumes that partial agonists can bind to and stabilize both forms of the receptor; the proportion in the active conformation would determine the extent of partial agonist activity.

In time-course studies, progesterone associated more rapidly with the receptors than dexamethasone, even though it has a lower affinity for the receptor. This observation is consistent with the hypothesis that, for the high-affinity agonist binding, the receptor must shift toward the active conformation. The time required for this could then explain the slower kinetics of agonist binding. Conversely, we assume that the antagonist can bind the inactive receptor directly and will therefore associate with it more rapidly.

Receptor-stability studies provided additional support for the existence of two different conformation states of the receptor. Receptors bound by the agonist dexamethasone were more stable than unoccupied receptors or those bound by the antagonist progesterone (similarly to the destabilization of unoccupied receptors) (9).

The interpretations of the on-rate studies might be questioned if steroids with higher affinities associate more slowly than those with lower affinities. Indeed, the rate of receptor association of these steroids classified as optimal inducers, does show such a correlation. Therefore, to determime whether a faster on-rate is also an intrinsic characteristic of antagonists vs agonists, it would be cri-

tical to compare compounds with similar affinities. This analysis has previously not been performed.

In the current studies, we have extended these examinations and have employed an agonist (aldosterone) that does not suffer from the above objections, since it has an affinity for the glucocorticoid receptor that is similar to progesterone.

A second problem with the previous studies relates to stability. These studies were performed before it was known that molybdate, DTT and other agents could stabilize the receptors; therefore, enhanced unstability in the presence of progesterone as compared with dexamethasone could result in an apparent on-rate that is faster than the actual on-rate. Therefore, in the current studies DTT were used to stabilize the receptors.

2.0 Materials

(6, 7 ^{3}H)-Dexamethasone (57 Ci/mmole), (New England Lab) (1, 2, 6, 7 ^{3}H)-aldosterone (90 Ci/mmole) and (1, 2 ^{3}H)-progesterone (57 Ci/mmole) were obtained from New England Corp. (Boston, MA). Unlabeled dexamethasone was obtained from Merck Co., Inc., Rahway, N.J.

Buffers: The homogenization buffer (medium #1) consisted of 20 mM Tris HCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 3 mM DTT (dithiothreitol), 10 mM sodium molybdate (Na_2Mo_4) and 10% glycerol, freshly prepared. Phosphate-buffered saline consisted of 0.1 M NaCl and 0.25 M potassium phosphate pH 7.6. Activated charcoal (Norit A, Fischer) was

prepared as described (8). Growth medium pH 7.6 to 7.8 was Swims' 77 (Grand Island Biological Company, New York) supplemented with Na HCO_3 0.05 g Tricine, 0.002 M glutamine and 10% calf serum.

Preparaton of Cytosol: HTC cell cytoplasmic extracts were prepared by washing cells with ice-cold phosphate buffered saline. The washed cells were disrupted in one volume of homogenizaton buffer medium #1, using a Teflon glass tissue glinder and the resulting homogenate was centrifuged at 100,000 g for one hour. The supernatant fraction was collected for the binding study.

3.0 Methods

3.1 Binding affinities of dexamethasone, aldosterone and progesterone to HTC cell cytosol receptors

Radiolabeled progesterone, aldosterone and dexamethasone at concentrations from 10^{-10} to 10^{-8} M were incubated with a constant amount of cytosol in the presence and absence of a thousand-fold excess of non-radioactive steroid; sodium molybdate to 10 mM and dithiothreitol to 3 mM were added to the medium to minimize receptor degradation. The incubation was allowed to reach maximum binding (8 hours for dexamethasone, 13 hr for aldosterone and 45 min for progesterone. The temperature was 0°C). The specifically-bound steroid was then measured by subtracting the total bound from the background as mentioned in Section 3.0 Part I. Scatchard plots were generated from these data.

3.2 Measurement of agonist and antagonist receptor complex stability

In a manner similar to the above, the cytosol fraction was incubated with radiolabeled dexamethasone, aldosterone and progesterone at concentrations of 1×10^{-8} M for dexamethasone, 1×10^{-7} M for aldosterone and 2×10^{-7} M for progesterone in the presence and absence of 10^{-5} M competing nonradioactive dexamethasone. At time intervals, the amounts of specifically bound steroid were determined as described above.

3.3 Measurement of the association rates

At 0°C, HTC cell cytosol fractions in medium #1 with 10 mM Na_2MoO_4 and 3 mM DTT were incubated with radiolabeled steroids at saturation concentrations (1×10^{-8} M for dexamethasone 1×10^{-7} M for aldosterone and 2×10^{-7} for progesterone) in the absence and presence of a 2,000-fold excess of unlabeled dexamethasone. At the indicated time intervals, 100 µl aliquots were collected into a centrifuge tube containing 10 µl of activated charcoal, 100 mg/ml, and agitated in a vortex mixer for 10 seconds. After standing for 2-4 minutes at 0°C, the charcoal was removed by centrifugation at 25,000 g for 3 min and 80 µl of the supernatant was used to determine the specifically-bound radioactivity. The concentration of total binding sites was determined by the maximum binding at equilibrium from the saturation curve derived from Scatchard measurements or from the binding of the labeled hormone at a concentration ten times that of its K_d value for saturation.

3.4 Measurements of the dissociation rate

The above cytosol solution was incubated for a prolonged time to allow equilibration. At various times, a 2,000-fold excess amount of unlabeled dexamethasone was added. At various time intervals thereafter, 100 µl aliquots were pipetted into centrifuge tubes containing 10 µl of the activated charcoal and agitated in a vortex mixer for 10 sec. After standing at 0°C for 2 minutes, the charcoal was removed by centrifugation and 80 µl of the supernatant was taken for radioactivity determination. The radioactivity obtained in sample without added excess of unlabeled steroid was used to correct any eventual denaturation of the binding activity.

4.0 Results

4.1 Binding affinities of dexamethasone, aldosterone and progesterone for the HTC cell cytosol receptors

The binding of steroid hormones to receptors will reach equilibrium when the association rate is equal to the dissociation rate. By this definition, the apparent equilibrium dissociation constant (K_d) of the reaction could be directly obtained from the ratio of dissociation rate and association rate constants. The values obtained are 1.0×10^{-8} M for progesterone and 1.2×10^{-8} M for aldosterone at 0°C. Scatchard techniques (10) have been employed to determine hormone affinities to the receptors. Derivation from the above reaction (2.2, part I) gave:

$$\frac{RS^*}{(S)} = -\frac{1}{K_d} \times RS^* + \frac{RT}{K_d}$$

Figure 1 shows the Scatchard plots for the three steroids. The K_d's extracted from the negative reciprocal of the slopes were 1.62 x 10^{-8} M for progesterone, 1.45 x 10^{-8} M for aldosterone, and 2.1 x 10^{-9} M for dexamethasone.

The K_d values for aldosterone and progesterone measured by the equilibrium technique shows reasonable agreement with those obtained from the kinetic studies (Table 2). The proximity in values of total receptor site for agonist binding in the study (Rt = 625 fmole/5 µg and 700 fmole/5 µg receptor protein for dexamethasone and aldosterone respectively) indicates that the system has reached equilibrium. The lower number in the total receptor site for antagonist progesterone (R_t = 470 fmole/5 µg) is due to some receptor degradation of inactive receptors that bind progesterone. The protein contents of the cytosol extraction was measured by the method of Lowry (11) and they are varied from 4.5 to 5 mg/ml.

4.2 Glucocorticoid-receptor complex stability

During periods shown in Figure 2, we obtained increasing binding of dexamethasone and aldosterone to the receptor. Similar values were found for the two steroids. By contrast, progesterone binding to the receptor decreased after two hours and insignificant amounts were detected by 12 hours. Thus, receptors complexed with dexamethasone and aldosterone were stable whereas the receptor-progesterone complex appear to be unstable.

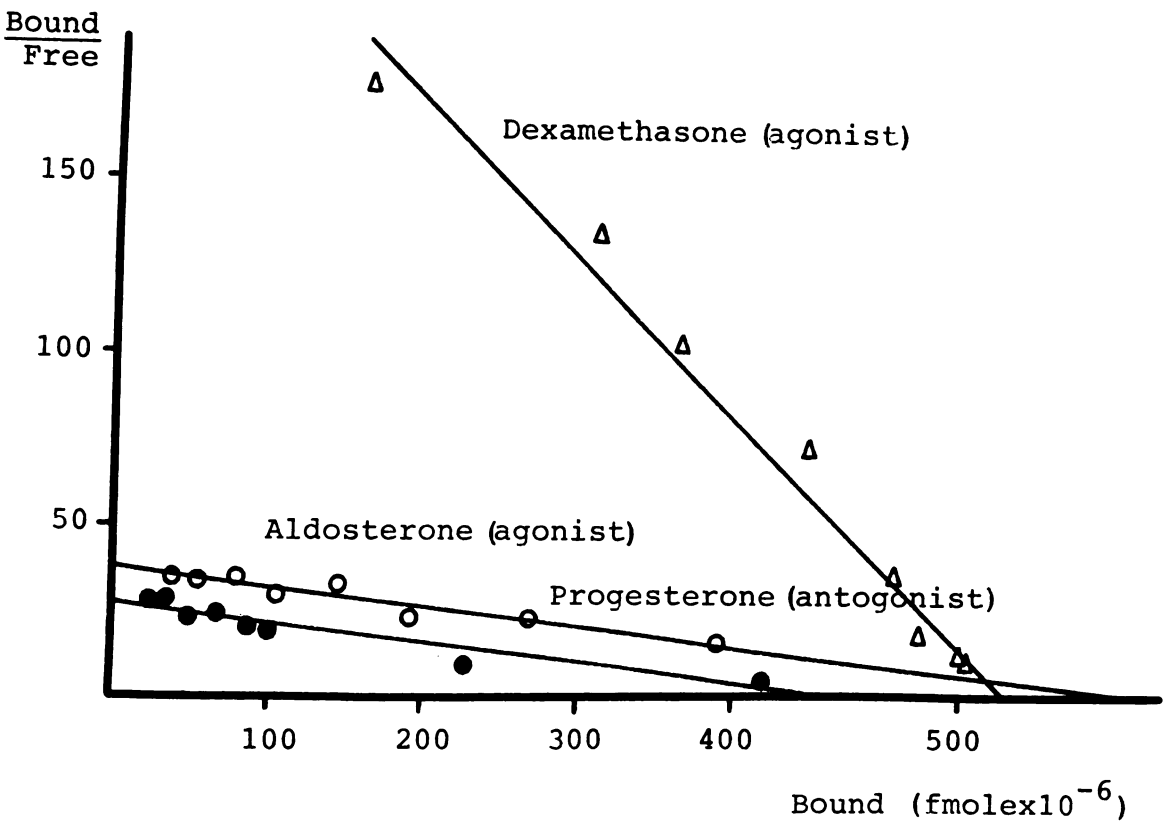

Figure 1 : Scatchard plots of agonist ^{3}H-dexamethasone, ^{3}H-aldosterone and antagonist ^{3}H-progesterone binding to HTC cytosol receptors. The cytosol receptor protein is stabilized by 3mM dithiothreitol and 10mM sodium molybdate.

PARAMETER	ANTAGONIST PROGESTERONE	AGONIST	
		ALDOSTERONE	DEXAMETHASONE
AFFINITY (nM) $0^{o}C$			
Scatchard	16.2	14.5	2.1
Kinetics	10.0	12.0	
Mean	13.1	13.2	
ON-RATE, $0^{o}C$ (10^{4} $M^{-1}sec^{-1}$)	35.0	7.0	
OFF-RATE, $0^{o}C$ (10^{-5} sec^{-1})	35.0	8.4	
OFF-RATE, $18^{o}C$ (10^{-5} sec^{-1})	238.0		21.0

Table 2 : Kinetics and affinity properties of the antagonist progesterone and the agonist aldosterone, dexamethasone with HTC cell cytosol receptors.

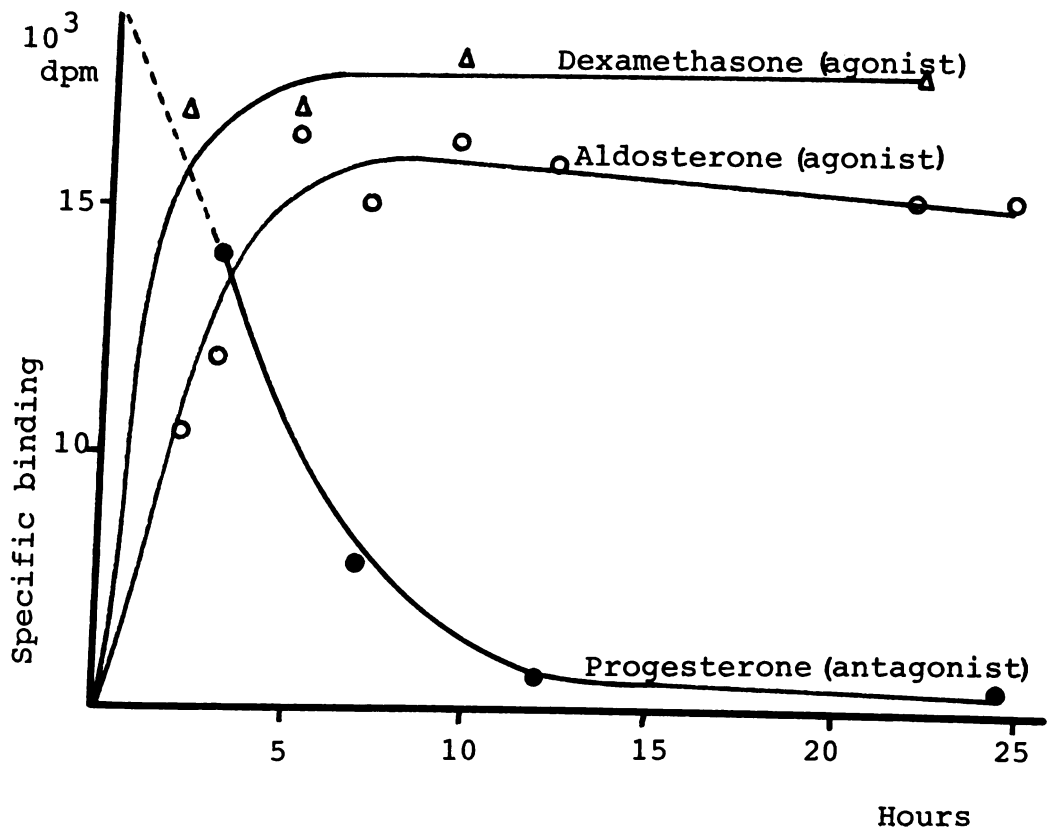

Figure 2 : Receptor stabilization study of agonist ^{3}H-dexamethasone, ^{3}H-aldosterone, antagonist ^{3}H-progesterone to HTC cytosol receptor at 0°C. The cytosol receptor protein is stabilized by 3 mM dithiothreitol and 10 mM sodium molybdate.

4.3 Association rate

Previous studies by Baxter and Rousseau, have shown that steroids associated with glucocorticoid receptors in a simple second order kinetic process as follows (9):

$$S + R \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} RS$$

For short reaction times, the dissociation rate (k_{-1}) of RS is negligible and the association rate (k_1) of S and R is:

$$\frac{d(RS)}{dt} = k_1 (R) (S)$$
$$= k_1 (R_t - RS) (S_t - RS)$$

total steroid concentration (obtained by direct measurement of radiolabeled steroid at each time indicated to avoid errors caused by losses due to absorption of steroid on the test tube).

RS: amount of steroid specifically bound to the receptor

R_t: total receptor binding site obtained from saturation curve

[R] and [S] are free receptor and steroid concentratons.

The integrated form is:

$$\frac{1}{S_t - R_t} \times \left(\ln \frac{R_t}{S_t} \right) \frac{S}{R} = k_1 t$$

A plot of the above equation (Figure 3) directly gives the association rates (k_1) of the steroid to receptor at 0°C from the slope. The values were $k_1 = 35 \times 10^3$ M^{-1} sec^{-1} for the antagonist progesterone and $k_1 = 7 \times 10^3$ M^{-1} sec^{-1} for the agonist aldosterone.

4.4 Dissociation rate measurements

The binding of hormone with receptor is a continuous process of association and dissociation between these two elements. In the presence of a large excess amount of nonradiolabeled hormone(s), as the radiolabeled hormone receptors (RS^*) dissociates, the association will favor the formation of nonradiolabeled hormone receptor complex. As a result, the changes of radiolabeled hormone receptor complex with time represent the dissociation rate of this complex expressed as following:

$$RS^* + S \rightleftharpoons RS + S^*$$

The above reaction is pseudo-first order and the rate law is:

$$\frac{dS^*}{dt} = k_{-1}(RS^*)$$

The integrated form is:

$$\ln \frac{RS^*}{R_T} = k_{-1}t$$

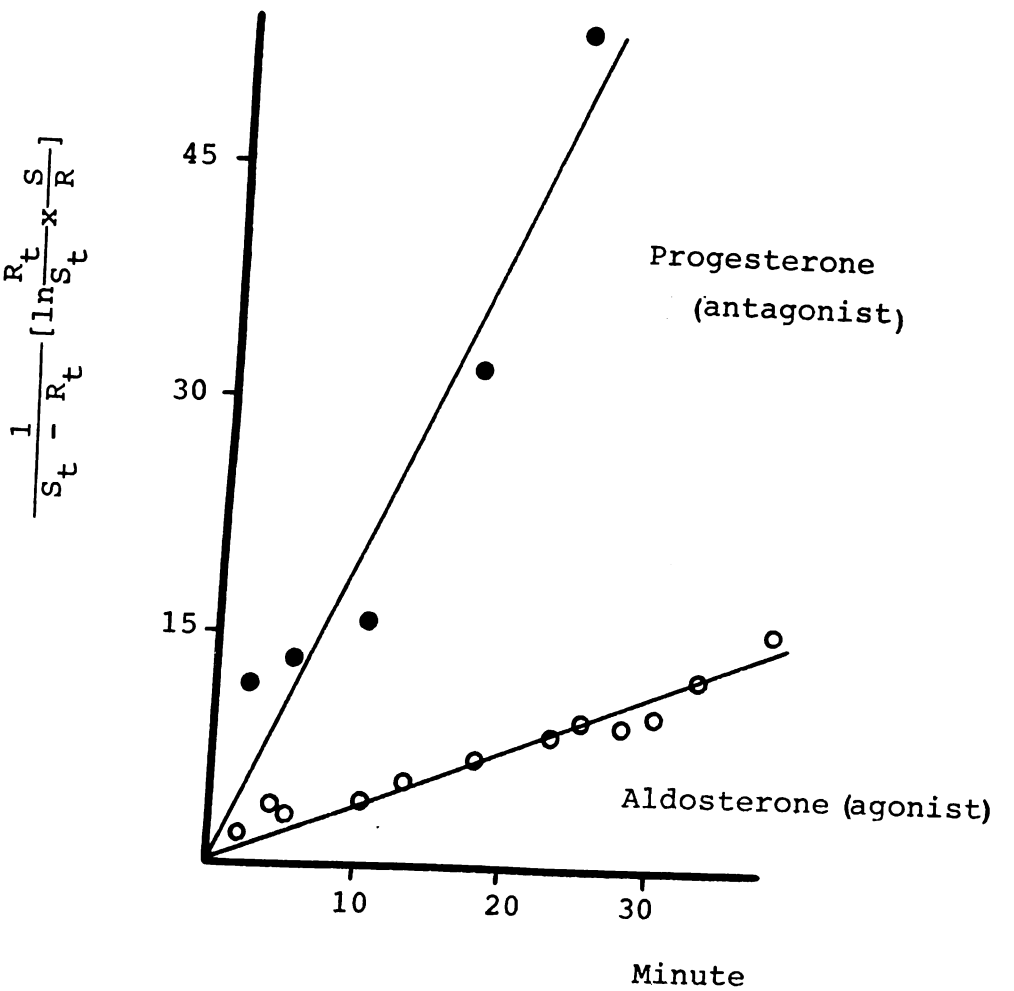

Figure 3 : Association rate (k_1) of aggonist ^{3}H-dexamethasone and antagonist ^{3}H-progesterone binding to HTC cytosol receptors measured at 0°C. k_{-1}(prog.)=3.5×10^{4} M^{-1} sec^{-1} and k_{-1}(dex.)=7×10^{3} M^{-1} sec^{-1}.

Figure 4 shows from the slope at 0°C the dissociation rate k_{-1} = 35 x 10^{-5} sec^{-1} for the receptor-progesterone complex and k_{-1} = 8.4 x 10^{-5} sec^{-1} for the receptor-aldosterone complex. Similarly, Figure 5 shows at 18°C that k_{-1} = 238 x 10^{-5} sec^{-1} for the receptor-progesterone complex and k_{-1} = 21 x 10^{-5} sec^{-1} for the receptor-dexamethasone complex. Because of the long half-life of receptor-dexamthasone complex at 0°C, it was convenient to study the dissociation rate of receptor-dexamethasone complex at higher temperatures (e.g. 18°C).

Table 2 summarizes our results of the kinetic properties of agonist and antagonist glucocorticoids in their associations with the receptors. The agonist aldosterone and the antagonist progesterone are observed to have similar apparent affinities (K_d) determined either by Scatchard analysis or kinetic measurements. However, at 0°C, the antagonist progesterone associates with the receptors five times faster than does the agonist aldosterone and progesterone remained associated with the receptors much more briefly (one fourth as long) than aldosterone. The table also shows that the dissociation of progesterone from the receptors is ten times faster than that of dexamethasone studied at 18°C.

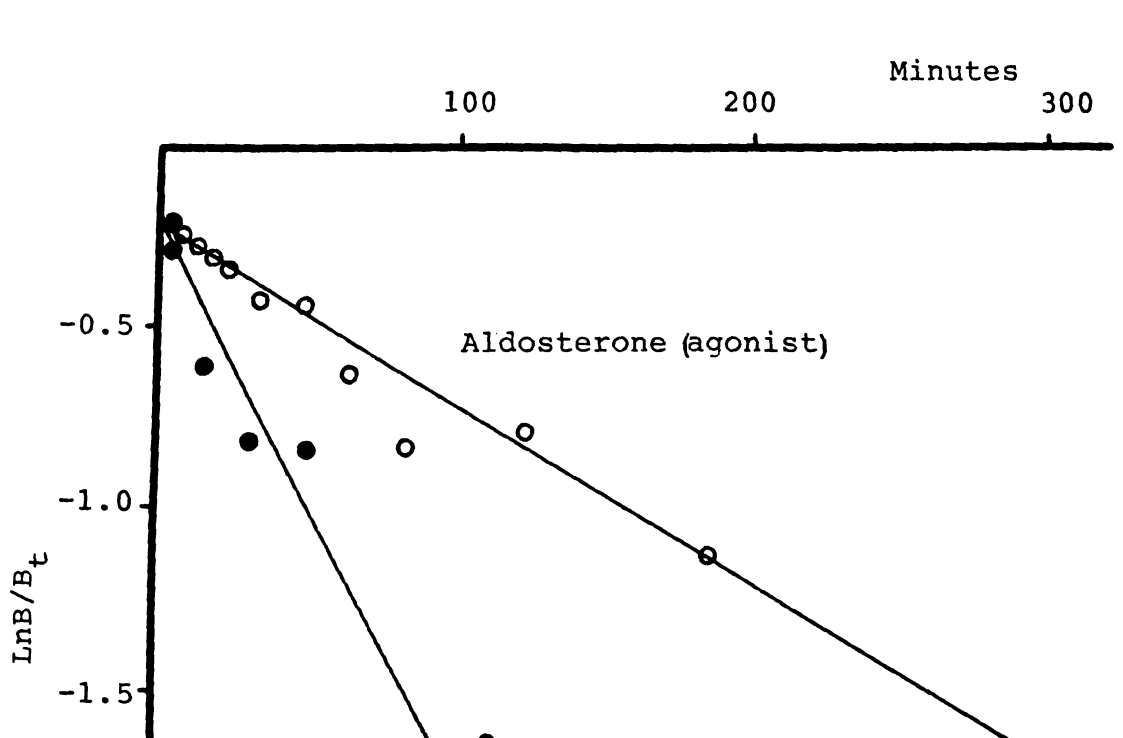

Figure 4 : Dissociation rates (k_{-1}) of agonist ^{3}H-aldosterone and antagonist ^{3}H-progesterone from HTC cytosol receptors mearsured at 0°C. k_{-1}(prog.)= 3.5×10^{-4} sec^{-1} and k_{-1}(aldo.)= 8.4×10^{-5} sec^{-1}.

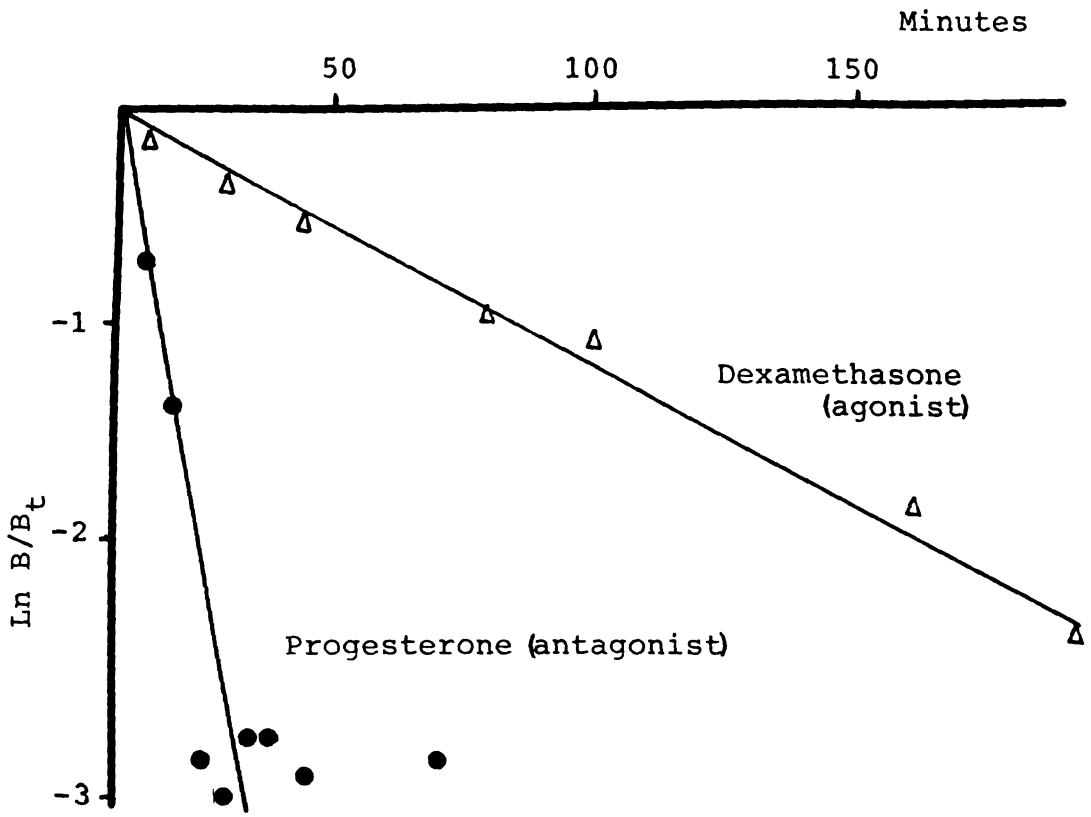

Figure 5 : Dissociation rates (k_{-1}) of agonist ^{3}H-dexamethasone and antagonist ^{3}H-progesterone from HTC cytosol receptors measured at 18°C. k_{-1}(prog.) =$2.38 \times 10^{-3} sec^{-1}$ and k_{-1}(dex.) = 2.16×10^{-4} sec $^{-1}$.

DISCUSSION

These studies were directed at elucidating more clearly the mechanism of glucocorticoid antagonist and agonist action. To do this, the kinetics of the interaction with glucocorticoid receptors of two agonists dexamethasone and aldosterone, were compared with those of the antagonist progesterone.

Samuels and Tomkins proposed that the steroid regulation of enzyme synthesis is mediated by an allosteric receptor system (1). Their model, comprising agonists, partial agonists, and antagonists, was proposed prior to the identification of receptors. Earlier, in the mid 1960's, Monod et al. (12) had hypothesized a similar allosteic model for regulatory protein. In 1972, Rousseau, Baxter, and Tomkins (9) demonstrated that the antagonist progesterone competitively inhibits binding of the agonist dexamethasone to glucocorticoid receptors.

According to the model, the receptors are assumed to equilibrate in two conformational states. The inactive state exists predominantly with the unoccupied receptors. This state is in equilibrium with the active state present at a lower concentration (responsible for the basal level of enzyme activity in the absence of the hormones). Binding of agonists to receptors in the inactive state will shift the equilibrium to the active state. Antagonists bind to the inactive receptors, stabilize them in this form and prevent agonist binding. Rousseau et al. (9) proposed that the shift in the receptor from the

inactive to the active state to permit high affinity agonist binding explains the slow association rate of agonists such as dexamethasone and cortisol. By contrast, the more rapid association rate of the antagonist progesterone was attributed to the fact that this steroid associates more rapidly with the form of the receptor prevalent in the unoccupied state.

It was also noted, however, that of the two agonists, cortisol and dexamethasone, cortisol exhibited more rapid binding, and in general (21) the rate of association of various glucocorticoids agonists inversely proportional to their rates of association (21). Therefore it is possible that the differences between progesterone and dexamethasone or cortisol can be attributed simply to the lower affinity of progesterone for the receptor than of cortisol or dexamethasone.

Rousseau et al. (9) also found a second line of evidence suggesting that there exist two conformational states of the receptors in the unactivated state. Unoccupied receptors or those bound by progesterone were found to be much less stable to heating than those occupied by cortisol or dexamethasone. However, again, the studies could be critical because the effects might solely be due to the differences in off-rate kinetics which were more rapid for progesterone than cortisol or dexamethasone.

In the current studies, an attempt was made to diminish these problems by comparing the kinetics (of binding and stability of bound receptors) of the agonist aldosterone that has an affinity for the receptors that is similar to that of progesterone. The relative affinities (K_d's) of aldosterone, progesterone, and dexamethasone for the HTC cell cytosol receptor (stabilized by 3 mM DTT, 10 mM Na_2MoO_4), determined by Scatchard analysis, were found to be 14.5×10^{-9} and 16.2×10^{-9} M, respectively (Figure 1). The linearity of the Scatchard data for all three glucocorticoids suggests that they all bind to a single class of receptor sites. Additionally, in competitive binding studies it was shown that dexamethasone prevented specific progesterone binding to the receptor to the extent predicted from the relative affinity for the two steroids for the receptors, lending support to the theory that agonist and antagonist glucocorticoids bind to the same site on the HTC cell cytosol receptors (9).

In receptor-stability studies involving measurement of radiolabeled hormone-receptor complexes incubated for up to 26 hours in the presence of 3 mM DTT and 10 mM Na_2MoO_4 to minimize receptor protein degradation (as discussed in Section 3.3.1, Part I), we found that the amount of receptor stabilzied by the low-affinity agonist aldosterone was as high as that for the high-affinity agonist dexamethasone. Conversely, receptors binding the antagonist progesterone underwent thermal inactivation, and the amount of radiolabeld progesterone receptor complex decreased with time; after 12 hr, little remained when compared

with the amount of receptor bound to either of the agonist (Fig. 2). It is unlikely that these marked difference can be explained by the slower off-rate of aldosterone, since its dissociation rate is intermediate between that of dexamethasone and progesterone. Thus, the findings support the notion that there are two classes of receptors, an active receptor form that complexes tightly with agonists and is more thermally-resistant, and an inactive form that is more subject to thermal inactivation and binds to antagonists. This classification of receptors is also supported by the fact that receptor-agonist, but not receptor-antagonist complexes have a greater compatibility to bind to the nucleus (12).

When the association rate (k_1) for aldosterone was compared with those for progesterone and dexamethasone, the findings indicated that, like dexamethasone, aldosterone associated slowly with the receptors, whereas progesterone associated rapidly. At 0°C, we obtained $k_1 = 7 \times 10^3$ M^{-1} sec^{-1} for aldosterone and $k_1 = 35 \times 10^3$ M^{-1} sec^{-1} for progesterone (Figure 3). Thus, the data are consistent with the notion that progesterone binds directly to the receptor form that pre-exists in an inactive state, since progesterone associates five times faster than aldosterone, which would bind preferentially to the active receptor forms available only after they shift from the inactive state.

Conversely, at 0°C, aldosterone dissociates nearly five times more slowly than progesterone ($k_{-1} = 8.4 \times 10^{-5}$ sec^{-1} and 35×10^{-5} sec^{-1}, respectively) (Figure 4), and dexamethasone was shown to dissociate

from the receptors ten times more slowly than progesterone (k_{-1} = 21 x 10^{-5} sec^{-1} and 238 x 10^{-5} sec^{-1}, respectively) (Figure 5). Thus, the equivalent aldosterone affinity for the receptors is due to a slower off and on rate as compared with progesterone.

In general, two allosteric models have been proposed to account for such behavior: The allosteric equilibrium model (Figure 6) and the induced fit model (Figure 7). Rousseau et al. (9) discussed their data in terms of the allosteric equilibrium model, and this has been the case with the preceeding discussion. However, these models cannot be distinguished by the current findings. Nevertheless, Pratt (14) has discussed glucocorticoid-receptor binding data in terms of the induced-fit model. This model assumes that receptors exist originally in the inactive form and that active receptors are the product of agonist binding (Figure 7). Supporting this model is the proposal that there exists an intermediate kinetic event before the formation of a stable hormone-receptor complex by the agonist-induced conformational change. This step would escape detection by the usual equilibrium assay method because of the rapid rate of dissociation of the agonist from this intermediate complex. The slow transition of this intermediate complex to a stable complex could explain the slow association rate of agonists to the receptors. Antagonists would only bind to the inactive receptors but would not promote a conformational change in the receptor. However, Pratt's data could also be explained by either model providing one assumes that the agonists do

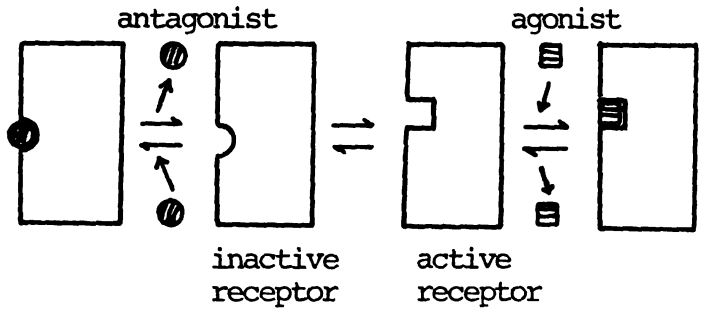

Figure 6: Allosteric model proposed by Samuels and Tomkins (1).

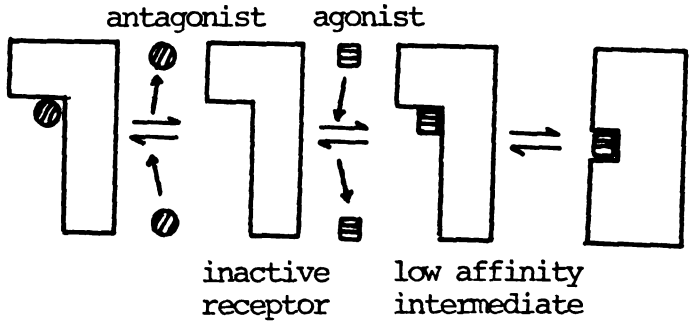

Figure 7: Induced fit model by Pratt et al. in Glucocorticoid Receptor system (14).

not, as we propose, bind to the inactive form with high affinity and that the time delay is forming the high-affinity agonist-receptor state could be due to the time required for allosteric equilibration.

As mentioned above, the agonist and antagonist glucocorticoids are assumed to compete for the same binding site of the receptor. However, a two-site model for receptor interaction with agonists and antagonist glucocorticoids was first proposed by Suthers et al., Fig. 8 (15) through their demonstration in rat liver cytosol that progesterone can bind the receptor that is still occupied by dexamethasone and that progesterone increased the rate of dissociation of dexamethasone and aldosterone from their receptor complex. Consistent with this observation is the recent report of Svec et al. (16) with both A_tT-20 cytosol receptors and intact cells of the enhancement of the dissociation rate of the agonists dexamethasone and triamcinolone acetomide from their receptor complex by progesterone and synthetic progestin R5020. In this model, the receptor is postulated to have two classes of binding sites: agonist site(s) and antagonist site(s). Progesterone, upon binding to the antagonist site, will induce a physical change in the glucocorticoid receptor to the inactive configuration, reducing the stability of dexamethasone in the agonist site as reflected in an increase in its dissociation rate. Binding of agonists will result in the active configuration, possibly reducing the affinity of the antagonist stage for antagonists and partial agonists. (Partial agonists can bind both sites, and this will produce equal por-

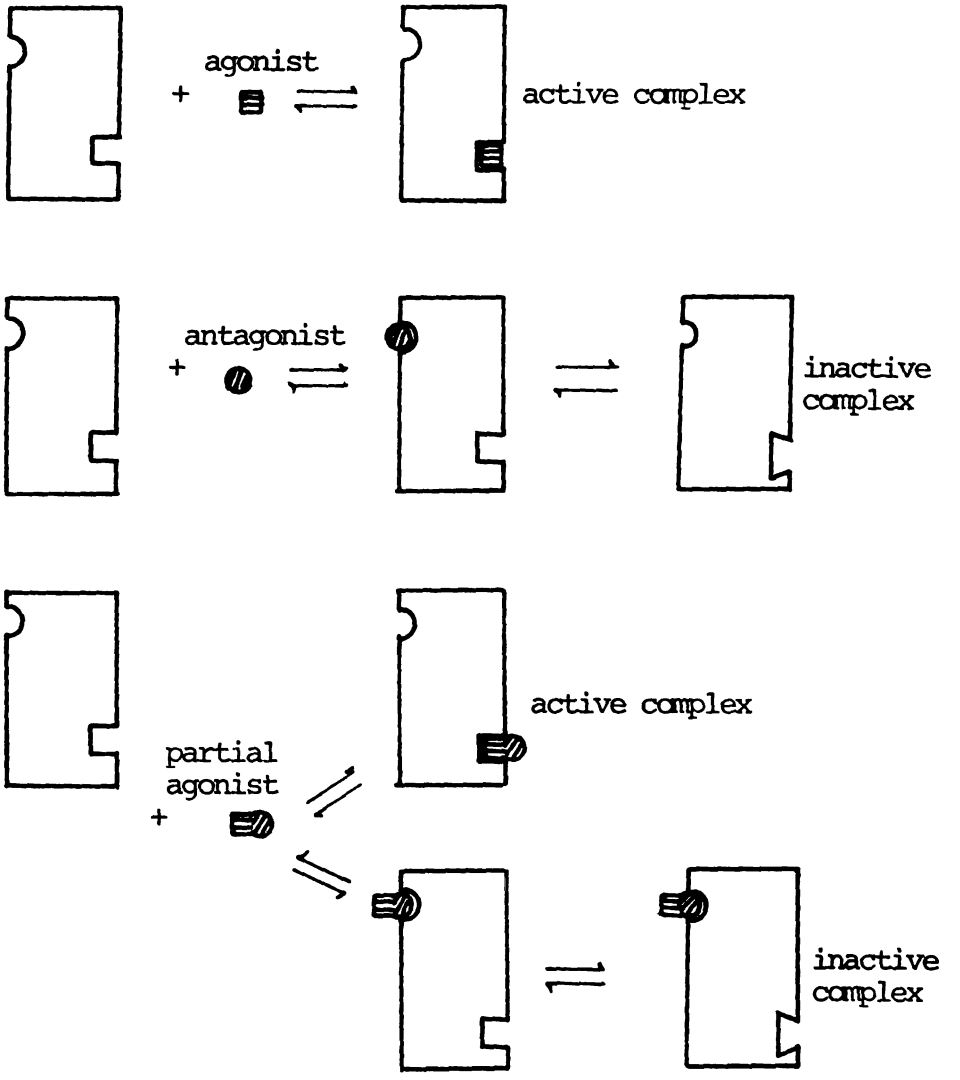

Figure 8 : Schematic picture of two site model proposal by Sutherland et al. (15).

tions of active and inactive receptors). This model cannot be excluded by the present data. However, it seems unlikely that progesterone, for instance, does not bind to the same site as cortisol, considering the near identity of their structures. Instead, it appears that there may be a second, lower affinity steroid-binding site on the receptor that may not be of biological importance that explains how concentrations of various steroids several order of magnitude above the physiologic can bind to and affect receptor properties.

To accommodate the discrepancies of the above model, the entry-site model was established by Bell and Jones (17; Figure 9). This is, in fact, a combination and extension of the allosteric, induced-fit, and two-site models. It extends the difference between agonist and antagonist at the activated receptor level. The steroid first weakly associates with the receptor on an entry site; this is followed by trapping of steroid at a higher affinity site. The binding on this site has two effects: first, it reduces the affinity of the second entry site for the steroid; and second, it activates the receptor if it is occupied by an agonist (but not by an antagonist). At the entry site the antagonist will associate with the receptor faster than the agonist, and it can displace the agonist from the high-affinity site (as observed by Suthers). Because the antagonist does not activate the receptor, its dissociation rate is higher compared with that of the agonist. The activated complex is believed to be more stable than the activated receptor can exist in the absence of steroids (as proposed

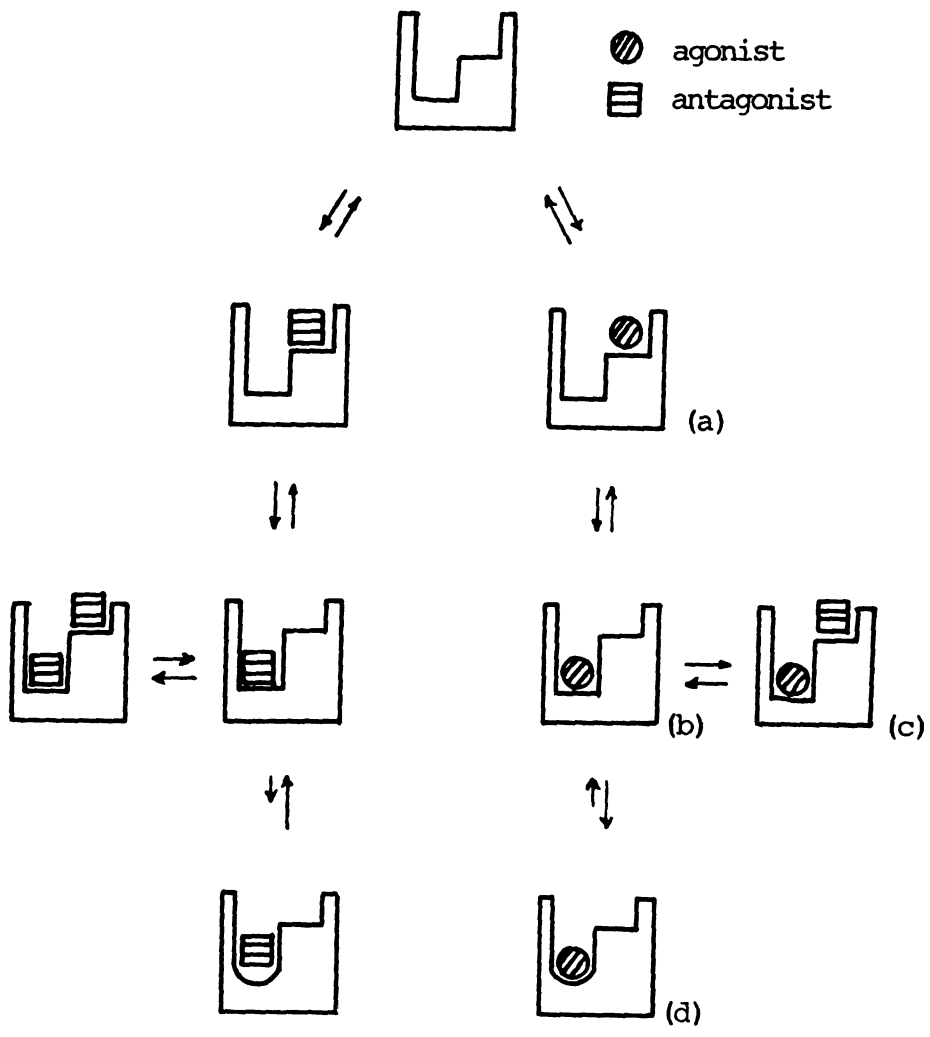

Figure 9: Pictorial representation of the entry site model. Form (a) of the steroid receptor complex is rapidly produced , but can be detected only indirectly by the kinetics of association. Form (b) is the slowly dissociating species detected by conventional assays. Form (c) is that which gives rise to enhanced dissiciation and form (d) is the activated steroid receptor complex. (From Antihormone, Agarwal M.K. ed. Elselier/North-Holland,pg. 47, 1979).

by Tomkins in the allosteric model). Again, validation of this model in terms of whether there are one or two sites depends on the considerations discuseed in the preceeding paragraph. An additional problem with the model is the fact that some antagonist (i.e. RU-38486, discussed below) can dissociate quite slowly from the receptor.

The findings with RU-38486 obtained by M. Moguilewsky and D. Philibert (unpublished data) has shown that the compounds is of particular interest with respect to the current findings. This glucocorticoid antagonist is a synthetic estrogen derivative, and is also a progesterone antagonist. Interestingly, this antagonist does induce n activation and nuclear binding of the progesterone receptor, but the complex does not activate subsequent events in progesterone action. RU-38486 was shown to dissociate at a much slower rate ($t_{\frac{1}{2}}$ = 150 min at 25°C and at least five days at 0°C) for non-activated receptors than the agonists dexamethasone, cortisol and corticosterone. However, after this complex was activated by heating for 30 min at 25°C, the dissociation rate of RU-38486 remarkably increased ($t_{\frac{1}{2}}$ = 70 min at 25°C). This increase is not associated with a significant increase in the ability of the RU-38486-receptor complex to bind to DNA cellulose and cell-free nuclear uptake of the ^{3}H-RU-38486-receptor complex at 25°C and 37°C, was found to be minimal. In their study, the rate of dissociation of progesterone from the receptor before and after heat treatment was the same ($t_{\frac{1}{2}}$ = 70 min at 0°C).

These findings indicate that slow off-rate, per se, cannot explain the mechanism of antagonists action in all cases. However, the studies indicate some differences in the mechanism of RU-38486 and progesterone interaction since the former steriod, but not the latter, can induce some change in the receptor as detected by differences in off-rate. These observations may reflect the fact that the conformational state induced by an agonist involves changes in addition to those reflected by increased nuclear or DNA binding or a change in the elution profile from DEAE columns. Indeed, the receptor is known to have domains other than those required for steroid and DNA or nuclear binding; for instance mutant receptors smaller in size than "wild-type" receptor can bind steroid and nuclei but are inactive in inducing glucocorticoid effects in S49 cells (19-20). It is possible, therefore, that some compounds can affect receptor properties to the extent that some, but not all of the necessary conformational changes in the receptors can occur and RU-38486 and progesterone could differ in this respect.

In summary, the finding that the antagonist progesterone exhibits a faster on-rate than an agonist, aldosterone, which has a similar affinity for the receptors and that progesterone-receptor complexes are much less stable than are aldoterone-receptor complexes is consistent with the notion that there are two states of the unactivated receptors. One state, the "inactive" one does not undergo the subsequent changes in conformation required for "activation" and nuclear

binding, whereas the other "active" state can. Agonists may bind more slowly to the receptors because some time is required for the allosteric shift to the active form for which they have a higher affinity. Whether the agonist binds loosely to the inactive form and then there is a transition to a higher affinity state or whether the agonist only binds to the active form cannot be ascertained from the current data.

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