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City University of New York PH.D. 1984

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BIOCHEMICAL REGULATION OF ESTRADIOL BINDING IN HUMAN ENDOMETRIUM

BY

ROSALYN DIANE BLUMENTHAL

A DISSERTATION SUBMITTED TO THE GRADUATE FACULTY IN BIOMEDICAL SCIENCE IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY, THE CITY UNIVERSITY OF NEW YORK.

1984

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2/10/1984
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Supervisory Committee

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Abstract

Biochemical Regulation of Estradiol

Binding in Human Endometrium

By

Rosalyn Diane Blumenthal

Advisor: Dr. Erlio Gurpide

The addition of molybdate to intact or homogenized cells of the endometrial adenocarcinoma line, HEC-1, or to homogenates of normal endometrium during incubation with 3H-estradiol at 4C caused significant increases in specific cytoplasmic E2 binding. The effects of molybdate appear to involve activation of E2 binding rather than protection from destabilization of binding. Fractionation of cell homogenates and recombination of subfractions revealed that molybdate ($MoO_4^=$) requires cytosolic factors as well as factors associated with the cell membrane to exert its effect. The addition of ATP, GTP or cGMP to homogenates of Human Endometrial Cancer (HEC) cells, normal or neoplastic endometrium, increased E2 binding to levels comparable to those obtained by addition of $MoO_4^=$. In contrast, the addi- tion of cAMP lowered E2 binding and counteracted the effects of $MoO_4^=$, ATP, GTP and cGMP. The binding sites generated by the addition of cGMP were found to sediment in the 8S and 4S regions of low salt glycerol gradients. The effects of ATP and GTP were elicited only in the presence of cell membrane factors, whereas both cyclic nucleotides exert their

respective effects when added directly to cytosol. It is hypothesized that MoO4=, ATP and GTP affect specific estrogen binding primarily by increasing the cGMP concentration through processes involving a plasma membrane bound guanylate cyclase. The cNMP effects were rapid, reaching completion in <15 minutes in the presence of Mg++, Mn++ or Ca++. Changes in estrogen binding (EB) levels were not observed if cNMPs were added to ATP depleted cytosol but responsiveness to the cNMPs was restored upon addition of exogenous ATP.

Fluctuations in estrogen binding by HEC cells in culture have been correlated with rapid changes in the ratio of the levels of cAMP/cGMP. In addition, the increases in EB induced by molybdate have been inversely related to changes in the cAMP/cGMP ratio.

The ATP requirement for cNMP activity and the pattern of dependence on divalent cation concentrations suggest that cGMP and cAMP effects on EB may be mediated by the action of cyclic nucleotide dependent protein kinases.

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This thesis is dedicated
to my family

- To my parents for always supporting my endeavors.

- To my brother- Andy- for being my friend as well as my sibling-- for the maturity and perceptivity that go far beyond your years.

- To my grandparents (Oma & Opa) for laying a solid foundation and for offering your love while asking nothing in return.

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INTRODUCTION

I do not know what I may appear to the world;
but to myself I seem to have been only like a boy playing
on the seashore, and diverting myself in now
and then finding a smoother pebble or a prettier shell
than ordinary, whilst the great ocean of truth lay
all undiscovered before me.

Sir Isaac Newton

Introduction

I Mechanism of Action of Steroids: (Figure 1)

A. Characterization of Cytoplasmic Binding Sites

The mechanism by which steroids enter target tissue still remains an open question. Many investigators believe that, because of their lipophilic nonionic nature, steroids freely diffuse through cell membranes. This belief is supported by superfusion studies of human endometrium and the measurement of unidirectional flow of steroid tracers (E. Gurpide and M. Welch, 1969). Evidence for a carrier system has been presented based on differential effects of sulfhydryl blockers on estradiol (E2) entry and E2 binding (E. Milgrom et al., 1973). Recently E2 binding proteins on the surface of rat endometrial cells have been shown and the investigators suggest that these binders may act as carriers (R J. Pietras and C.M. Szego, 1977).

The binding of steroid hormone to the cytoplasmic receptor protein has been implicated as the initial step in the action of the hormone on its respective target cell. The hormone binds to high affinity (10^{-10} - 10^{-8} M), limited capacity (approximately 15,000 sites/cell) cytoplasmic sites that show tissue specificity and result in a biological response. In general the concentration dependence for receptor binding and for responsiveness to steroid in target

cells is similar; the kinetics of binding is compatible to the time course of response. Cells that are deficient in cytosolic steroid receptor will be unresponsive to the hormone. A striking correlation exists between the ability of hormone antagonists to inhibit induction of response and the ability to inhibit binding of radiolabelled ligands (J.H. Clark et al.,1978).

Saturation analysis of rat uterine cytosol over a concentration range of 0.5-80nM estradiol (E2) indicates the presence of two binding proteins which can be resolved by the method of Scatchard and Rosenthal (G. Scatchard, 1945; H. E. Rosenthal, 1967). Both proteins are specific for estradiol; diethylstilbestrol can compete with labelled estradiol while progesterone, testosterone and cortisol at physiological concentrations are unable to compete. Both binding proteins show tissue specificity, they can be detected in uterus breast and vagina but not in spleen, kidney or serum. The first protein (type I) is a high affinity, low capacity binder. It is saturated with 20nM E2, has a Kd of 0.8nM and exists at a concentration of 1pmol/300ug DNA. The second protein (type II) is a low affinity, high capacity binder. It saturates with 80nM E2, has an approximate Kd of 30nM and is found in a 3-4 fold greater concentration than the type I site (J.H. Clark et al. 1978). The type I site sediments in low salt sucrose gradients as an 8S entity and is depleted under conditions that result in cytoplasmic receptor translocation. The type II site sediments as a 4S

entity and is unable to translocate to the nucleus (J.H. Clark et al., 1978). The significance of the type II site is uncertain. It has been suggested that this site may be involved in retention of steroid for subsequent binding to the type I site. Alternatively, it may serve as a precursor to the type I site (J.H. Clark et al., 1978). Such secondary sites are usually ignored and/or considered to be of no physiological significance However the proper evaluation of secondary binding sites is necessary for the valid measurement of estrogen receptors.

Two estrogen binding proteins have been purified from calf endometrium (Puca et al.,1974) and both of these two proteins possesses specific physical characteristics that have been well documented. These characteristics include isoelectric point, molecular weight, Stokes radius and frictional ratio (Table 1). The characteristics of each protein can be altered by changes in the salt concentrations or the presence or absence of protease inhibitors (A.C. Notides,1981).

Heterogeneity of cytosolic estrogen binders differing in their affinity for estradiol and perhaps in their translocatability to the nucleus have been found in human endometrium (R G. Smith et al., 1979) and in the human endometrial adenocarcinoma cell line HEC-1 as well (O. Friedman et al., 1980).

Table 1:

Characterization of Estradiol Binding Proteins From Calf Uterus

Characteristic:	Binding Protein #1:	Binding Protein #2:
Molecular Weight	238,000	61,000
Sedimentation Coefficient	8.6 S	4.5 S
Stokes Radius	67 °A	33 °A
Frictional Ratio	1.65	1.25
Axial Ratio		
Prolate	8.30	3.40
Oblate	0.09	0.31
Isoelectric Point	6.20	6.60, 6.80

(From G.A. Puca at al., 1981)

B. Transformation and Translocation

The binding of steroid to the cytoplasmic receptor results in a "transformation" or "activation" of the steroid receptor complex thus increasing the affinity of the receptor for chromatin and allowing for the translocation of the complex to the nucleus. There is a concommitant decrease in cytoplasmic binding and an increase in nuclear binding. Transformation is often associated with detectable changes in physical parameters of the binding protein. Changes in molecular weight, quarternary structure and sedimentation properties can be subtle or major depending on the the system in question. For the estrogen receptor, an increase in molecular weight (80K→ 130K) and sedimentation coefficient (4S→5S) have been reported.

A number of investigators have found that "activation" of the receptor is dependent upon the temperature (0C→30C), the concentration of salt (50mM→ 400mM KCl). Transformation can also occur by subjecting the cytosolic preparation to gel filtration or to dialysis (E.V. Jensen and E.R. Desombre, 1973).

A number of mechanisms for the transformation process of the estrogen receptor have been suggested. These mechanisms include:

(1) An association of the 4S form of the binding protein with another molecule resulting in the 5S form; either a dimerization of receptor monomers or addition of a nonsteroid binding subunit (A.C. Notides and S. Nielson, 1974).

(2) A conformational change of the untransformed receptor that would expose positively charged residues on the exterior surface of the receptor protein, thus increasing its affinity for negatively charged DNA. This mechanism is compatible with the claim that transformation is a first order reaction (E. Milgrom, 1981) and that it is hormone dependent and reversible (A. Bailly et al., 1980).

(3) Removal of a cytosolic factor, a putative inhibitor of the process. This suggestion would be consistant with the finding that transformation can be induced by dialysis and gel filtration of cell cytosol (B. Sato et al., 1979)

(4) A proteolysis of the receptor protein. A calcium activated transforming factor (RTF) with protease activity has been identified in calf uterine cytosol that cleaves the 5.3S form of the receptor into the 4.5S nuclear-binding form. (G.A. Puca et al., 1977).

(5) A phosphorylation of the steroid-receptor. Nuclear binding of the estrogen receptor complex is enhanced by incubation with 5-10mM ATP. ADP and AMP were not effective in causing transformation (R.J.B. King et al., 1977).

The process of transformation may simply be an IN VITRO artfact brought about by the artifactual association of various proteins, enzymes or other factors with receptors as a result of cell disruption and cytosol preparation procedures. The act of IN VITRO transformation may be simply to remove these inhibitory factors or aggregated proteins from the receptor, restoring it to its native IN VIVO state. (See W.W. Grody et al., 1982 for review).

C Nuclear and Post-Nuclear Effects

Nuclei contain nonhistone acceptor sites for the transformed complex (T.C. Spelsberg et al., 1971). The consequence of receptors relocating to the nucleus is an alteration in the pattern of gene transcription (A.R. Means & T.H. Hamilton, 1966). It has been observed that RNA polymerase activity and chromatin template activity are enhanced by the administration of estrogens (C. Raynaud-Jammet et al., 1971). O'Malley's laboratory has reported that the number of initiation sites increases in response to steroid. This result has been determined by the amount of binding of the antibiotic rifampicin to the B-subunit of RNA polymerase (M.J. Tsai et al. 1975). As a result of steroid, quantitative changes in the rate of elongation of RNA chains have been found. In addition, a stimulation of hnRNA rRNA and tRNA have all been demonstrated. Cycloheximide, actinomycin D and puromycin have all been shown to prevent hormone action by inhibiting mRNA and protein synthesis (L. Chan and B.W. O'Malley, 1976). The rate of protein synthesis has been shown to be modulated by (1) a change in the concentration of translatable mRNA, (2) a change in the rate at which ribosomes attach to mRNA and initiate protein synthesis as evidenced by an increase in polysome size and (3) a change in the rate of ribosomal movement along the mRNA (elongation) (R.P. Palmiter 1972). Estrogens do not have any effect on protein degradation (S.J. Higgins & V. Gehring, 1978).

D. Processing of the Nuclear Receptor Complex

The "off reaction" or fate of receptors after gene induction is completed has been minimally explored. A calcium activated protease with a low Km for the progesterone receptor has been found in chick oviduct nuclei (W.V. Vedekis et al., 1980). The proteolytic fragments of the receptor are no longer able to bind to DNA. In another report, polyribonucleotides have been shown to promote the release of androgen receptor from DNA. The receptor might therefore he released from chromatin by the nascent RNA chain whose synthesis it has just induced (S. Liao et al., 1980).

A study on the processing of the estrogen receptor has demonstrated that the filled nuclear receptors (RnE) are progressivly depleted by 3-5 hours following estrogen administration. This event is inhibited by actinomycin D (AcD) or chromomycin A3, compounds that can intercalate into G-C base pairs of DNA. Other intercalators that lack G-C specificity (e.g. adriamycin, ethidium bromide or quinicrine) or translation inhibitors (e g. cordycepsin) did not prevent estrogen receptor (ER) processing, suggesting that inhibition of RNA or protein synthesis was not involved (K.B. Horwitz and W.L. McGuire, 1978), but rather that AcD and chromomycin A3 have a direct effect on DNA conformation and thereby effect the interaction on RnE with chromatin.

In another report (F. Auricchio et al., 1981), it has been suggested that the processing is a result of a nuclear phosphatase that has a high affinity for the estrogen receptor complex. The enzymatic activity is inhibited by several phosphatase inhibitors including fluoride and molybdate, as well as by 4-nitrophenyl phosphate, a phosphatase substrate. Similarly, progesterone has been shown to antagonize estrogen activity presumably by promoting nuclear estrogen receptor dephosphorylation through an increase in nuclear acid phosphatase activity leading to inactivation of the binding protein (G. MacDonald et al., 1982).

FIGURE 1: Mechanism of Action of Steroid Hormones:

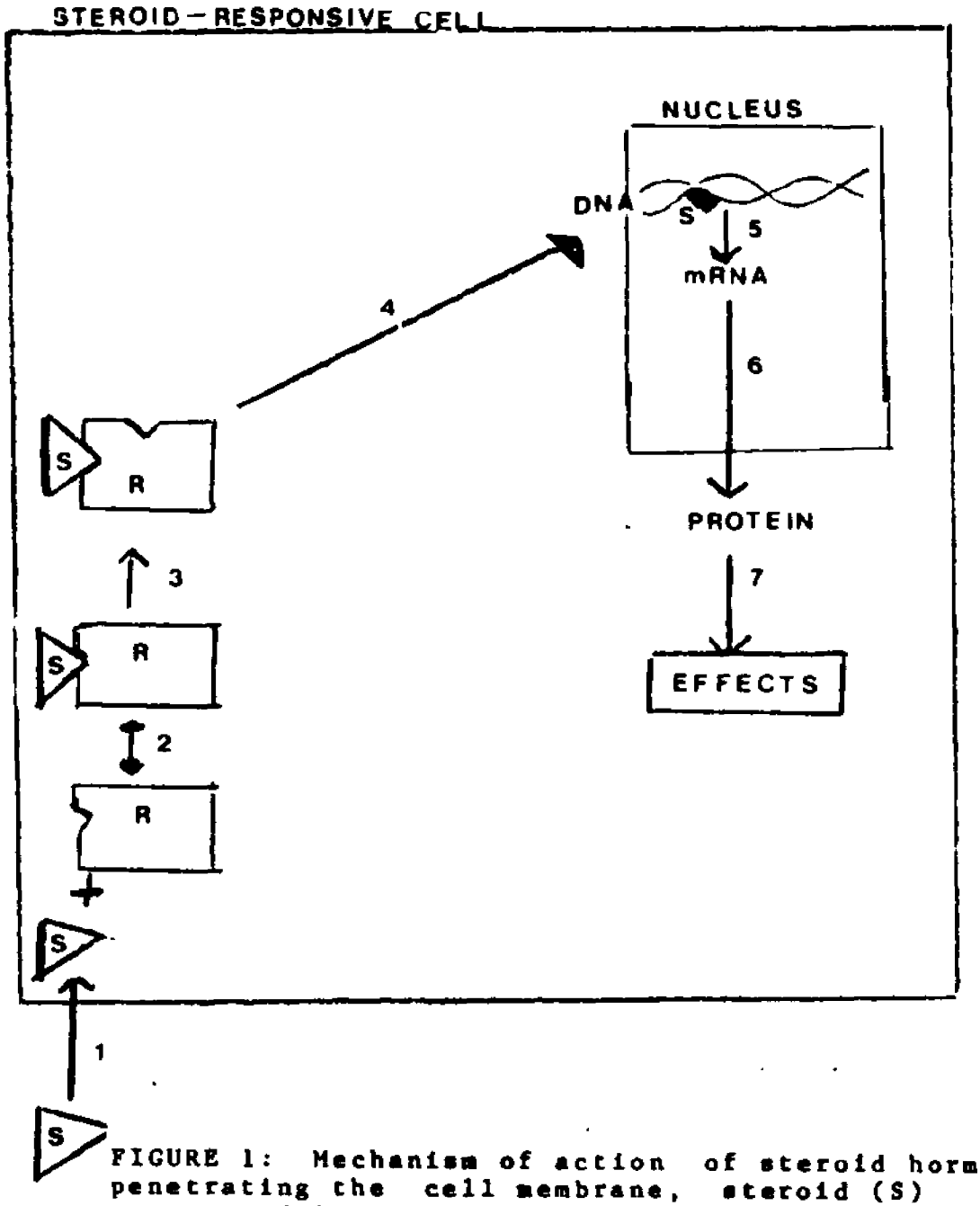


FIGURE 1: Mechanism of action of steroid hormones: After penetrating the cell membrane, steroid (S) combines with receptor (R) thus altering its conformation. S*R then binds to chromatin and stimulates (or possibly represses) mRNA synthesis. The changes in mRNAs result in alterations in the synthesis of specific proteins that mediate or reflect the steroid hormone response. 1. steroid entry; 2. cytoplasmic binding 3. transformation; 4. translocation; 5. transcription; 6. translation; 7. physiological response.

II Regulation of Cytoplasmic Steroid Binding

A. Significance of Receptor Regulation

The "Occupation Theory" of drug action, a direct application of the law of mass action, states that the magnitude of effect elicited by a drug (hormone) is directly proportional to the degree of occupancy of the receptors by the drug (hormone), with a maximal response corresponding to occupancy of all receptors (A. Goldstein et al., 1974). This concept can be applied to the relationship of estrogen binding to biological responses induced by estrogen. A number of years ago, two mechanisms for estrogenic action were postulated. The "Domino Theory" suggests that the steroid-receptor complex sets off a sequence of events such that one change leads to the next and each change depends only on the one directly preceeding it. The "Sustained Output Theory" states that the immediate and long term responses to steroid require the sustained presence of the steroid-receptor complex (B.S Katzenellenbogen & J. Gorski, 1979) (Fig. 2). Both theories are consistant with the idea that a cytoplasmic binding protein is essential for hormone action.

Over the past decade work has been directed towards elucidating how steroid receptor levels change within the menstrul cycle and what regulates these changes. The amount of endometrial cytoplasmic estrogen receptor shows a marked

Figure 2:
Mechanism of Estrogen Action: The Domino Theory and the Sustained Output Theory

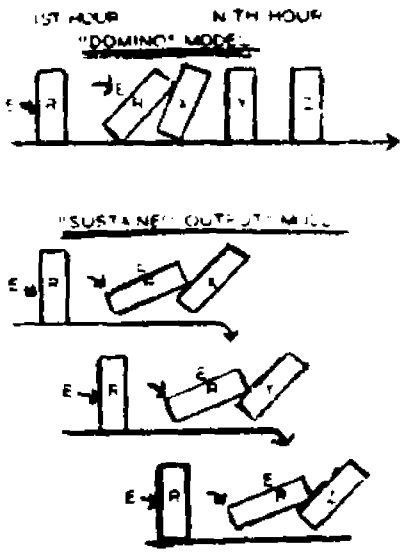


Figure 2: Models of the domino or sequential effect theory and the sustained output theory. E represents estrogen and R the receptor. X,Y and Z represent various tissue responses that occur at different times following estrogen administratin. (From Gorski and Raker, 1974).

decline during the luteal phase of the cycle (F. Bayard et al., 1978; P M. Martin et al., 1979; K. Pollow et al., 1980). This finding suggested that progesterone, the dominant ovarian hormone following development of the corpus luteum, might be responsible for the fall in estrogen receptors in secretory endometrium.

Administration of medroxyprogesterone acetate (MPA) for a few days to patients in the proliferative phase of their cycle resulted in lower estrogen receptor levels in the endometrial samples from these patients as compared to levels measured in untreated patients (P.M. Martin et al., 1979; K. Pollow et al., 1980; O Janne et al., 1980). There is suggestive but inconclusive evidence that estrogens may increase the levels of their own receptors (L. Tseng et al., 1977; P.M. Martin et al., 1979).

The changes in binding just described represent one form of regulation in which the amount of measurable receptor proteins varies over a period of many days. It has been suggested that any measurable changes in the concentration of receptor are dependent upon receptor synthesis and degradation (J. Mester & E.E. Baulieu, 1975) and on recycling of receptor proteins from the nucleus back into the cytoplasm (J A. Cidlowski and T.G. Muldoon, 1974). Recent results however, have suggested that the concentration of unoccupied steroid receptors in cultured cells fluctuate significantly over hourly intervals. Binding can triple within one hour

and decline just as rapidly, a rate of change that is much greater than would be expected from half life measurements of the steroid receptor protein (3-4 hours) and therefore, could not be explained by protein synthetic mechanisms alone (H. Fleming & E. Gurpide, 1981) (Fig. 3). It has been suggested that the receptor molecule can undergo a rapid and reversible biochemical process regulating the equilibrium between active (unmasked) and inactive (masked) binding forms of the receptor.

MODEL: $R(i) \longrightarrow R(a) + S \longrightarrow RS$

(inactive) ? (active)

Steroid binding fluctuations are not unique to this particular system. In WI138 fibroblasts, a reproducible pattern of changes in glucocorticoid binding has been reported. Two hours after replating these cells, GC binding declines by 50; binding quadruples after six hours and declines once

again by the tenth hour. These binding changes show an excellent temporal correlation with hormone responsivenes (V.J. Christafolo et al.,1979). In another study, glucocorticoid binding was measured during the cell cycle in HeLa cells that had been synchronized with a double thymidine block procedure. Binding doubled between the late G1 and S phase and was significantly reduced during the G2 to M part of the cycle (J.A. Cidlowski and G.A. Michaels, 1977).

The mechanism underlying these rapid changes in the levels of steroid binding has been the subject of intense investigation over the past few years

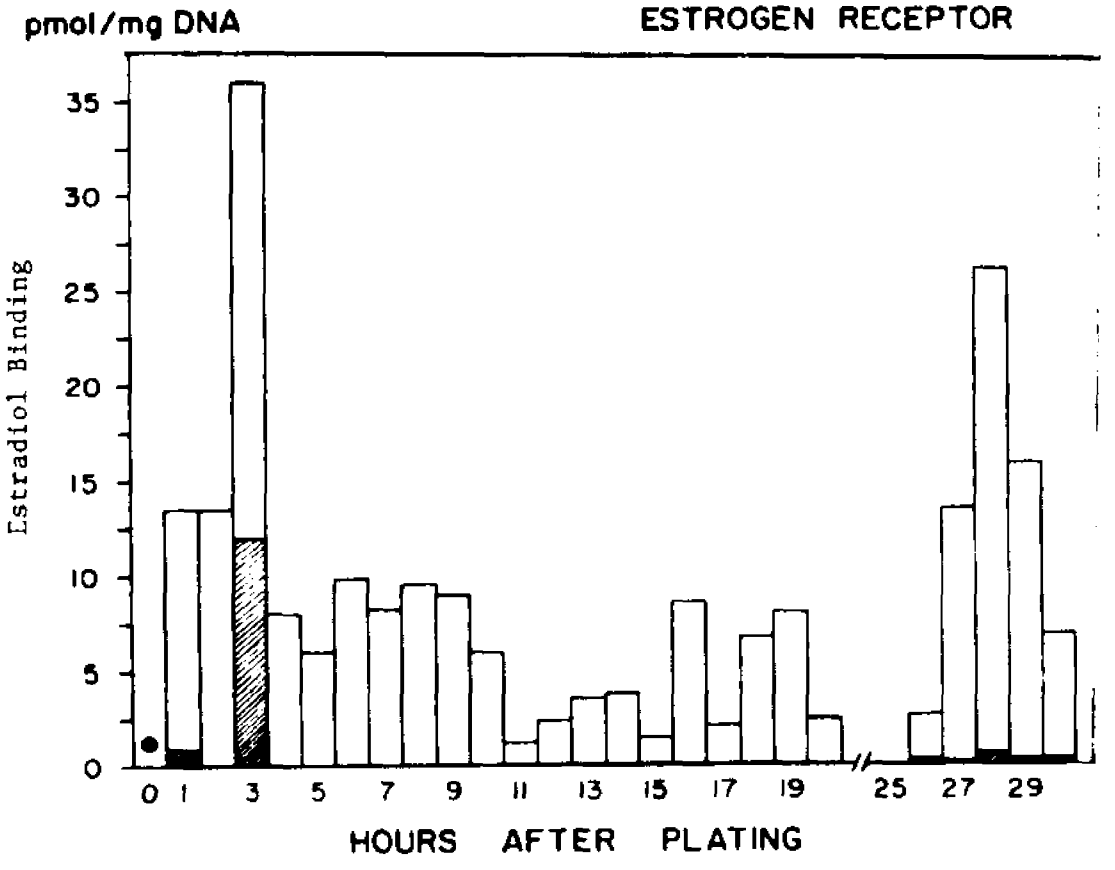


FIGURE 3 Fluctuations of estradiol binding in HEC cells in culture. Cells were maintained in serum free medium for 3 days prior to the start of the experiment and were refed the morning of the experiment. Samples of cells were assayed for total binding before replating and refeeding (time zero) and hourly from 2-20 hours and 25-30 hours. Four dishes were pooled for each experiment. (From Fleming and Gurpide 1981).

B Proteolysis and Receptor Stability

Physiological reactions are thought to be regulated by four mechanisms: (1) allosteric modification and (2) covalent modification of a protein; two rapid and often reversible types of changes, (3) transcriptional control; a slow and irreversible change and (4) proteolytic conversion of an inactive precursor protein to an active form; a mechanism which produces rapid and irreversible changes. Examples of physiological systems regulated by limited proteolysis include blood coagulation and complement formation, production of hormones, regulatory peptides and pancreatic zymogens (e.g. insulin, kinins and trypsin), collagen assembling processes and fertilization (H. Neurath and K.A. Walsh, 1977).

Proteases have been reported to alter the sedimentation profile of steroid receptors (A.C. Notides et al., 1973 E.M. Wilson and F S French, 1979). A large body of conflicting evidence, is now available describing proteolytic effects on steroid binding capacity. In a number of reports, the addition of serine or thiol-serine protease inhibitors such as diisopropyl fluorophosphate (DFP) or leupeptin to the homogenization buffer prior to labelling with steroid (G. Daxenbicher et al. 1977; G.S. Prins and C. Lee 1982) or after the labelling process is complete (T. Hazato and A. Murayama, 1981) resulted in a greater quantity of estrogen

receptor (ER) and progesterone receptor (PgR) in uterus and androgen receptor (AR) in prostate. These results are explained as a protective effect against proteolytic breakdown of the receptor protein. In contrast, there exists a number of reports which state that addition of protease inhibitors such as tosyl-lysine chloro-methyl ketone (TLCK), tosylamide phenyl ethyl chloromethyl ketone (TPCK) and phenylmethylsulfonylfluoride (PMSF) or protease substrates (Tosyl-arginine methyl ester and tryptophan methyl ester) decreases the amount of measurable steroid binding while the addition of trypsin increases binding capacity (M.E. Baker et al. 1978 K. Pettersson et al., 1982) although the mechanism of action is unclear. The reason for the difference in results between reports is also uncertain, however protease inhibitors tend to be somewhat nonspecific in their action. Reports in which protease inhibitors do not have any effect on steroid binding also exist (C.J. Nielson et al., 1977b).

C. Sulfhydryl Groups and Receptor Stability

The unoccupied steroid receptor in many tissues is unstable IN VITRO presumably due to oxidation of the receptor sulfhydryl groups. The rapid temperature-dependent inactivation of receptor protein can be prevente or reversed by addition of sulfhydryl reducing compounds such as dithiothreitol, mercaptoethanol, thioglycerol or glutathione, resulting in a stimulation in steroid binding activity (J.P. Granberg and P.L. Ballard, 1976). Scatchard analysis indicates that compounds like dithiothreitol influence the number of binding sites, rather than the affinity of the steroid for the binding protein (W.L. McGuire and M. DeLa-Garza, 1973). Addition of oxidizing compounds such as N-ethylmaleimide or p-chloromercuri phenylsulfonic acid inactivates the receptor; the presence of bound steroid protects the receptor against inactivation. These results have been reported for receptors for glucocorticoids, androgens, estrogens and ecdysone. Stabilization by reducing agents is distinct from stabilization by EDTA, a divalent cation chelator, since the two agents have an additive effect (A.M. Rees and P.A. Bell, 1975).

Dithiothreitol and molybdate have a synergistic effect on receptor stabilization (J. Hubbard and M. Kalimi, 1982), although it is unknown whether they act directly on the receptor or on receptor-modulating enzymes or factors. The two agents together increase the sedimentation coefficient

cient of the steroid receptor complex, possibly by causing aggregation or by preventing subunit dissociation. Together they also prevent dialysis induced inactivation, perhaps by preventing removal of a small molecular weight stabilizing factor or by eliminating the requirement for a stabilizing factor. In rat pancreas, estradiol binding has been shown to be stimulated by a water soluble, heat stable, dialyzable oligopeptide (A.M. Boctor et al.,1981) referred to as "accessory factor". An endogenous heat stable glucocorticoid activating factor has also been identified. This endogenous activating factor (EAF) appears to be thioredoxin and is dependent upon temperature and NADPH and is blocked by arsenite (J.F. Grippo and W.B. Pratt, 1983). A molecule that inhibits cytosolic androgen binding has been identified in a mouse Leydig cell tumor; dialysis removes the factor and converts the inactivated receptor form to a receptor binding form (B. Sato et al., 1981).

D. Phosholipids and Receptor Stabilization

Soluble steroid binding capacity can be inactivated by incubation with phosholipase A2 (PLA2), an effect that is calcium dependent and is blocked by addition of phospholipids (H.F. Schulte et al., 1976) and by the addition of molybdate (K.L. Leach et al., 1983). It is possible that the receptor protein is associated with a phospholipid component which is required for steroid binding. Alternatively, PLA2 may form detergent products which in turn may inactivate the steroid receptor. This second possibility is supported by the finding that lysophosphatides can inactivate both glucocorticoid and progesterone receptors (H.M. Westphal et al., 1978). However, phospholipase C, which does not produce lysophosphatides, is also able to inactivate steroid binding. In addition, molybdate can block inactivation of glucocorticoid receptors by PLA2 but it can not inhibit the conversion of phosphatidylcholine to lysophosphatidylcholine by PLA2 (K.L. Leach et al., 1983). These two findings taken together argue against the possibility that PLA2 mediates steroid binding by forming detergent products. Rather, it appears to act by disrupting phospholipids that are associated with the steroid binding protein.

E. ATP is Required for Receptor Stabilization

Energy related reversible changes in steroid binding have been demonstrated in cell culture (A. Munck et al., 1972; G.P. Rossini and S. Liao, 1981; R. H. Wheeler et al., 1981) and IN VITRO using in a cytoplasmic preparation (J.J. Sando et al., 1979). Under anaerobic conditions, the ability of cortisol to decrease the uptake of glucose by thymus cells is abolished. Thymus cells that are incubated in the absence of glucose and oxygen also show a significant decrease in the amount of specific cytoplasmic glucocorticoid binding from levels present under aerobic conditions, a result which correlates well with a loss in cellular ATP. Binding is restored when cells are returned to an aerobic enviroment, even in the presence of a protein synthesis inhibitor such as cycloheximide. Nonspecific binding is virtually unaffected by these metabolic alterations (A. Munck et al. ,1972; P.A. Bell & A. Munck, 1973).

In another set of experiments, Liao demonstrated that the prostatic androgen receptor is rapidly inactivated to a nonbinding form (t1/2=2 minutes) in the presence of the oxidative phosphorylation uncoupling agent 2,4-dinitrophenol (DNP). The observed decrease in androgen binding did not result from (1) a decrease in the stability of the androgen-receptor complex, (2) a decrease in the uptake of steroid or (3) an increase in the metabolism of steroid (G.P. Rossini and S. Liao, 1980). Androgens appear to have a stabilizing

affect on the receptor molecule since DNP is unable to inactivate the protein once it is occupied by steroid. The inactive receptor can be efficiently reactivated by addition of nucleotide triphosphates (ATP is most efficient), even if protein synthesis is blocked (G.P. Rossini & S. Liao, 1980).

Pratt and his associates have shown that when IM-9 human lymphoblasts are incubated in a glucose free, nitrogen rich atmosphere, both the ability to bind steroid and the ATP levels declined. When glucose and oxygen were reintroduced, ATP levels and receptor activity returned. Activation is rapid (10 minutes to reach maximum levels), independent of protein synthesis and directly correlated with cellular ATP content (R.H Wheeler et al., 1981). Lastly, in another study by Pratt's group, specific glucocorticoid binding capacity in cytosols prepared from L929 mouse fibroblasts could be inactivated by increasing the temperature from 4C to 25C (T1/2=2 hours). ATP slows the rate of inactivation. Following complete inactivation, addition of molybdate, a recognized phosphatase inhibitor and ATP together results in a 40-70% reactivation of steroid binding. The effect is inhibited by EDTA and this block is overcome by the addition of magnesium. Receptor reactivation is temperature dependent, suggesting that an enzymatic process is involved (J.J. Sando et al., 1979). Pratt's group has postulated that a kinase may be mediating the reactivation process but in pilot experiments were unable to show effects of cAMP, cGMP or the Walsh protein inhibitor of cAMP-dependent protein kinase on steroid binder reactivation.

In all of these studies, the correlation between specific binding and amount of cellular ATP suggests that ATP may be required to maintain and/or restore the structure of the steroid receptor.

F. Is the Steroid Receptor a Phosphoprotein ?

A number of direct demonstrations of steroid receptor phosphorylation have recently been published. The calf uterine estradiol receptor appears to be a substrate for a nuclear phosphatase (see introduction-IX-1E) thus resulting in a loss of binding ability. The receptor can be rephosphorylated IN VITRO with a purified cytosolic enzyme that has a high affinity for the inactive receptor, is ATP dependent and is stimulated by MgCl2 and CaCl2 (A. Migliaccio et al., 1982). In the same study, this group treated the (32P-)ATP labelled cytosol preparation with a monoclonal antibody to the receptor and examined the immunoprecipitated receptor by gel electrophoresis under denaturing and non-denaturing conditions and by centrifugation through sucrose gradients, showing that the receptor was phosphorylated.

Progesterone receptor subunits, purified by ammonium sulfate precipitation and 3 succesive chromatographic steps (phosphocellulose, DNA-cellulose and heparin-sepharose), have been phosphorylated with γ-32P-ATP and physiological concentrations of a cAMP dependent protein kinase isolated from bovine heart. The phosphorylation reaction was 50% complete in 5 minutes (N.L. Weigel et al.,1981).

In another study, IN VIVO phosphorylation of the oviduct progesterone receptor was demonstrated by injection of 32P-orthophosphate into chickens (J.J. Dougherty et al.,1982). The receptor was purified by affinity chromatography, gel

filtration and ion-exchange chromatography and a isotopically labelled 90,000 molecular wei component was identified on SDS gels. Partial acid hydrolysis and thin-layer electrophoresis of this protein showed that the only phosphoamino acid was phosphoserine.

Housley and Pratt have recently published results demonstrating phosphorylation of glucocorticoid receptor by culturing intact L-cells with 32P-orthophosphate. The receptor was purified by affinity chromatography and analyzed by SDS-polyacrylamide gel electrophoresis and isoelectric focussing. A 92,000 phosphoprotein was identified as the receptor by covalently radiolabeling it with a site-specific affinity ligand. Cytosol from 32P-labelled glucocorticoid resistant L-cells which contains 5% of the steroid binding capacity of the sensitive cells contains very little 92,000 phosphoprotein. The glucocorticoid receptor in this study has also been shown to contain a phosphoserine moiety (R. Housley and W.B. Pratt, 1983).

The results described regarding (1) the necessity for ATP to stabilize binding (section 2E) and (2) the finding that receptor proteins in a number of different systems can be phosphorylated IN VITRO (section 2F) are consistant wit the hypothesis that steroid receptors can serve as substrates for phosphorylation. It is uncertain, however, whether this phosphorylation affects steroid binding.

G. Is Steroid Binding Affected by Phosphorylation ?

Much supporting evidence now exists in favor of the hypothesis that the reversible ATP dependent stabilization of binding described in section 2E is due to a phosphorylation reaction and that the inactivation or destabilization of binding is a result of a dephosphorylation of the receptor. Addition of purified alkaline phosphatase to cytosol prepared from L929 cells inactivated unoccupied glucocorticoid binding sites, a reaction that requires zinc and is inhibited by arsenate (C.J. Nielson et al.,1977). In two similar studies, a decrease in cytoplasmic glucocorticoid binding in Xenopus Laevis liver (F.E.B. May and B.R. Westley, 1982) and a decrease in cytoplasmic estradiol binding in rabbit corpus luteum (K.M. Yuh and P.L. Keyes, 1981) are observed upon addition of exogenous alkaline phosphatase. These results suggest that one or more phosphate groups are necessary to maintain the hormone binding capacity of the receptor protein.

A second approach which suggested that steroid binding is affected by phosphorylation was the copurification by DEAE-cellulose chromatography of endogenous membrane associated "receptor inactivating activity" in L-cells with endogenous dephosphorylating activity by DEAE-cellulose chromatography (C.J. Nielson et al.,1977).

A third approach used to address this issue has been to show that inhibitors of phosphatase activity such as molybdate and vanadate (K. Paigen, 1958), when added to steroid binding assays, permit detection of larger quantities of steroid binding, presumably by inhibiting a dephosphorylation reaction and thus preventing or slowing the inactivation of unoccupied steroid binding sites. This result has been demonstrated for glucocorticoid receptors (C.J. Nielson et al.,1977b), estrogen receptors (K.M. Anderson et al.,1980; L.K. Miller et al.,1981), progesterone receptors (W.W. Grody et al., 1980; T.J. Chen et al.,1981), androgen receptors (C.M. Gaubert et al.,1980; W.W. Wright et al.,1981). aldosterone receptors (D. Marver 1980) and vitamin D receptors (D. Feldman et al., 1979). Addition of molybdate ($MoO_4^=$) to the steroid binding assay does not affect the affinity of the steroid for the binding protein; the association and dissociation constants are unchanged (C.M. Gaubert et al.,1980; G. Shyamala and L. Leonard, 1980).

Figure 4 represents a model suggested by Pratt and his associates for the mechanism of regulation of cytoplasmic steroid receptors.

FIGURE 4: MODEL FOR RECEPTOR REGULATION BY PHOSPHORYLATION:

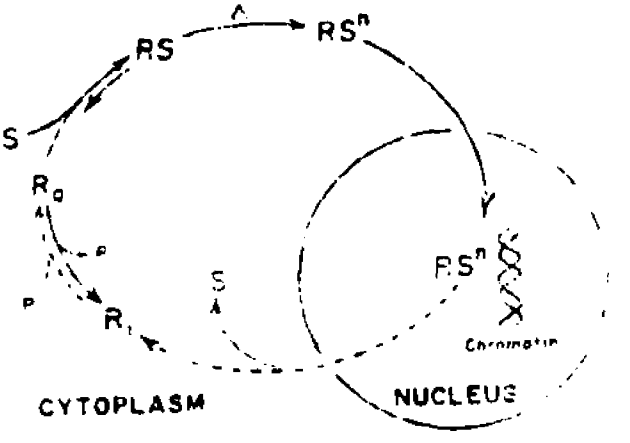


FIGURE 4: Proposed cycle of events controlling the binding state and cellular location of the glucocorticoid receptor. Ri= dephosphorylatd receptor that is inactive; Ra= phosphorylated receptor that is active. RS= steroid-receptor complex; RSn= complex that binds to nucleus. (From Wheeler et al.,1981)

In summary, a direct role for phosphorylation in receptor regulation is suggested by: (1) the use of exogenous alkaline phosphatase to inactivate binding capacity, (2) the copurification of receptor inactivating activity and dephosphorylating activity, (3) the finding that nuclear processing of the estrogen receptor involves nuclear phosphatase activity (F. Auricchio et al., 1981) and (4) the use of putative phosphatase inhibitors to block binder inactivation.

H. Activities Associated with Molybdate

It has been thought that molybdate stabilizes steroid binding by its ability to inhibit phosphatase activity. However, four observations have recently been made that challange this hypothesis.

In the first observation, inactivation of unoccupied cytoplasmic receptors in L929 cells appears to be a nonenzymatic process. Inactivation can be induced at 0C by 1) exposure to salt (50-150mM NaCl), 2) precipitation by $(NH_4)_2SO_4$ or 3) Sephadex G-50 gel filtration. Molybdate can prevent all three forms of inactivation (K.L. Leach et al.,1979). A similar finding has been reported for the rat liver glucocorticoid receptor and the rat prostate androgen receptor (K. Noma et al.,1980). Both of these receptors could be inactivated by dialysis or gel filtration at 0C and inactivation could be blocked by $MoO_4^=$. It is questionable whether a phosphatase could be involved at nonphysiological temperatures. Many, but not all enzymatic activities are reduced or totally abolished at 0C. Therefore, the criterion of temperature dependence alone is not sufficient to exclude the possibility that a phosphatase is involved in the process of steroid receptor regulation.

The second observation that challanges the idea that molybdate stabilizes steroid binding by its phosphatase inhibitory activity is based on the finding that molybdate will prevent inactivation of the chick oviduct progesterone

receptor only if the receptor is in the aggregated state. Molybdate is able to stabilize binding if the cytosol preparation has been pretreated with KCl or ammonium sulfate or exhaustive dialysis (W.W. Grody et al.,1980). A model that states that $MoO_4^{=}$ is acting by preventing phosphatase activity, would require the dimer form of the receptor to be a much more favorable substrate for a hypothetical stabilizing protein kinase than the monomer form. This could only be true if dimerization involves a conformational change of one or both subunits of the receptor, thereby, exposing essential sites that can be phosphorylated.

That third observation that addresses the mechanism of $MoO_4^{=}$ activity centers around the finding that other phosphatase inhibitors such as fluoride, glucose 1-phosphate and tungstate do not protect the glucocorticoid receptor from inactivation like molybdate does (K.L. Leach et al.,1979). A proposal has been put forth suggesting that molybdate prevents inactivation of steroid receptors by interacting with a phosphate moiety on the receptor protein (see section 2-H). Substances such as tungstate form much weaker complexes with phosphate and therefore, do not protect steroid binding as effectively as molybdate.

The fourth relavent observation concerning the stability of the unoccupied androgen receptor in mouse kidney cytosol at various temperatures (W.W. Wright et al.,1981). An Arrhenius analysis of the effect of temperature on receptor

inactivation indicated that the enthalpy (ΔH) of inactivation in the presence of molybdate was 20% gretaer than in the absence of molybdate. The result was surprising since energy should not be expended in degradation if molybdate is competitively inhibiting a phosphatase. Since competitive inhibition does not affect rate constants, molybdate should not change any of the thermodynamic properties of the reaction. The increase in ΔH found with molybdate is more consistent with uncompetitive or noncompetitive inhibition but these forms of inhibition are uncommon with phosphatases. Wright et al have also shown that molybdate stabilizes androgen binding only in a pH range of 6.0-7.6. Most phosphatases, however, are active in the acid or alkaline pH range as well.

A number of other mechanisms have been suggested to explain how molybdate is able to prevent inactivation of steroid binding. One possibility is that $MoO4^{=}$ can directly interact with the receptor (K. Noma et al.,1980; W.W. Wright et al.,1981). Such an interaction could block proteolytic cleavage of a vulnerable site. In such a case, additional energy would be required to remove the molybdate before proteolysis could occur. The observed increase in the ΔH of receptor inactivation in the presence of $MoO4^{=}$ is compatible

with this hypothesis and the pH dependence may reflect constraints on molybdate binding to a specific site on the receptor (W.W. Wright et al.,1981). molybdate could interact with receptor proteins in a number of different ways. Molybdate can react with thiol groups of cysteine and with imidazole groups of histidine (B.J. Weathers et al.,1976). Molybdate can also interact with heavy metals and there is some evidence that steroid receptors are metalloproteins (M.R. Sherman et al.,1970). Evidence has already been provided in favor of steroid receptors being phosphoproteins and molybdate can form polycomplexes with inorganic phosphate. It has been demonstrated that a solution of dithiothreitol (Dtt) will turn yellow in the presence of MoO4=, suggesting potential sulfhydral-molybdate interactions. Addition of glucose-1-phosphate can reverse the yellow color produced by MoO4= and Dtt while glucose alone is ineffective. This indicates that molybdate can bind phosphate in this valence state (K.L. Leach et al., 1979). Another mechanism suggested to explain how molybdate can stabilize steroid binding relates to its ability to stimulate a number of enzymes involved in phosphorylation. The cyclic nucleotide independent Epidermal growth factor-stimulated kinase in murine leydig cell tumors is stimulated by MoO4= (M.H. Melner and D. Puett, 1981). In another set of studies, adenylate cyclase activity in many tissues was shown to be stimulated by molybdate (J.M. Richards and W.I. Swislocki, 1979), tungstate (P.L. Hwang and R.J. Ryan, 1981)

and vanadate (W. Krawirtz et al., 1982). These findings may explain the need for ATP in the reactivation process of glucocorticoid binding in L-cells upon heat inactivation and the reactivation of estrogen binding in calf uterus upon inactivation in the nucleus. ATP may act as a substrate for a putative cyclase or kinase. (N.L. Weigel et al., 1981; A. Migliaccio et al.,1982) This postulated mechanism does not explain how MoO4= could have its stabilizing effect in cytosol since adenylate cyclase is a particulate enzyme.

This last suggestion to explain the mechanism by which molybdate acts to stabilize binding (phosphokinase or adenylate cyclase stimulation) involves effects on enzymatic processes. Both studies were performed at physiological temperatures. However, MoO4= effects on steroid binding have been observed at 4C. It remains to be determined whether these enzymes (or any others) could be active at this temperature.

III Metabolic Interconversion of Proteins

A. Phosphorylation-Dephosphorylation Biochemistry

Reversible posttranslational covalent modification of proteins has come to be a well known biochemical phenomenon. Examples of such reactions involving derivatization of individual amino acid residues include: acetylation-deacteylation (E.L. Gershey et al. 1968), adenylation-deadenylation (E.R. Stadtman et al. 1977), uridylylation- deuridylylation (S.P. Adler et al. 1973), methylation-demethylation (W.K. Paik and S. Kim 1980), sulfhydral oxidation-reduction interconversion (B.L. Hobecker et al.1973) and phosphorylation-dephosphorylation (E.G. Krebs and J.A. Beavo 1979). Much emphasis in the literature has been placed on the last regulatory mechanism, which involve protein kinase and phosphoprotein phosphatase catalyzed reactions. Control of these two opposing reactions may be mediated by adaptive changes altering the ratio of protein kinase and phosphoprotein phosphatase within the cell. Alternatively, rapid control of these reactions may be mediated through immediate fluctuations in the levels of molecules or ions (e.g. cyclic nucleotides, RNA, proteases, Ca++) that affect these two enzymes.

The criteria to establish that a protein undergoes physiologically significant phosphorylation-dephosphorylation include: (1) demonstration both IN VITRO and IN VIVO that the protein can be stoichiometrically phosphorylated by a

a protein kinase and dephosphorylated by a phosphoprotein phosphatase at a significant rate and with a concomitant change in the functional properties of the protein; (2) correlation of the cellular levels of kinase and phosphatase affector molecules with the extent of phosphorylation of the protein and (3) demonstration that the kinase and the phosphatase shown to catalyze the phosphorylation of a given protein are located in the cell type and/or subcellular component from which the protein was derived.

Over the past 20 years, more than 20 metabolic enzymes and regulatory proteins have been reported to undergo phosphorylation-dephosphorylation reactions. Covalent phosphorylation can result in an increase in activity of some enzymes and a decrease in activity of other enzymes (J. Larner et al., 1973; O.H. Wieland et al., 1973) Some of the metabolic enzymes and regulatory proteins whose activities are regulated by this mechanism are listed in Table 2:

Table 2:

Metabolic Enzymes and Regulatory Proteins Whose Activity or Function is Modified by Phosphorylation-Dephosphorylation

	Substrate	Effect of Phosphorylation	Reference
Metabolic Enzymes	1. Phosphofructokinase	Increase Activity	I.A. Brand, 1975
	2. Pyruvate Kinase	Decrease Activity	O. Ljungstrom, 1974
	3. Glycogen Synthetase	Decrease Activity	D.L. Friedman, 1962
	4. Glycogen Phosphorylase	Increase Activity	E.H. Fischer, 1955
	5. Fructose-1,6-Biphosphatase	Increase Activity	J.P. Riou, 1977
	6. Acetyl-COA- Carboxylase	Decrease Activity	C.A. Carlson, 1973
	7. Hormone Sensitive Lipase	Increase Activity	J.D. Corbin, 1970
	8. Cholesterol Ester Hydrolase	Increase Activity	W.H. Trzeciak, 1974
	9. HMG- COA- Reductase	Decrease Activity	Z.H. Beg, 1973
Regulatory Proteins	10. Phospholambden	Ca^{++} Sequestration by Cardiac Sarcoplasmic Reticulum	M. Tada, 1974
	11. Myosin Light Chain	Mg^{++} ATPase activity (muscle contractility)	A. Gorecka, 1976
	12. EIF-2	Polypeptide Chain Initiation	R.S. Ranu, 1976
	13. Histones	Regulate Gene Expression	T.A. Langan, 1969
	14. 34K Viral Protein	Transformation	E. Erikson, 1980
	15. Acetylcholine Receptor	?	A.S. Gordon, 1979
	16. EGF Receptor	?	S. Cohen, 1980
	17. Insulin Receptor	?	M. Kasuola, 1982

(Based on E.G. Krebs and J.A. Beavo, 1979)

B. Cyclic Nucleotide Physiology

Numerous agents and conditions have been identified that alter the steady state levels of cyclic 3′,5′-adenosine monophosphate (cAMP) and cyclic 3′,5′-guanosine monophosphate (cGMP) including polypeptides biogenic amines, cholinergics, steroids, fatty acids, vitamins, oxidants and nucleophiles. The two cyclic nucleotides are considered to be key regulatory effectors in view of the long list of cellular events that they appear to influence. These include secretion, muscle contractility, neuronal excitability, inflammatory and immune processes and cellular growth. Many of the cellular responses produced by cGMP are antagonistic to those that occur when the concentration of cAMP is increased in the same cells From this observation, the Yin Yang or Dualism hypothesis of biological regulation thru two opposing forces, cAMP and cGMP, has been suggested and the emphasis has been placed on the ratio of the two cyclic nucleotides (N.D. Goldberg et al., 1974). Examples of systems for which this hypothesis is applicable are listed in Table 3 (Reviewed in I.H. Pastan et al., 1975; N.D. Golberg and M.K Haddox, 1977). Thus, there appears to be at least two types of bidirectionally controlled systems; those stimulated by an elevation in the level of cellular cAMP (A-type) and suppressed by an elevation in cGMP and those suppressed by an increase in the concentration of cAMP and stimulated by an elevation in cGMP (B-type).

Table 3:

Physiological Systems that Exhibit Bidirectional Regulation by the Cyclic Nucleotides

System	cAMP Effect	cGMP Effect
1. Lysosomal enzyme release from leukocytes	inhibition	stimulation
2. Rate and intensity of cardiac muscle contractility	stimulation	inhibition
3. Firing of post-ganglionic neurons	hyperpolorization	depolorization
4. Chemotactic response in monocytes	stimulation	inhibition
5. Antibody production by B-lymphocytes	suppression	induction
6. Synthesis and Expression of specific mRNA molecules	inhibition	stimulation
7. Transport of small molecules (e.g. uridine, leucine and 2-deoxyglucose) in fibroblasts	inhibition	stimulation
8. Proliferation of many cell types	inhibition	stimulation

(From N.D. Goldberg and M.K. Haddox, 1977)

Fluctuations in steroid binding in cultured cells in response to refeeding, replating and cell cycle have been discussed (section 2A). It is interesting to note that cyclic nucleotides have been shown to fluctuate with the cell cycle in fibroblasts, liver, and lymphocytes (D.L. Friedman et al., 1976), such that cAMP is highest during the S phase and cGMP is highest during the G2/M phase. Cyclic nucleotides have also been shown to fluctuate in rat embryo fibroblast cells in culture following replating and refeeding or refeeding alone. Following replating and refeeding cAMP is elevated after 10 hours and cGMP is elevated after 3 hours and once again after 18 hours. Following refeeding cAMP is elevated after 3 hours and cGMP is elevated at 12 hours. In addition mitogenic treatment of these cells results in fluctuations in cyclic nucleotides as well. Mitogenic treatment of lymphocytes resulted in fluctuations in glucocorticoid receptors and glucocorticoid sensitivity (K.A. Smith et al., 1977). A number of reports have appeared in the literature demonstrating both coincident and direct relationships between cyclic nucleotides and steroid binding. Endogenous uterine cGMP and cAMP concentrations monitored in each stage of the rat estrus cycle. cGMP was highest and cAMP lowest during proestrus when uterine estrogen receptors are highest (F.A. Kuehl et al., 1974). In another report, cytosolic estrogen receptors in the rat anterior pituitary shows a dose dependent decrease in response to exogenous cAMP (P. Singh and T G. Muldoon,1982).

A third study using MCF-7 human breast cancer cells showed a growth arrest and a decrease in estrogen binding capacity after 3 days in response to micromolar concentrations of dibutaryl cAMP (Y.S. Cho-Chung et al., 1981). In summary, there is some inconclusive evidence for both a short term and long term relationship between cyclic nucleotides and steroid binding the nature of this relationship is unclear.

Summary of Introductory Material and Purpose of the Studies Which Follow:

For many years it was thought that changes in the concentration of steroid receptors within a target tissue occurred quite slowly and was dependent upon changes in the rate of synthesis and/or degradation of receptor proteins and on the process of recycling of receptors from the nucleus back into the cytoplasm. Recent results have suggested that the concentration of unoccupied steroid binding proteins could fluctuate rapidly. These findings suggested that a rapid reversible biochemical process was involved in inactivating (converting to a nonbinding form) and activating (converting to a binding form) a pool of receptors. A few reports have appeared demonstrating that proteases, sulfhydryl reagents and reagents directed at phospholipids could affect steroid binding ability. However some investigators have suggested that phosphorylation and dephosphorylation may be a key underlying mechanism controlling the amount of "active" cytosolic binding proteins. Included in the evidence offered by these investigators is:

(1) the direct demonstration that steroid receptors are phosphoproteins,

(2) the finding that steroid levels decline under anaerobic conditions or in the presence of oxidative phosphorylation uncoupling agents when cellular ATP levels would be low,

(3) the finding that addition of alkaline phosphatase results in a decrease in steroid binding while the addition of putative phosphatase inhibitors such as molybdate prevents any loss in steroid binding,
(4) the demonstration that receptor inactivating activity within the cell copurifies with dephosphorylating activity on DEAE-cellulose chromatography and
(5) the finding that recycling of receptor from the nucleus back into the cytoplasm involves nuclear phosphatase activity.

To further explore the mechanism of steroid receptor regulation the following studies have been performed:
(1) An investigation of the effect of molybdate, the putative phosphatase inhibitor, on estradiol (E2) binding in human endometrial cells.
(2) An evaluation of the site within the cell contains the "catalytic" component(s) that regulates changes in steroid binding by using the method of differential centrifugation and reconstitution of specific subfractions. Specific fractions are incubated with molybdate and radiolabelled estradiol to see if molybdate is effective in altering binding when particular cell components have been removed

(3) A search for physiological substances that can provoke changes in binding IN VITRO (in a broken cell preparation) that are similar to the rapid changes in binding that have been obtained IN VIVO (in endometrial cell cultures) Substances involved in phosphorylating reactions such as nucleoside triphosphates, cyclic nucleotides and divalent cations have been tested for their effect on E2 binding.

(4) An elucidation of the biochmical signal(s) which regulate(s) fluctuations in binding in cell cultures by correlating the changes in steroid binding in these cells with changes in the concentration of important physiological substances (#3 above).

EXPERIMENTAL

The devices which science has given us are neither good nor evil in themselves. Their capacity for good or evil lies in the use we make of them. Thus, not in the laboratory but in the human heart in the realm of the spirit, lies the challenge of the future.

David Sarnoff

MATERIALS AND METHODS

Cell Culture

Experiments were performed on cells from four established cancer lines: HEC-1B HEC-50, CG5, and T47D cells and on fresh normal and neoplastic human endometrial specimens Cell cultures were maintained at 37C in a humidified atmosphere of 95% 02 and 5% CO2. Cells were used after they reached confluence in Corning T75 or T125 flasks. HEC-1B cells (Fig. 5A) were established from an explant of moderately differentiated papillary adenocarcinoma (Kuramoto et al.,1972) and have proliferated in continuous culture since May 1968. These cells appear morphologically to be polygonal epithelial cells. They contain cytosolic estrogen receptors with a characteristic 4S sedimentation coefficient and a dissociation constant of 5nM (P.G. Satyaswaroop et al., 1978). These cells are unresponsive to estrogen as determined by lack of changes in protein synthesis in response to steroid (S.S. Shapiro et al., 1975) The receptor in these cells is unable to be activated to a nuclear binding form. HEC-1B cells were grown in Ham's F10 medium (Flow Laboratories) supplemented with 10% calf serum (HF10-CS), 4.0 mg/ml glucose, 10ug/ml insulin (Squibb) and 1% antibiotics and antimycotics (10,000U/ml penicillin, 10mg/ml streptomycin and 25ug/ml fungizone). For some studies, when the cells reached confluence, the HF10-CS medium

was replaced by Minimal Essential Medium (MEM) without serum (cell starvation) for 2-4 days prior to use.

The HEC-50 cell line (Fig. 5B), an estrogen responsive line, was established from a second specimen of moderately differentiated endometrial adenocarcinoma. These cells were grown in Minimal Essential Medium supplemented with 15% fetal calf serum and 1% of the antibiotic antimycotic mixture.

CG5 cells (Fig. 5C) are a new estrogen sensitive variant established by Iacobelli from the MCF-7 human breast cancer cell line. These cells were grown in Dulbecco's modified Eagle Medium (Grand Island Biological Company) with 20uM HEPES 10% fetal calf serum and 0.38% of the antibiotic antimycotic mixture.

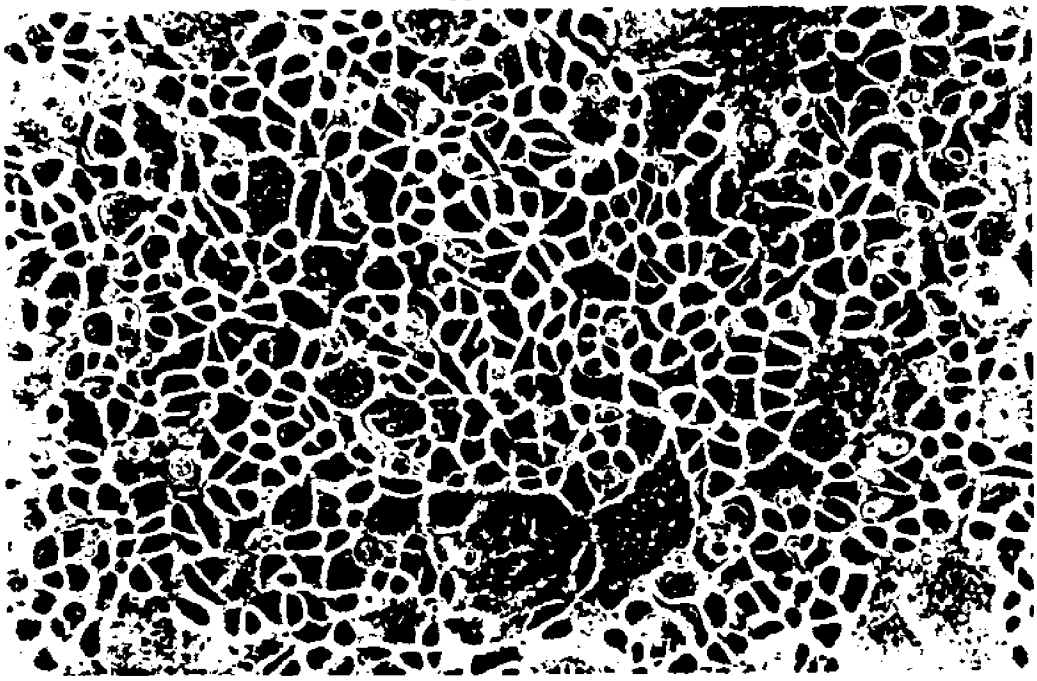
T47D cells (Fig. 5D), another human breast cancer line, obtained from the Rockville cell bank (Rockville Md.), were grown in RPMI 1640 medium (GIBCO) supplemented with 15% fetal calf serum and 1% of the antibiotic antimycotic mixture.

HEC-1B cells were harvested by incubating with 1.5mM EDTA in Hank's Balanced Salt Solution (HBSS) at 37C for 15 minutes. All other cells were removed by adding 0.05% trypsin and 0.03% EDTA in HBSS to the flask. Trypsin action was stopped after a 5 minute incubation at 37C by addition of serum. Cells were collected, pelleted and resuspended in their respective growth medium.

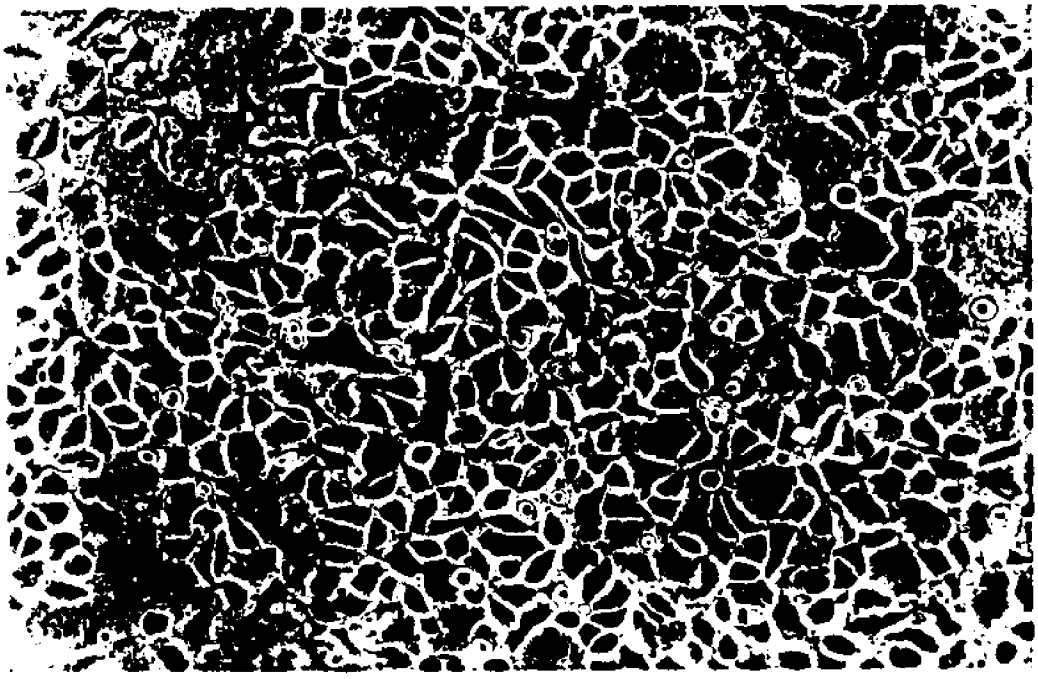
Surgical specimens of fresh human endometrium (Fig. 5E-F) or fresh human endometrial adenocarcinoma (Fig 5G) were obtained from patients undergoing vaginal and abdominal hysterectomies or dilatation and curattage procedures. A sample of fresh tissue was fixed in 10% formalin and stained with Harris's hematoxylin and eosin solution (H & E preparation). The day of the menstrul cycle for normal endometrium was determined (R.W. Noyes et al. 1950) thus providing an indication of the hormonal status of the patient from whom the tissue comes and helped explain differences in results from individual tissue samples. The remaining normal and neoplastic endometrial tissue was minced in HBSS. Tissue fragments were pelleted and resuspended in the assay buffer.

FIGURE 5: PHOTOMICROGRAPHS OF FOUR CELL LINES

A. HEC-1B



B. HEC-50



C. CCL

D. IMR

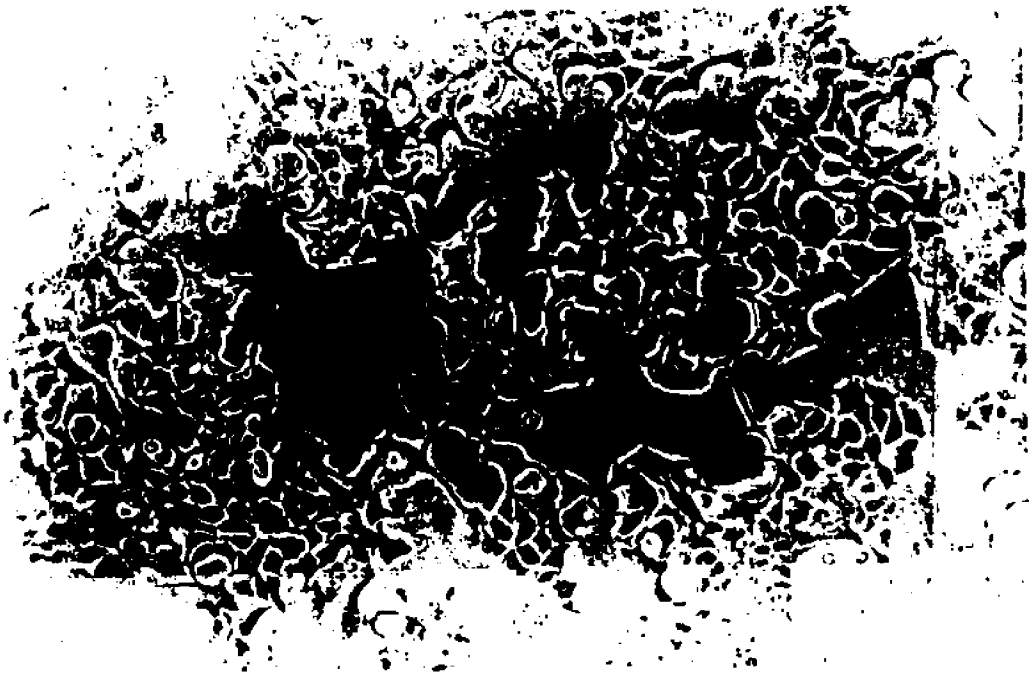
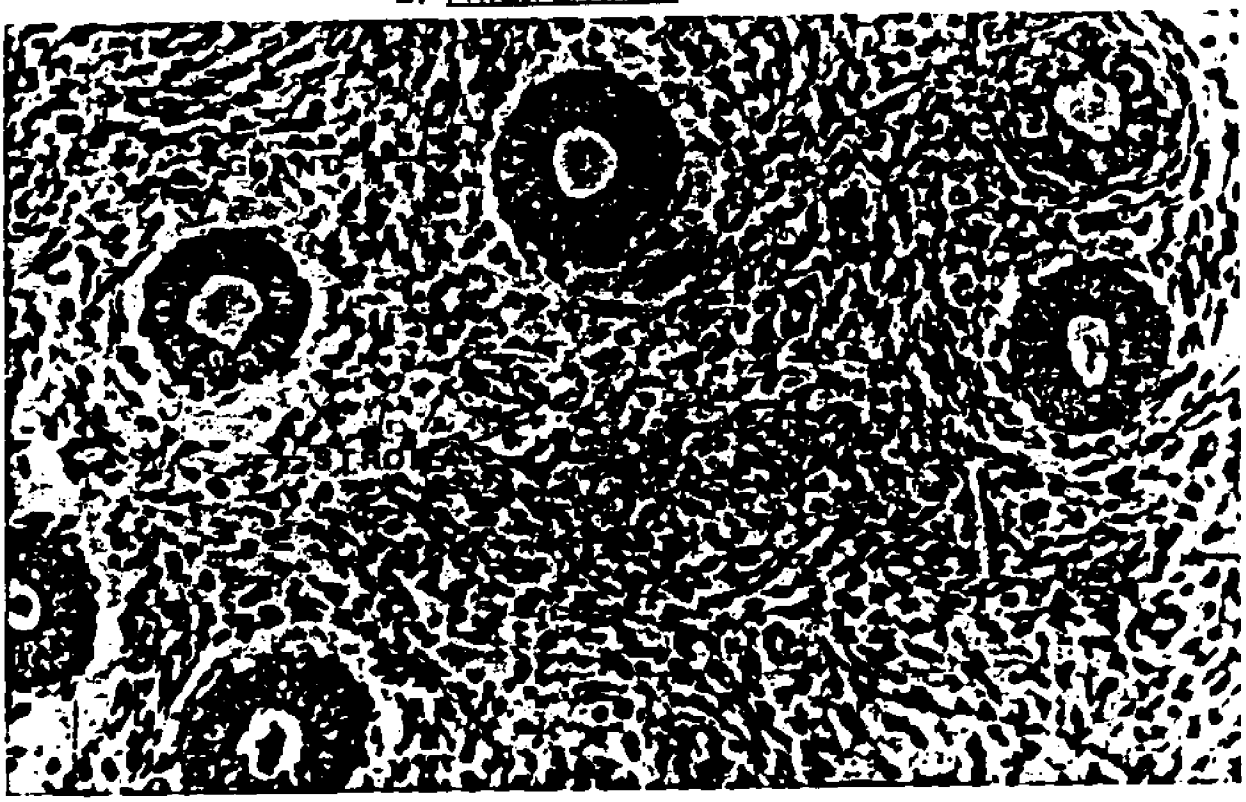


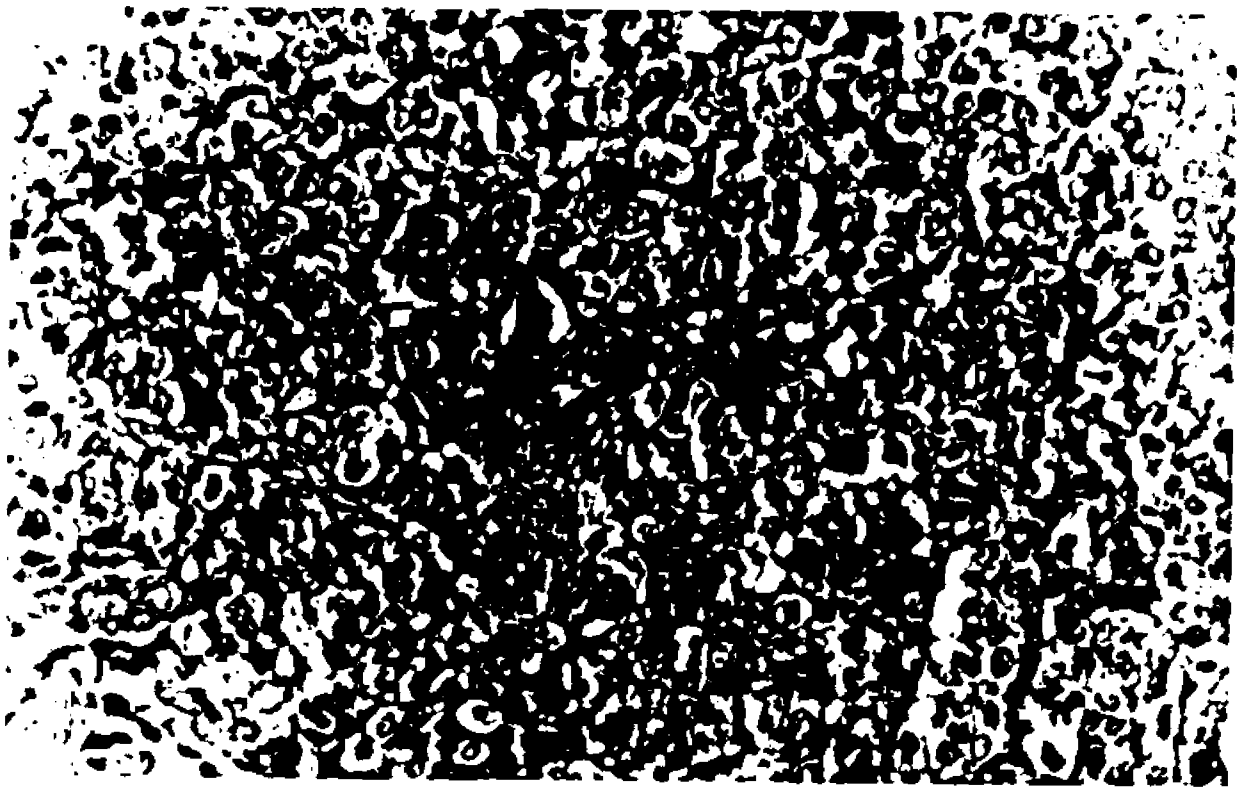
FIGURE 5: PHOTOGRAPHS OF FRESH HUMAN ENDOMETRIUM

E. PROLIFERATIVE



F. SECRETORY

FIGURE 5: MODERATELY DIFFERENTIATED ENDOMETRIAL ADENOCARCINOMA



Estradiol Binding Assay:

(1) Intact Cell Assay:

Aliquots of HEC-1B cells are suspended in MEM (with Earl's salts and glutamine) and incubated with (6,7 3H)-estradiol (52 Ci/mmol, New England Nuclear Corp., Boston. Mass.) in the presence or absence of a 100-fold excess of diethylstilbestrol (DES) for 1 hour at 4C. The cells were rapidly frozen in an alcohol-dry ice bath to stop the reaction. The cells were washed once with HBSS and then homogenized in GMTD buffer (30% glycerol, 1mM MgCl2, 10mM Tris-HCl pH 7.6 and 1mM dithiothreitol) with 0.05% Triton X-100 in a Kontes glass-glass homogenizer at 4C. The broken cell preparation was centrifuged at 800g for 5 minutes to separate the cytoplasmic supernatant fraction from the cell membrane/nuclear pellet. The pellet was washed once with 0.1% Triton in TE buffer (10mM Tris-HCl pH 8.0 and 1mM EDTA) and 3-4 times in TE buffer alone. Radioactivity was extracted from the pellet by incubating the pellet with 95% ethanol at 30C for 10 minutes and the precipitate was saved for DNA determination. The cytoplasm was treated with dextran coated charcoal (0.5% charcoal, 0.05% dextran in 10mM Tris-HCl pH 8.0) for 20 minutes at 4C and centrifuged at 1500 g for an aditional 20 minutes. Aliquots of the supernatant were assayed for tritium and the counts were normalized to sample DNA. Specific binding was calculated by subtracting nonspecific binding (in the presence of DES) from total binding (absence of DES).

(2) Broken Cell Assay

Cell or tissue samples were resuspended in GMTD buffer and homogenized in Kontes glass-glass homogenizers. Rupture of the cell was verified morphologically using a phase contrast microscope and by the loss of trypan blue exclusion in round cellular structures. The broken cell preparation was centrifuged at 800 g for 5 minutes to produce a supernatant cytoplasmic fraction. This fraction was centrifuged in an SW65 rotor at 105,000 g for 60 minutes to obtain a cytosolic supernatant. Aliquots of these subcellular fractions were incubated with (6,7 3H)-estradiol $\pm$ 100 fold excess DES Concentrations of steroid and experimental additions (molybdate, nucleotide triphosphates and cyclic nucleotides) were added as specified for each experiment. Incubations were carried out for 90 minutes at 4C unless otherwise indicated and the reaction was stopped by rapidly freezing each tube in an alcohol-dry ice bath. Upon thawing, 250ul of GMTD buffer was added to each tube to facilitate handling. This suspension was treated with dextran-coated charcoal as described previously for the intact cell assay. Radioactivity was measured in an aliquot of the supernatant and normalized to the concentration of protein in an equal size aliquot from the supernatant.

Separation of Plasma Membrane and Nuclei

Homogenates of HEC-1B cells or fresh human endometrial specimens were prepared in GMTD buffer and fractionated into a cytoplasmic (supernatant) and plasma membrane/nuclear pellet by centrifugation at 800g for 5 minutes. The pellet was washed three times with 0.25% sucrose prepared in Ca++ free HBSS and resuspended in 52% sucrose in HBSS (ρ=1.24g/ml) and 0.5ml were layered at the bottom of a Beckman pollyallomer tube (7/16" x 2 3/8"). Discontinuous sucrose concentrations were layered on top of this suspension as follows 0.5ml of 45% sucrose (ρ=1.20g/ml), 0.5ml of 41% sucrose (ρ=1.18g/ml) 0.5ml of 37% sucrose (ρ=1.16g/ml) and 0.7ml of 31% sucrose (ρ=1.13g/ml) all in HBSS. Gradients were spun in a Beckman SW65 swinging bucket rotor at 56,000g for 2.5 hours at 4C. The semipurified nuclei settled at a density of 1.24 (F5 fraction). The plasma membrane sedimented at a density of 1.16 (F2 fraction). The separation scheme is a modification of the method used for rat uterus (R.J. Pietras and C.M. Szego 1979). In their work the enrichment of 5′ nucleotidase and alkaline phosphatase activities are used as markers for plasma membrane along with electron microscopic observation. DNA appeared in the F5 fraction alone and not in the F2 fraction.

Sedimentation Analysis:

Continuous 16-41% glycerol density gradients (3.4ml)in TTE buffer (10mM Tris-HCl pH 8.0, 12mM thioglycerol and 1.5mM EDTA) containing 50mM KCl were prepared. Aliquots of cytosol were diluted 1:1 with TTE buffer and the diluted sample was layered on the preformed gradient. The gradients were centrifuged for 22 hours at 105,000g at 4C. The bottom of the tube was pierced and 100ul fractions were collected and quantitated for radioactivity. Five marker proteins, alcohol dehydrogenase (7.8S), bovine serum albumin (4.6S), Ovalbumin (3.5S), human hemoglobin (3.0S) and horse heart cytochrome c (2.2S) were layered on a separate gradient and run in parallel to determine the sedimentation coefficient of the radioactive peaks. Location of the marker proteins was determined by a protein assay (M. Bradford, 1976). The sedimentation coefficient of the marker proteins in each experiment correlated well with the relative mobility (Rm) of the proteins (R=0.97). Density gradients were prepared with glycerol rather than sucrose since glycerol has been reported to have a greater stabilizing effect on a variety of enzymes and receptors (M.R. Sherman, 1975). Glycerol might therefore decrease the dissociation of steroid from binding proteins over the prolonged centrifugation and thus increase the sensitivity of the measurement.

ATP Measurements

ATP was extracted from 200ul samples of cell homogenates with 50ul of 30% perchloric acid and neutralized with an equal volume of 1M KOH. Aliquots of the supernatant were assayed by the luciferin-luciferase firefly method. Luciferin reacts with ATP in the presence of the enzyme luciferase to liberate pyrophosphate. The Enzyme*Luciferin*AMP complex is then oxidized. The intermediate in this reaction produces the bioluminesence. The number of initial light flashes were measured in a Beckman LS-230 spectrometer in a tritium voltage interval for 6 seconds with the coincidence switch off Assays were done in a phosphate-arsenate buffer containing 0·1M KH_2PO_4, 0.1M arsenate, 40mM Mg_2SO_4 and 40mM MgCl (pH 7.4). A new ATP standard was established for each experiment with the sodium salt of ATP and a fresh firefly lantern extract. The assay was linear over a range of 1 to 100nM ATP. (P.E. Stanley and S.G. Williams, 1969). To insure that there was no chemical interference by the homogenizing buffer (GMTD), a control sample containing GMTD alone without cytosol was used in each experiment.

Cyclic Nucleotide Measurements:

Aliquots of cells, of cellular homogenates or of cytosol were extracted with acidic ethanol (1N HCl/100ml ethanol) in a ratio of 2 parts ethanol to 1 part cell sample and allowed to stand for 5 minutes at room temperature to precipitate protein. Following centrifugation, the supernatant was removed and the precipitate was washed once with ethanol:water (2:1) and centrifuged. The supernatants were combined, evaporated to dryness at 55C under a stream of nitrogen and diluted in 0.5ml of Tris-EDTA buffer (0.05M Tris pH 7.5 and 4mM EDTA). A competitive protein binding assay (Amersham kit #432) was used to measure cAMP in a 50ul aliquot of the above extract. Separation of the protein bound cAMP from the unbound nucleotide was achieved by adsorption of the free nucleotide with charcoal. Following centrifugation, an aliquot of the supernatant was assayed for tritium in a liquid scintillation counter. A 100ul aliquot of the cell extract was used to measure cGMP by radioimmunoassay (Amersham kit #500). Following competition between the unlabelled cGMP and a fixed quantity of the 3H-cGMP, the antibody bound radioactivity was separated from the unbound nucleotide by ammonium sulfate precipitation. The precipitate was dissolved in water and the radioactivity was determined by scintillation spectrometry. The concentration of cAMP was determined by comparison with a standard curve over

a range of 0-16 pmol cAMP. The concentration of cGMP was determined by comparison with a standard curve over a range of 0-8pmol. There was no crossreactivity in either assay between the 2 nucleotides or related compounds (ATP,GTP, ADP,GDP).

Thin-Layer Chromatography

Stock solutions of radiolabelled steroid or ethanol extracted steroid that had been bound to cytoplasmic proteins were chromatographed on silica gel GF plates (ANALTECH) to determine what percentage of the steroid remained in the original form of estradiol and what percentage had been metabolized. Approximately 200,000 DPM of 3H-steroid with 500ug cold estradiol carrier was dissolved in methanol, applied to each plate, air dried and developed for 45 minutes with chloroform:ethylacetate (4:1). The estradiol carrier was located using a short wave UV scan. One cm areas were scraped from the plate beginning at the origin through to the running front and eluted in methanol. Following centrifugatioh, aliquots of the supernatant were counted for tritium. The recovery of tracer was then established and the counts in each fraction were normalized to this factor.

Protein Assay

Protein was determined by a colorimetric method (M. Bradford, 1976), based on the binding of a dye, coomasie brilliant blue G-250. This binding causes a shift in absorption of the dye from 465nm to 595nm. The increase in absorption at 595nM is monitored. Bovine serum albumin (1mg/ml) was used as a reference standard. The response was linear over a range of 5-25ug protein.

DNA Assay

The 800g cellular pellet or the ethanol extracted pellet is incubated with 10% perchloric acid for 15 minutes at 70C and 1 ml of stock solution containing 4% diphenylamine in glacial acetic acid and 0.8ml of 4% aqueous acetaldehyde is added to each sample and incubated overnight at 30c to produce an unidentified chromophore with an absorbance maximum at 600nm. Calf thymus DNA (2.5mg/ml) was used as a reference standard. The response was linear over a range of 5-80ug DNA (K. Burton,1956 as modified by K.W. Giles and A. Myers, 1965).

Progesterone Receptor Assay

Cell samples of endometrial specimens were homogenized in GMTD buffer (25% glycerol, 10mM Tris pH 7.6, 1mM MgCl2 1mM Dtt). The homogenate was transferred to an ultracentrifuge tube and the homogenizer was washed with an equal volume of HBSS and the wash was added to the homogenate. The broken cell preparation was centrifuged at 4C for 1 hour at 105,000g to prepare a cytosolic supernantant. Aliquots of cytosol were incubated at OC with 20nM (1,2-3H)-progesterone (59Ci/mmol New England Nuclear) plus 2uM cortisol in the presence or absence of 2uM cold progesterone. After 3 hours the samples were rapidly frozen, then thawed. One hundred ul of sample buffer (GMTD) was added to each assay tube and incubated for an additional 10 minutes. Unbound steroid was removed with a 10 minute incubation with dextran coated charcoal (0.5% charcoal , 0.05% dextran in GMTD buffer) followed by a 10 minute centrifugation at 3000 RPM. Aliquots of the supernatant were assayed for radioactivity using 4.5 ml Dimiscint as the scintillant and 400ul glacial acetic acid to reduce quenching produced by the glycerol. Specific binding was calculated as total binding (absence of cold progesterone) minus nonspecific binding (presence of cold conpeting progesterone) and the specific counts were corrected for variations in the concentration of cytosolic protein between samples (a modification of the method of F Bayard et al., 1978).

Electrophoresis

Slab gel plates (R. Shadel Inc. San Fran , Calif) were sealed with 1% agarose solution. The lower running slab consisted of a 10% polyacrylamide prepared in 0.37M Tris buffer containing 0 1% SDS , 0.03% fresh ammonium persulfate and Temed (Biorad). The upper stacking gel consisted of a 3% polyacrylanide gel in a Tris bufer (pH 6.8) containing the same additions as the running gel and 4.8% glycerol.

HEC-1B cytosol prepared in GMTD buffer containing 200uM of the phosphodiesterase inhibitor, isobutyl-methylxanthine (IBMX) was incubated with 10mM KCl, 10mM MgCl2 50uM cold ATP and 20,000 CPM γ-32P-ATP/pmol unlabeled ATP (AMERSHAM) in the presence or absence of 1uM cAMP or cGMP at 0C for 30 minutes. The reaction was stopped by adding 0.5 volumes of sample buffer which contained 3% SDS, 10% glycerol and 3% mercaptoethanol in Tris-HCl pH 8.0 and heating for 5 minutes at 100C. Aliquots of each sample were layered on top of individual lanes of the stacking gel and run at 2mA/plate using a Buchler Instrument power source. The running buffer contained 0.025M Tris base, 0.192M glycine and 0.1% SDS. Bromophenol blue was used to mark the running front. Six protein standards (Pharmacia Fine Chemicals) were run sil-

multaneously on a separate lane. The standards consisted of phosphorylase B (94K), bovine serum albumin (67K), ovalbumin (43K), carbonic anhydrase (30K), soybean trypsin inhibitor (20.1K) and a-lactalbumin (14.4K).

Following electrophoresis the stacking gel and the running front were removed and the running gel was fixed in 10% TCA, stained for 1 hour with Coomassie blue prepared in 10% ethanol and 10% acetic acid. The gel was destained overnight in a solution of 10% methanol and 10% acetic acid, transferred to Whatman 1 filter paper and dried on a Biorad model 224 slab gel dryer using a Precision scientific pump model PV-35. Autoradiography was done on the dry gel using 5"x 7" x-ray film (Ewen Parker Corp). Following exposure for approximately 3 days the film was developed using Kodak D-19 developer and fixed with Kodak rapid fixer.

RESULTS

The most important motive for work in the school and in life is the pleasure in work, pleasure in its result and in the knowledge of the value of the result to the community. Such a psychological foundation alone leads to a joyous desire for the highest posession of men, knowledge and artist-like workmanship.

Albert Einstein

RESULTS:

1 Molybdate Generates E2 Binding IN VITRO

The effect of molybdate on estradiol binding in intact HEC-1 cells is shown in a series of experiments in Figure 6. Incubation of cells with 3H-E2 in the presence of 20mM MoO4= for 60 minutes results in E2 binding that is two to ten fold higher than control levels that were measured in the absence of MoO4=. These increases in binding sites can not be the result of protection of binding sites since addition of molybdate following the incubation with 3H-E2 and prior to homogenization of the intact cell sample results in little or no increase in EB while addition of molybdate to both the incubation buffer and the homogenization buffer, results in dramatic increases in binding (Figure 7). If the molybdate dependent increase in binding was due to protection against 'destabilization' of binding, then binding should be similar in all cells treated with molybdate irrespective of when MoO4= was added. In addition the bound receptor appears to be quite stable for many hours (Figure 8), suggesting that any changes in binding levels are not a function of binding protein stability rather MoO4= must be actively involved in generating new binding sites during the incubation with steroid The phenomenon appears to be independent of protein synthesis since it occurs at 4C reaches maximal levels within a 5-15 minute incubation (Figure 8) and is not inhibited by cycloheximide (Figure 9).

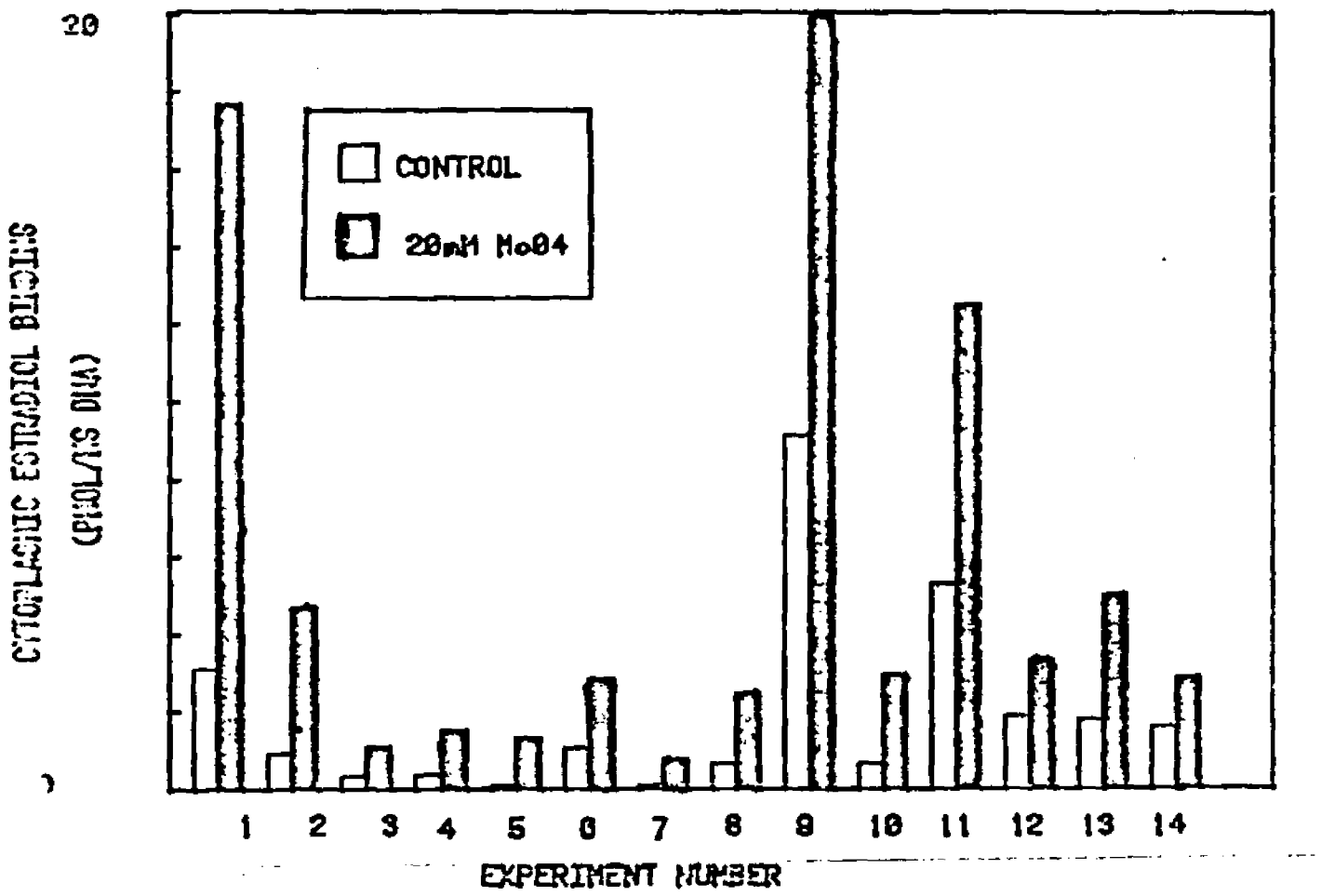


FIGURE 6: Stimulation of E2 binding by molybdate. Results from 14 experiments in which HEC-1B cells suspended in Minimal Essential Medium were incubated with 100nM 3H-E2 ± 10um DES in the presence or absence of 20mM MoO4= for 1 hour. Cells were rapidly frozen, thawed and excess steroid washed away with HBSS. Cells were homogenized in GMTD buffer (30% glycerol, 1mM MgCl2, 10mM Tris and 0.1mM Dtt pH 7.6). Molybdate was added to the homogenization buffer if it was present in the incubation buffer. The nuclear-plasma membrane fraction was removed by centrifugation at 800g for 5 minutes and the cytoplasm was treated with dextran-coated charcoal (0.5% charcoal, 0.05% dextran in 10mM Tris-HCl pH 8.0). Aliquots of the supernatant were counted. Nonspecific DPM (in the presence of DES) were subtracted from total DPM to get specifically bound counts and the results were normalized to DNA content in each sample.

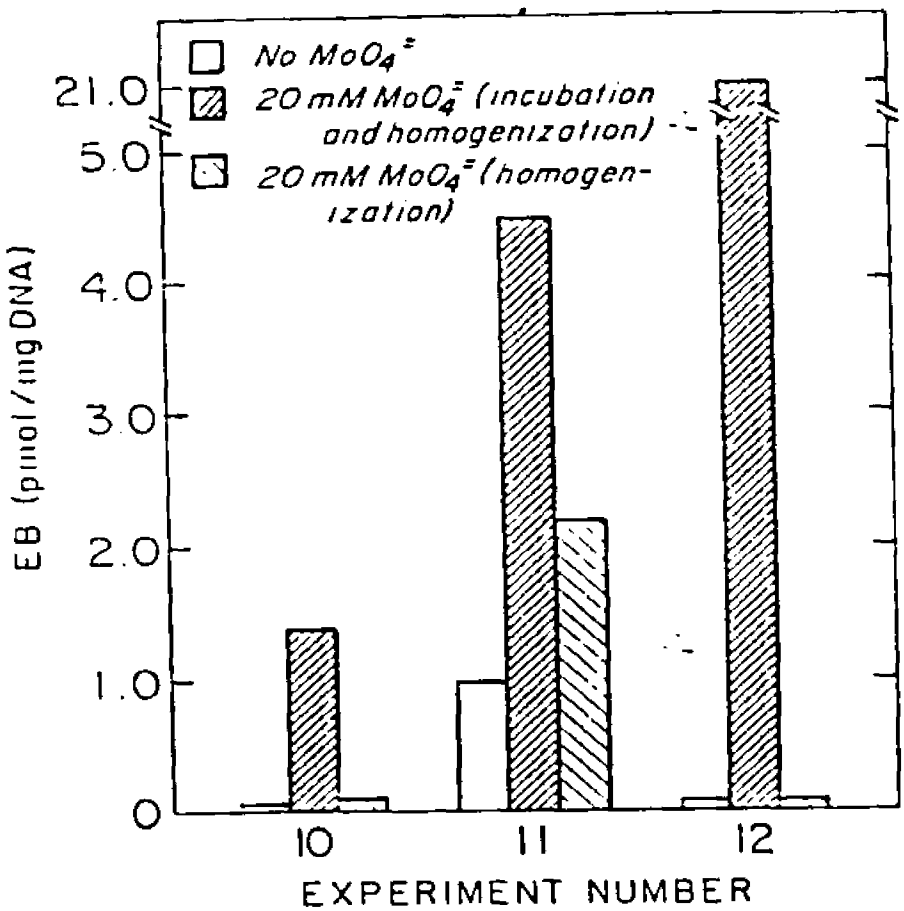


FIGURE 7: Stimulation and protection of E2 binding by molybdate. Results from 3 representative binding experiments performed as described in the legend to figure 6. The clear bar on the left of each experiment represents E2 binding in the absence of molybdate. The middle bar represents binding when 20mM MoO4= was added to both the incubation and the homogenization buffer. The bar on the far right in each set represents results when the cells were incubated in the absence of molybdate and homogenized in the presence of molybdate.

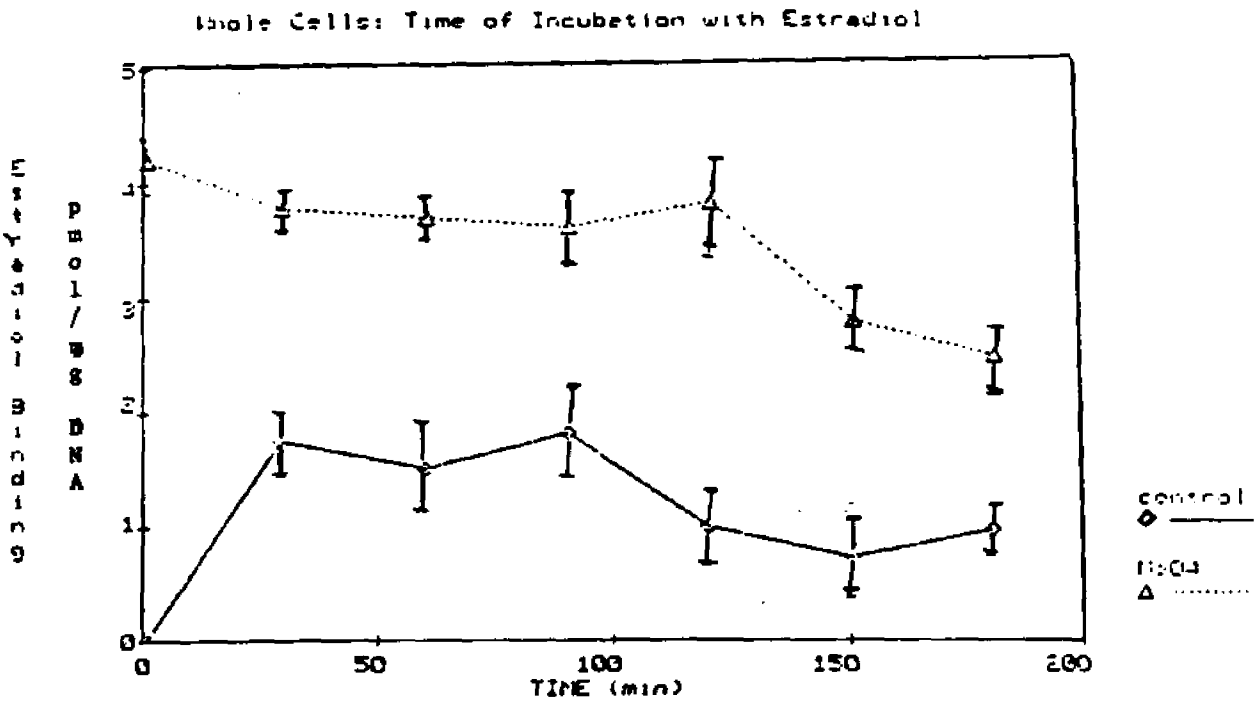


FIGURE 8: Time for basal and molybdate stimulated binding to reach equilibrium in intact cells. REC-1B cells were incubated with E2± DES for 5 minutes to 3 hours. Samples were processed as described in the legend for figure 6. Each point represents the mean + SE for 3 experiments.

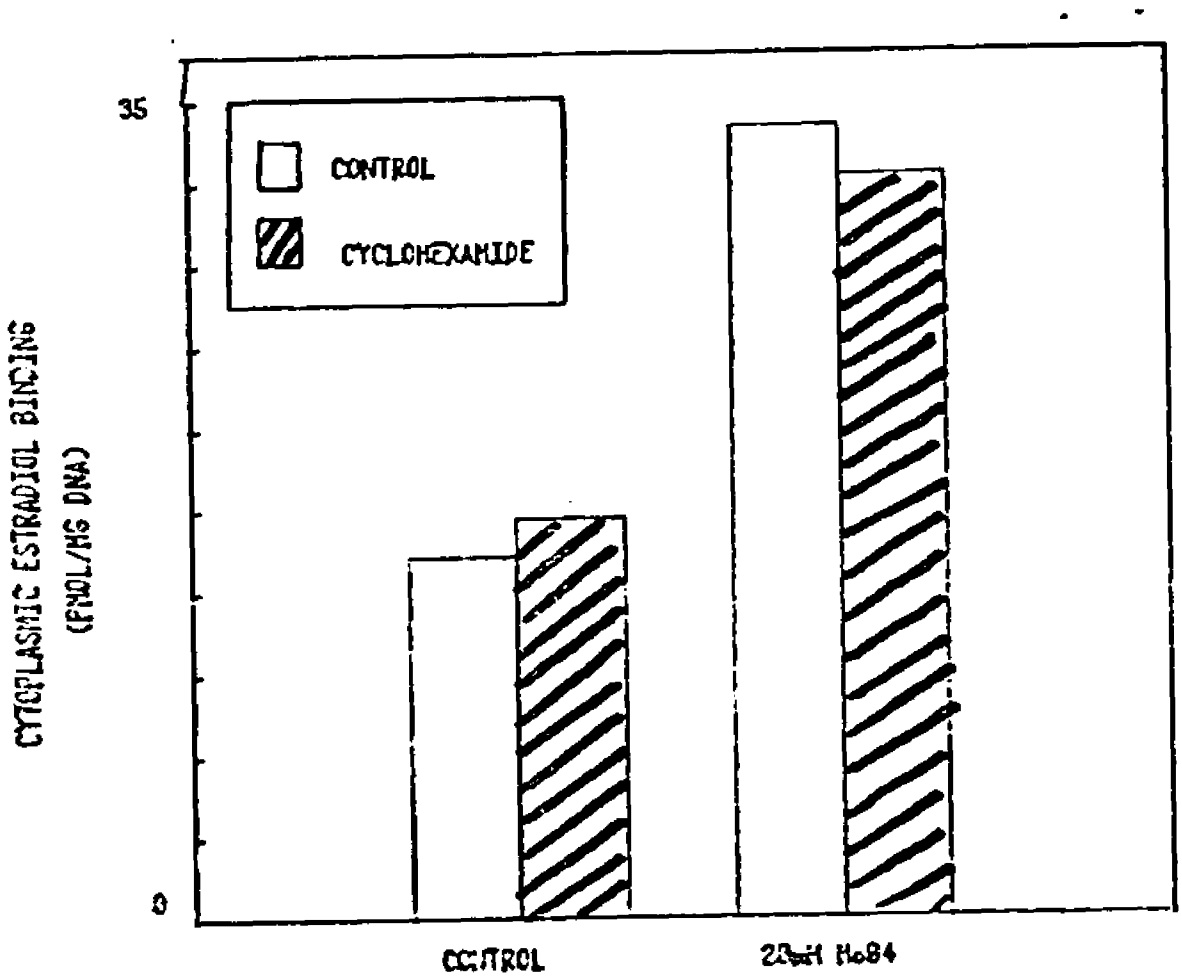


FIGURE 9: Effect of cycloheximide on molybdate dependent stimulation of E2 binding. HEC-1B cells were preincubated in the presence or absence of 15ug/ml cycloheximide for 30 minutes and then incubated for 1 hour with or without 20mM molybdate and 3H-E2 ± DES. Cells were processed as described in figure 6 and cytolasmic binding was normalized to DNA content in each sample.

2 Characterization of the MoO4= Dependent Increase in EB:

Figure 10 shows a dose dependent relationship between the concentration of MoO4= added to the incubation of intact HEC cells with 3H-E2 and the changes in EB. Maximal increases were produced with 20mM MoO4=; higher concentrations (30-40mM) resulted in a decrease in EB, sometimes to levels below basal values.

Saturation analysis in intact cells (Figure 11) reveals that both the high affinity (saturable with 20nM E2) and the low affinity (saturable with 80-100nM E2) binding sites are increased in the presence of 20mM MoO4=. Though the function of the second lower affinity site is unknown, it is clear that the pool of low affinity binders exceeds the pool of high affinity binders. All subseqent experiments unless otherwise stated were done with concentrations of E2 that would ensure saturation of both sites.

The next set of experiments were directed towards determining whether MoO4= could increase EB if the cells were first broken before incubating with steroid and molybdate and, if so, whether the conditions for this effect would be similar to those seen in the intact cell system. Cells were homogenized and the whole cell homogenate was incubated in the presence or absence of molybdate. Under these conditions, MoO4= was still able to increase cytoplasmic E2 binding. The effect was once again dose dependent (Figure 12),

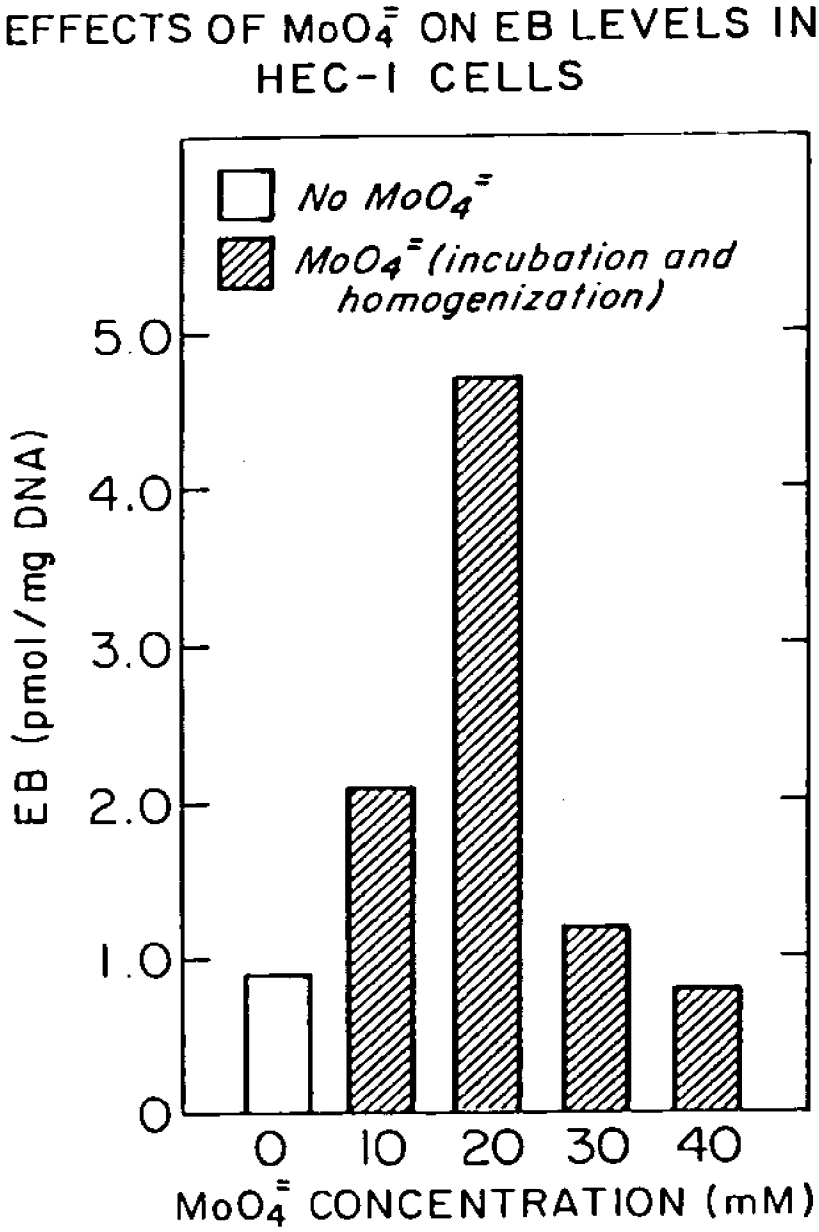


FIGURE 10: Dose response to molybdate in intact cells. HEC-1B cells were incubated with 3H-E2 ± 100x DES without molybdate or with varying amounts of molybdate for 1 hour at 4C. Identical amounts of molybdate were added to the homogenization buffer as was present in the incubation medium of each cell sample. Cells were processed as described in figure 6.

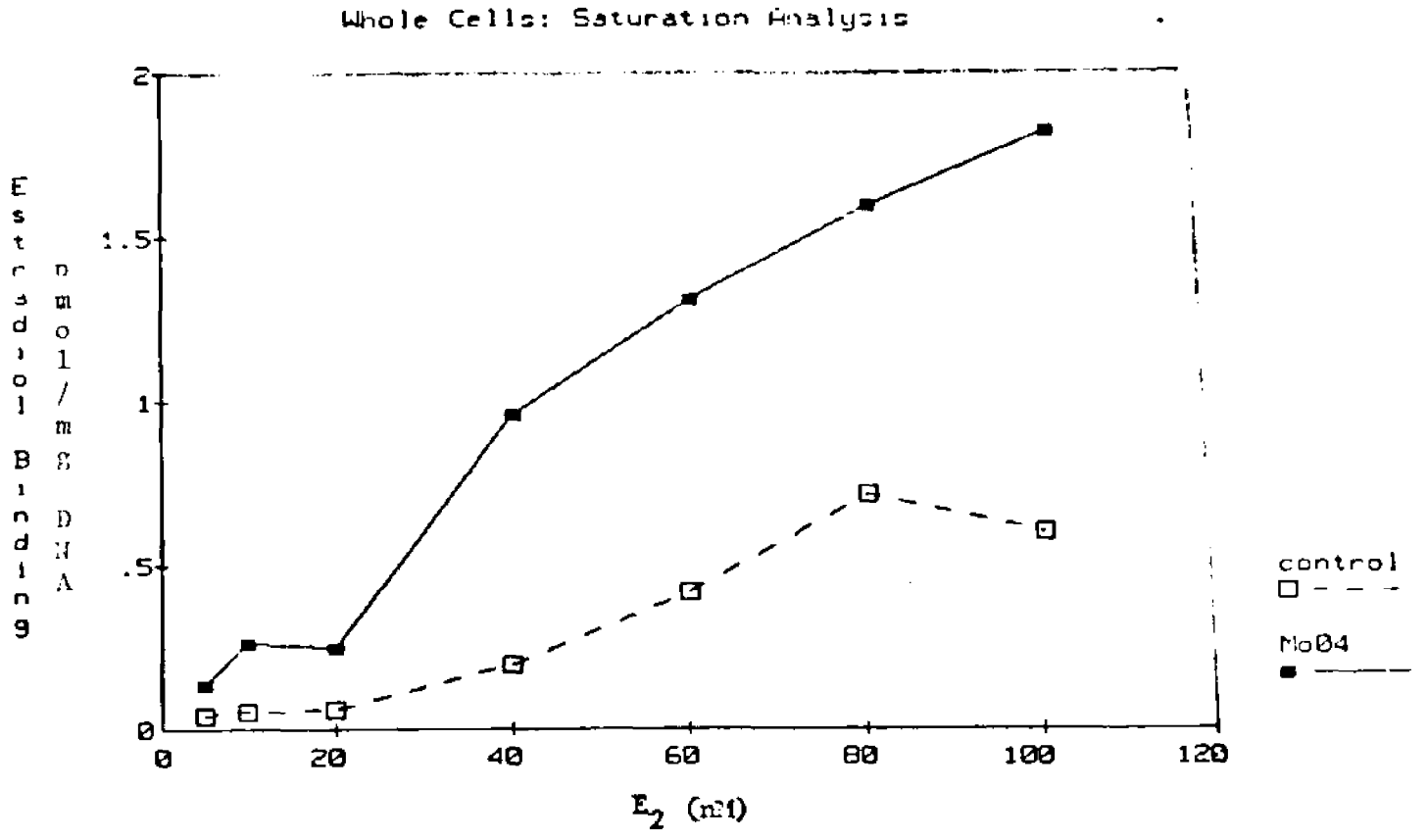


FIGURE 11: Saturation analysis of E2 binding in intact HEC-1B cells. Cells were incubated in the presence or absence of 20mM Mo04= and varying concentrations of estradiol from 5nM to 100nM ± 100x DES for 1 hour at 4C Samples were processed as described in figure 6.

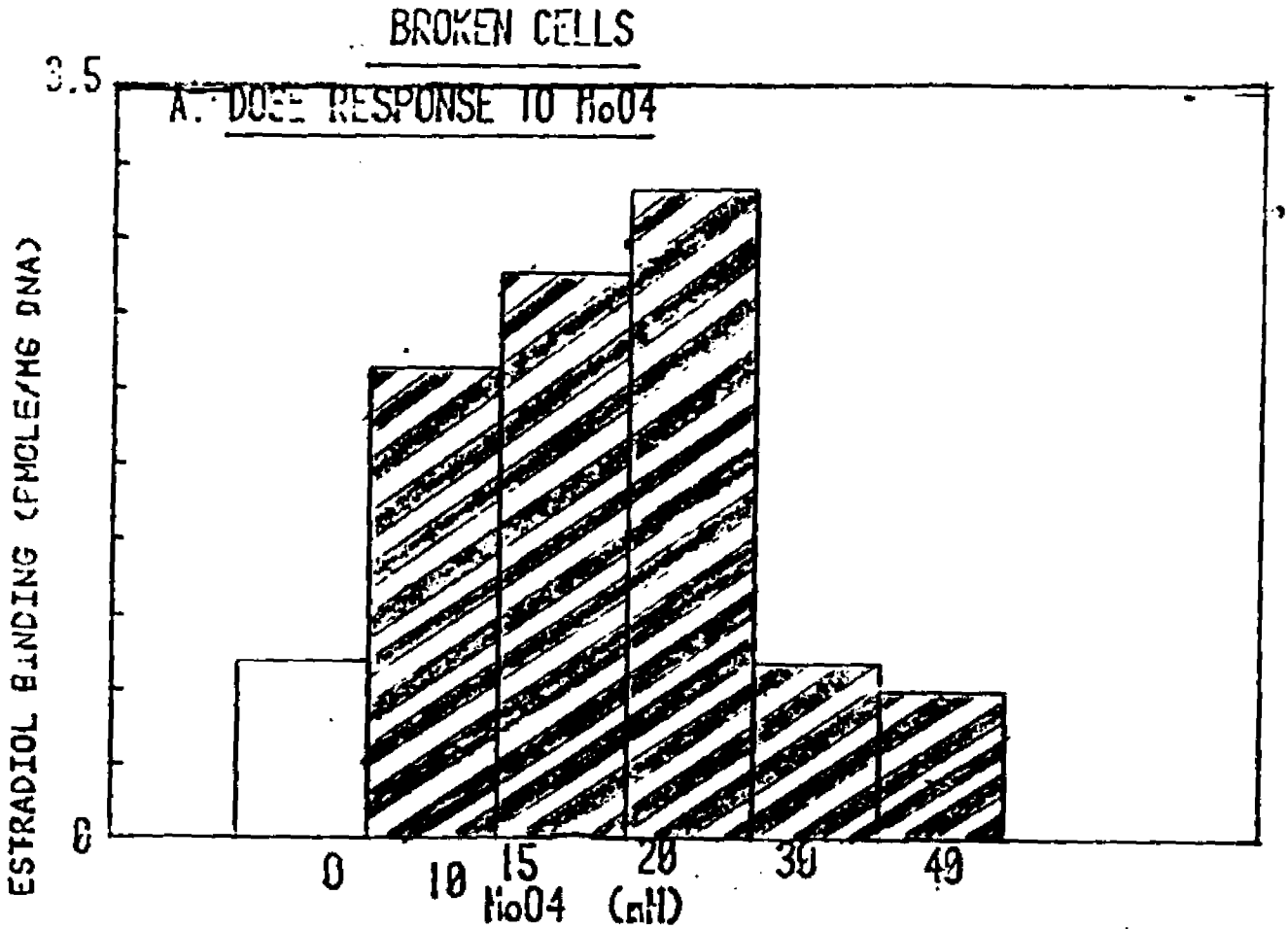


FIGURE 12: Dose response to molybdate in broken cell preparations. HEC-1B cells were homogenized in GMTD buffer and aliquots were incubated with 100nM 3H-E2± 10um DES in the presence or absence of varying concentrations of molybdate for 90 minutes. Samples were rapidly frozen, thawed and diluted in 350ul GMTD to facilitate handling. Each sample was treated with dextran coated charcoal for 20 minutes at 4C and centrifuged for an additional 20 minutes. Aliquots of the supernatant were assayed for tritium. counts were normalized to sample protein content and nonspecific binding was subtracted from total binding to determine specific binding. Each value is the mean of duplicate measurements.

with maximal effects occurring at 20mM MoO4=. Greater amounts of molybdate result in a reduction in binding.

Binding equilibrium for non-molybdate treated cells and for molybdate treated cells is reached rapidly, 80% of maximal binding is achieved after 5 minutes (Figure 13).

Both the high affinity and the low affinity binders are elevated in the presence of molybdate. The concentration of steroid required to saturate both binding proteins in the broken cell system is similar to what was seen in intact cells (Figure 14).

Studying the mechanism of action of molybdate and the biochemical regulation of E2 binding is greatly facilitated by the availability of a broken cell system that is responsive to molybdate. It is now possible to (1) fractionate the broken cell preparation and reconstitute subcellular fractions to determine the site(s) within the cell that molybdate acts upon and (2) add other non-diffusable substances to the cell preparation to see if any physiological compounds can mimic the molybdate effect without being additive with MoO4=.

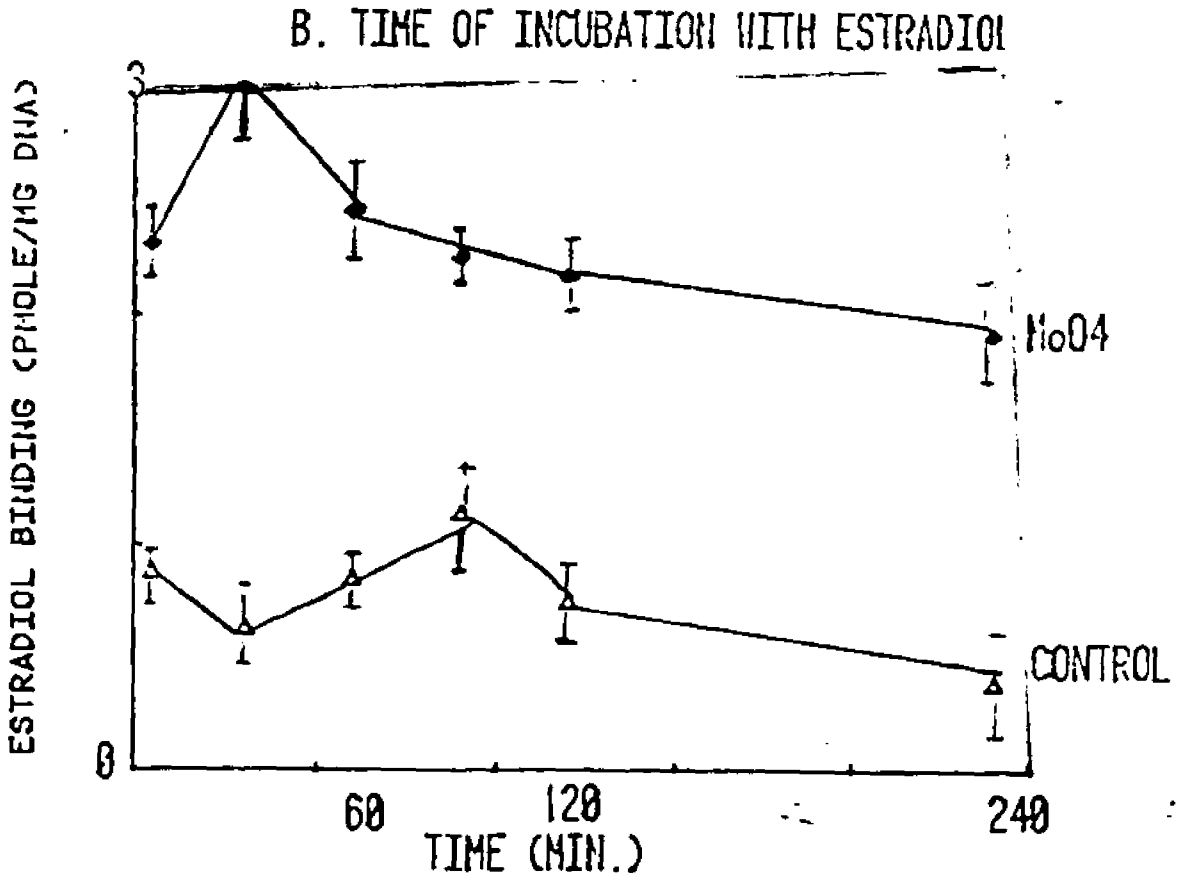


FIGURE 13: Time for basal and molybdate stimulated binding to reach equilibrium in broken cells. Cells were homogenized and aliquots were incubated with 100nM 3H-E2 ± 10ul DES for varying amounts of time from 5 minutes to 4 hours and then rapidly frozen and stored until all incubation were complete. Samples were processed as described in figure 12. Each point represents the mean of duplicates.

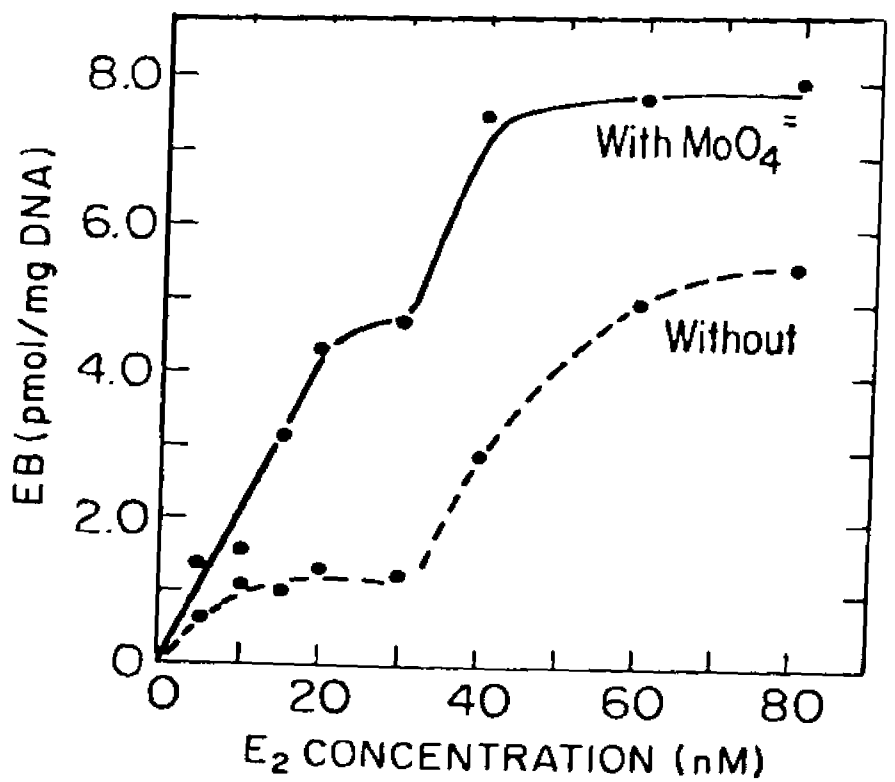


FIGURE 14: Saturation analysis of E2 binding in broken HEC-1B cells. Aliquots of the cell homogenate were incubated with varying concentrations of 3H-E2 ± 100x DES (5nM to 80nM). The reaction was stopped after 90 minutes by rapid freezing and samples were processed as described in the legend to figure 12. Results are representative of 4 separate experiments.

3. Fractionation and Reconstitution Studies

In Figure 15, a matched pair student t-test was used to evaluate the effect of $MoO4^{=}$ on EB in different cellular and subcellular preparations from HEC-1 cells and from fresh endometrium. $MoO4^{=}$ increases the level of specific cytoplasmic binding sites when added to the incubation of intact cells with 100nM 3H-E2 at 4C ($p<.02$) or when added to incubations of homogenates prepared from these cells ($p<.01$). The effect of molybdate was not observed once the homogenate was fractionated and the low speed supernatant (cytoplasm), the high speed supernatant (cytosol) or the low speed pellet (nuclear-plasma membrane fragments) were incubated with molybdate and radiolabeled estradiol. Resuspension of the low speed pellet with the cytosolic fraction restores the sensitivity of cytosolic binding to molybdate ($p<.02$). The low speed pellet was fractionated on a discontinuous sucrose gradient into enriched nuclei and plasma membrane portions (R.J. Pietras and C.M. Szego, 1979) and each portion was resuspended in cytosol. Significant increases in cytosolic estradiol binding levels in response to addition of molybdate were observed only in mixtures of cytosol and plasma membrane preparations ($p<.02$). This finding indicates that activation of soluble E2 binding sites by molybdate requires membrane bound factors.

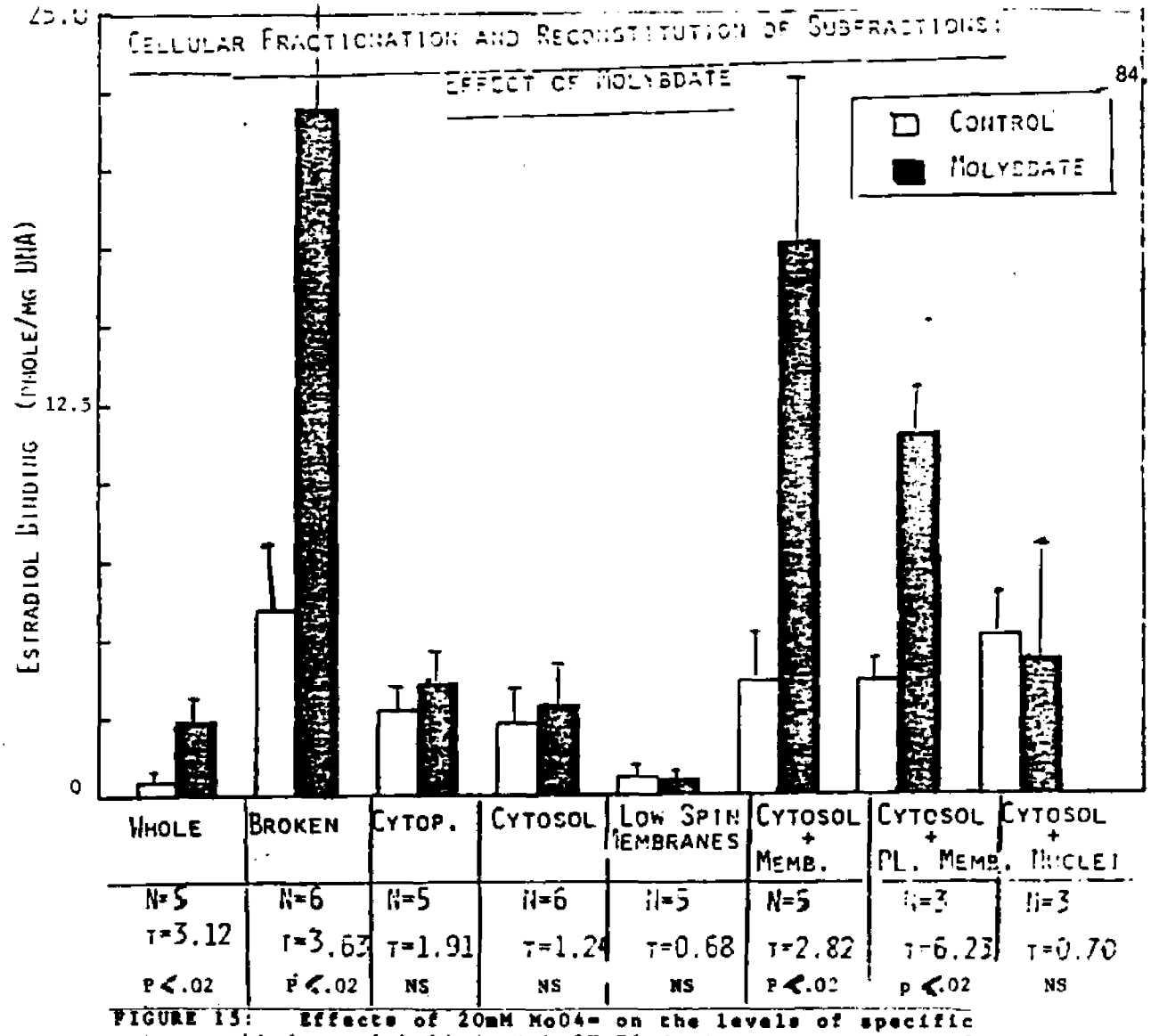


FIGURE 15: Effects of 20mM $MoO_4^=$ on the levels of specific estrogen binders labelled with 3H-E2 during incubations of intact HEC-1B cells, whole cell homogenates or different subcellular fractions. Cells were incubated with 100nM 3H-E2 ± 10uM DES in MEM, with or without $MoO_4^=$ for 1.5 hours at 4C. After washing the cells with HBSS, the cells were homogenized in GMTD buffer containing 0.05% triton X-100 (cells incubated with $MoO_4^=$ were homogenized with $MoO_4^=$). The homogenate was cntrifuged, treated with charcoal and radioactivity measured in the supernatant as described in figure 6.

Homogenates of unlabelled cells were prepared in GMTD buffer. The homogenate was fractionated into cytoplasm and a low speed pellet containing nuclei and plasma membrane fragments by centrifugation at 800g for 5 minutes. Cytosol was prepared from cytoplasm by centrifugation at 105,000g for 1 hour. The low spin pellet obtained from the 800g spin was fractionated into a nuclear and plasma membrane fraction by discontinuous sucrose density centrifugation as described in figure 12 and specific binding was determined in the presence or absence of 20mM $MoO_4^=$.

4 Regulation of E2 Binding by Physiological Substances

The requirement of plasma membranes in the activation of E2 binding sites by molybdate along with reports that adenylate cyclase could be stimulated 3-7 fold by molybdate tungstate and vanadate (J.M. Richards and W.I. Swislocki, 1979; P.L. Hwang and R.J. Ryan, 1981; M. Krawirtz et al., 1982) and reports that steroid binding may be regulated by phosphorylation (see introduction- IX-2E) suggested that cyclic nucleotides might be involved in the regulatory process. Figure 16 shows that addition of 1mM ATP, GTP or cGMP to HEC-1 cell homogenates results in activation of cytoplasmic E2 binding that is comparable to the levels found upon addition of 20mM $MoO_4^=$. In contrast 1mM cAMP caused levels of EB to decline below control levels. The effects of GTP and ATP on EB levels were not additive with the effect of molybdate (results not shown). These results provide some insight into the mechanism of action of molybdate, but more importantly, reveals a possible relationship between estradiol binding levels and metabolically important compounds. The stimulation of specific estrogen binding in cell homogenates by GTP may reflect its role as a precursor of cGMP. With regard to this possibility it is of interest that guanylylimisodiphosphate, a nonhydralyzable analog of GTP does not increase EB levels (results not shown).

It may seem surprising that ATP, a precursor of cAMP also activates E2 binding. In adition, in all but two sys-

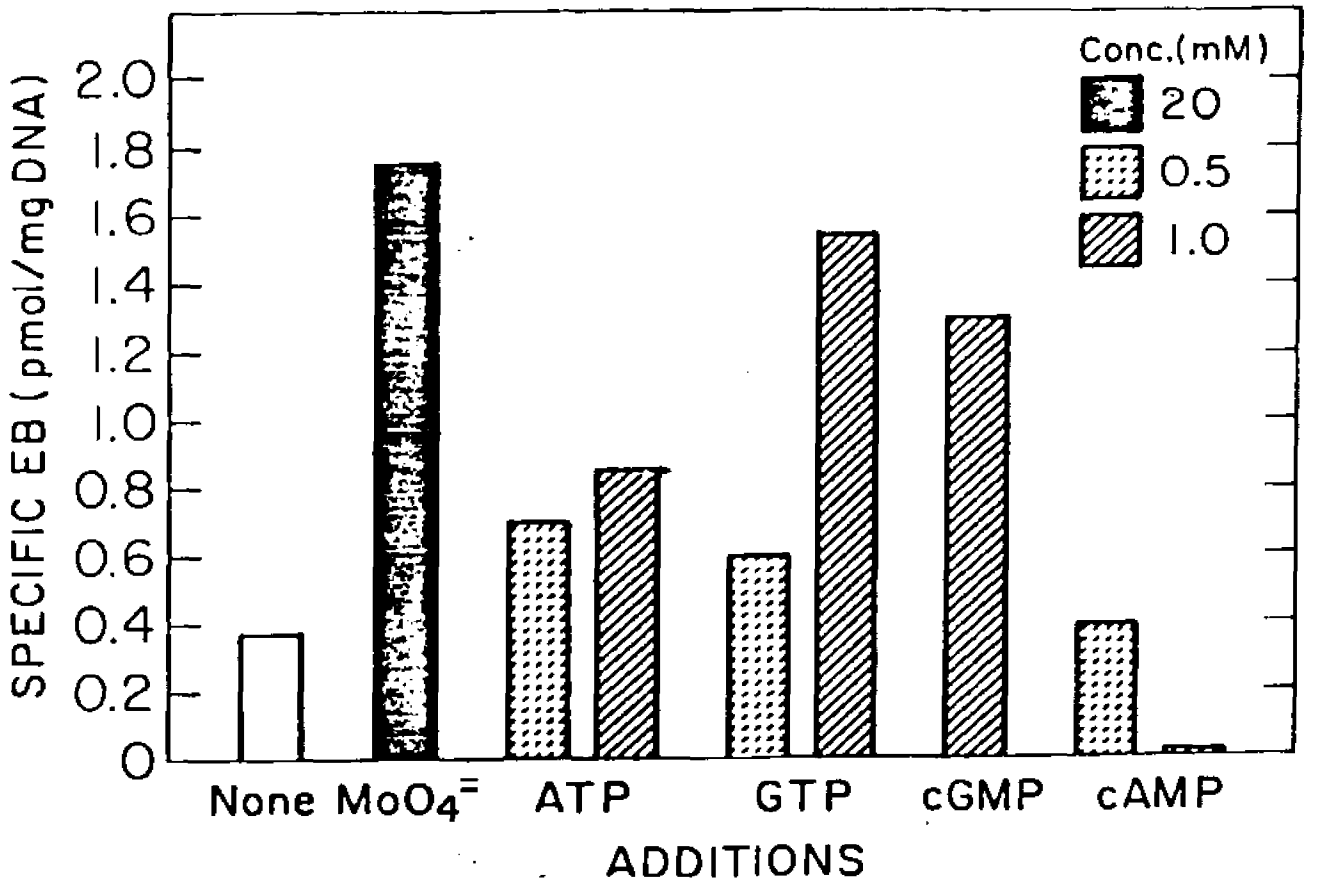


FIGURE 16: Comparison of the effects of MoO4=, ATP, GTP, cGMP and cAMP on cytoplasmic E2 binders in HEC-1 cells. Cells were homogenized as in figure 13 and 20mM MoO4= or three different concentrations of ATP, GTP, cGMP, or cAMP were added to the homogenate together with 80nM 3H-E2 with or without 8uM DES. Following a 90 minute incubation at 4C. cytoplasmic E2 binding levels were measured as described in figure 13. Each value represents an average of duplicate determinations. The data are representstive of experiments.

tems studied, ATP inhibits guanylate cyclase activity. However, in calf uterus, guanylate cyclase activity is stimulated in the presence of ATP (M.I Siegel et al., 1976). It is possible that ATP could be activating E2 binding by stimulating a particulate form of guanylate cyclase in this human uterine preparation or it may serve as a phosphate donor in phosphorylations catalyzed by a cGMP kinase.

cAMP was able to counteract the stimulating effects of $MoO4^{=}$, ATP and cGMP on binding (Figure 17). The finding that cAMP and cGMP have opposite effects on EB levels is characteristic of the action of these compounds on various other physiological events (N.D. Goldberg et al., 1974). Regulation of steroid binding may represent another bidirectionally controlled system by cyclic nucleotides.

As was shown with molybdate, neither ATP nor GTP had any effect on estrogen binding in cytoplasmic or cytosolic preparations (Figure 18). In contrast, both cyclic nucleotides maintained their respective effects in these fractions, cGMP increasing EB levels and cAMP decreasing binding levels. These observations extend previous findings on the involvement of components of the nuclear-plasma membrane pellet in the generation of E2 binding sites and once again suggests a common mode of action by molybdate and the two nucleotide triphosphates.

Table 4 summarizes the experiments performed in the HEC-1B cell line, in fresh human endometrium and in fresh

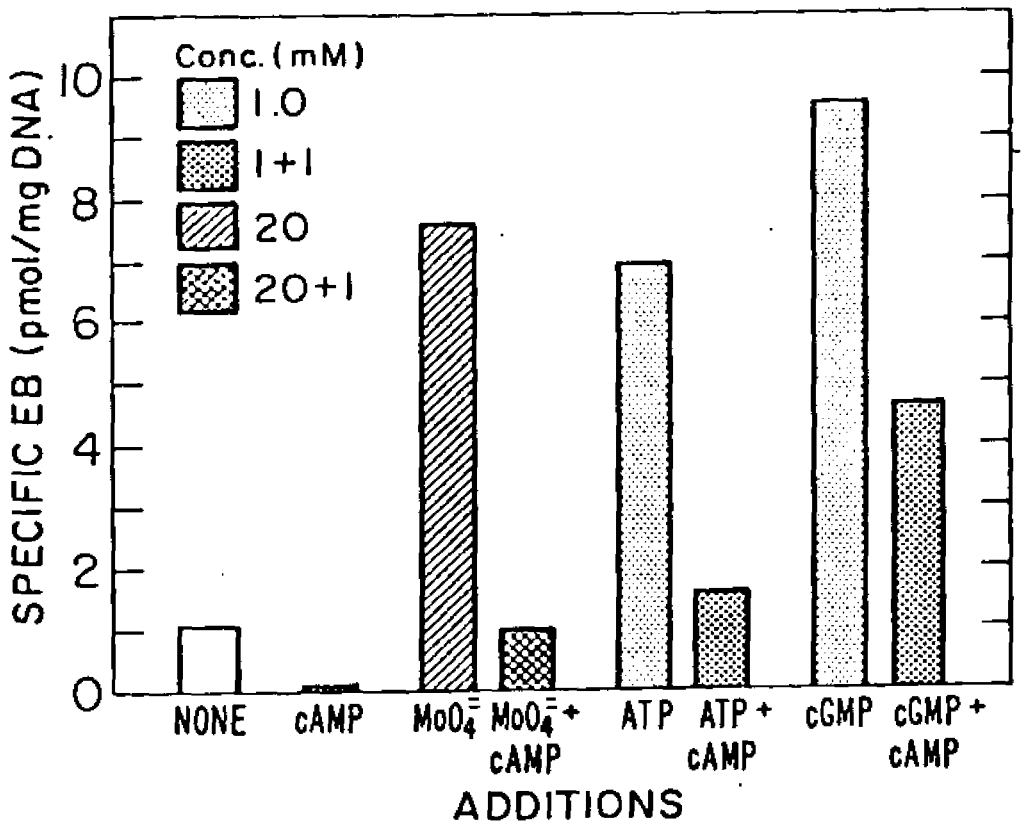


FIGURE 17: Comparison of the effects of cAMP on E2 binder activation affected by Mo04=, ATP, GTP or cGMP. HEC-1 cells were homogenized as described in figure 13 and 20mM Mo04=, lmM ATP, lmM GTP or lmM cGMP was added to the homogenate separately or together with lmM cAMP and incubated with 3H-E2. Specific cytoplasmic binding was measured as described in figure 13. Each value represents an average of duplicate determi The data are representative of three experiments which yielded similar results.

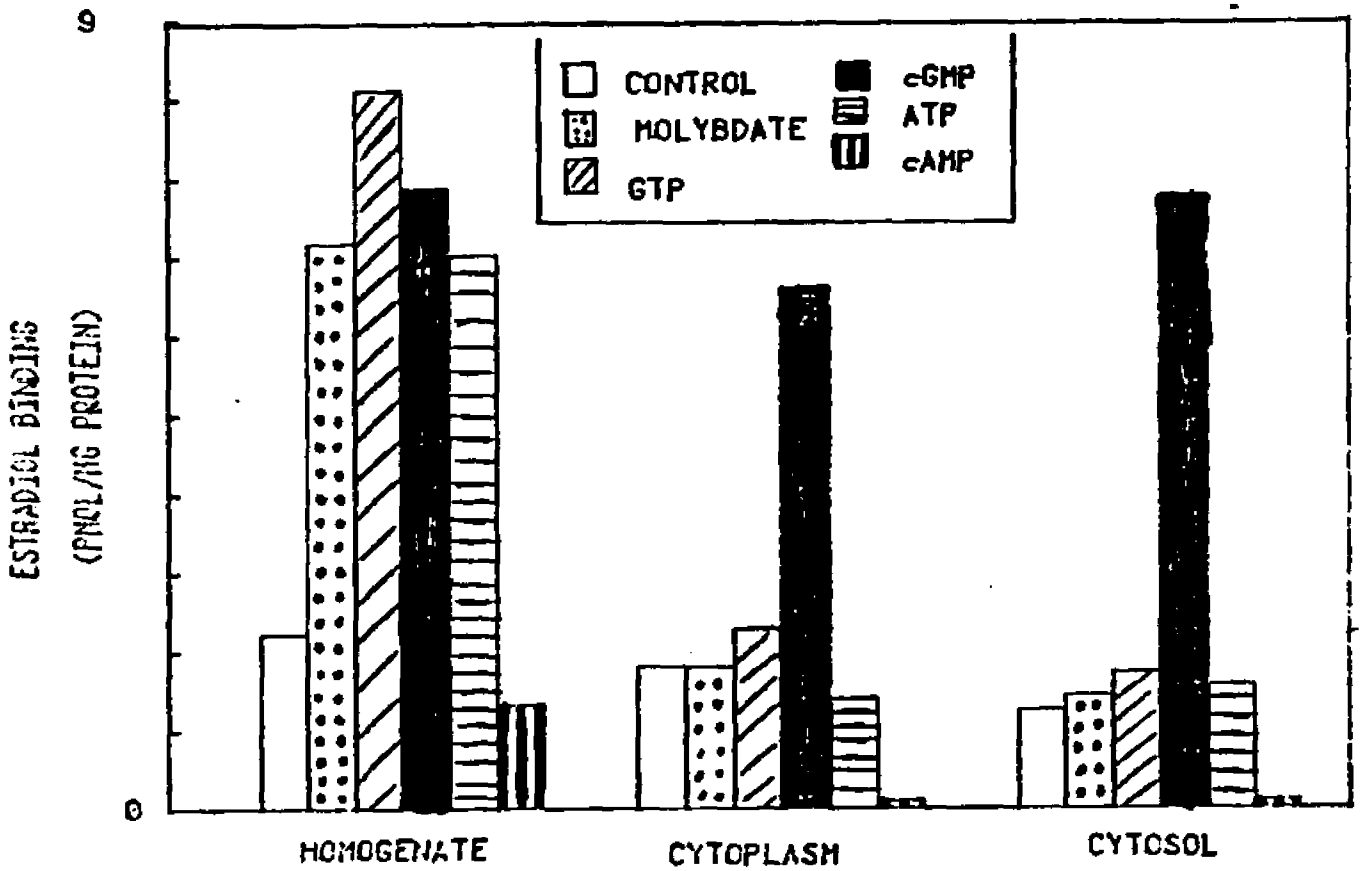


FIGURE 18 Effects of 20mM molybdate, 1mM GTP, 1mM ATP, 1mM cGMP and 1mM cAMP on the levels of specific estrogen binders labelled with 3H-E2 during incubations of whole cell homogenates, cytoplasm or cytosol prepared from HEC-cells. Cells were homogenized in GMTD buffer. A portion of the homogenate was centrifuged at 800g for 5 minutes to prepare cytoplasm and a portion was centrifuged at 105,000g for 1 hour to prepare cytosol. Each of these preparations were incubated with 80nM 3H-E2 with or without 8uM DES with no additions or with one of the above mentioned additions for 90 minutes at 4C. Radioactivity was measured in an aliquot of the charcoal treated supernatant and binding was determined as described in figure 12. These results are representative of 7 other experiments.

Table 4

Effect of Molybdate, Nucleotide Triphosphates and Cyclic Nucleotides on Total Cytoplasmic Estradiol Binding in Broken Cell Preparations:

Comparison	(N)	Mean (Cont)	Mean (Exp)	Significance
		HEC-1B Cells:		
Control vs. MoO4=	9	1.85	5.83	$P < .001$
Control vs. GTP	6	1.94	6.40	$P < .001$
Control vs. ATP	6	2.23	5.25	$P < .02$
Control vs. cGMP	9	1.65	6.14	$P < .001$
Control vs. cAMP	9	2.18	0.49	$P < .002$
		Fresh Human Endometrium:		
Control vs. MoO4=	8	1.24	2.79	$P < .05$
Control vs. GTP	7	0.77	2.69	$P < .05$
Control vs. ATP	6	0.85	2.22	$P < .05$
Control vs. cGMP	6	0.86	2.41	$P < .05$
Control vs. cAMP	4	0.84	0.41	$P < .02$
		Endometrial Adenocarcinoma:		
Control vs. MoO4=	6	1.05	1.87	$P < .002$
Control vs. GTP	6	1.05	2.14	$P < .002$
Control vs. ATP	6	1.05	2.17	$P < .02$
Control vs. cGMP	9	1.26	3.19	$P < .02$
Control vs. cAMP	7	1.67	0.31	$P < .02$

endometrial adenocarcinoma A significant increase in estradiol binding by MoO4=, GTP, ATP and cGMP and a significant reduction in binding in response to cAMP is seen in all three systems as evaluated by matched pair student t-tests.

Nuclear receptor were not affected by molybdate (Table 5) This indicates that the changes in cytoplasmic binding produced by ths compound is not due to intracellular redistribution of binding proteins between the cytoplasm and nucleus. This finding is further substantiated by the demonstration that cyclic nucleotides alter E2 binding in cytosol (in the absence of nuclei).

In preliminary trials, compounds that are known to stimulate guanylate cyclase activity, e.g. nitroprusside, nitric oxide and metcholine (F. Murad at el., 1978) all elevated estrogen binding levels 2-5 fold in cell homogenates but were ineffective when added to cytosol (results not shown).

The steroid binding proteins in HEC-1B cells were analyzed by sedimentation equilibrium on low salt glycerol density gradients in the presence of the serine protease inhibitor, phenylmethylsulfonyl fluoride (PMSF). Figure 19 shows that the radioactivity under both the 4S peak and the 8S peak sedimentation coefficients that are characteristic of E2 binding proteins under these conditions, are increased when 1mM cGMP is present during the labelling of cytosol with 80nM 3H-E2. Similar results have been obtained in the presence of 1mm GTP and 20mM MoO4=. Both peaks can be resolved

TABLE 5. (X ± SE of N-6)

EFFECT OF MOLTBDATE ON HEC-1B ESTRADIOL BINDING

CONTROL		20mM MoO4	
CYTOPLASMIC	NUCLEAR	CYTOPLASMIC	NUCLEAR
1.62	0.17	2.12	0.00
0.63	0.00	4.85	0.09
3.11	0.00	17.67	0.13
0.79	0.00	4.85	0.00
0.34	-0.00	-1.12	-0.08
0.09	0.12	1.25	0.00
0.98 ± 0.49	0.05 ± 0.03	5.31 ± 2.57	0.02 ± 0.02

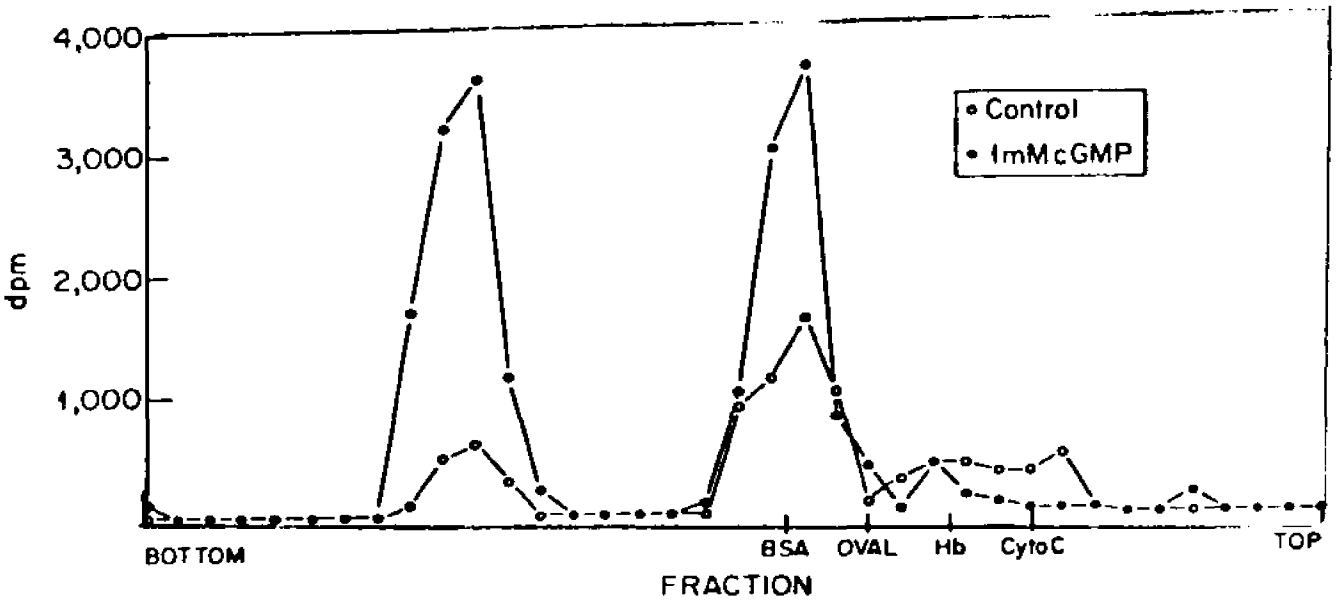


FIGURE 19 Low salt glycerol density gradients of HEC-1 cytosol prepared from cellular homogenates labelled with 3H-E2 in the presence or absence of cGMP. Equivalent aliquots of whole cell homogenate were labelled in the presence (•) or absence (o) of 1mM cGMP using 80nM 3H-E2 with or without 8uM DES for 2 hours at 4C. Labelled cytosol was prepared from the homogenate as described in figure 12, treated with charcoal and analyzed on low salt gradients as described in the methods section. Binding to the 8S and 4S binders was completely eliminated by DES. CytoC= cytochrome c; Hb= hemoglobin; OVAL= ovalbumin; BSA= bovine serum albumin.

if cytosol is labeled with 10nM E2. It is uncertain whether the two peaks represent two distinct binding proteins or whether the 4S form is a subunit or a proteolytic product of the 8S form of the binding protein.

The cGMP dependent increase in cytosolic binding (labeled with 10nM E2) is due to an increase in the number of binding sites rather than to a change in affinity of the ligand for the binding protein as determined by Scatchard analysis (Figure 20). In this particular experiment the number of cytosolic binding sites doubles in the presence of 1mM cGMP with E2 while the affinity constant (Kd) remains constant at 1nM. The second binding protein which saturates at 80nM can not be analyzed by this method, it exhibits positive cooperativity as represented by a concave plot.

The effects of cGMP and cAMP on cytosolic EB levels were found to be concentration dependent (Figure 21). In the presence of 100uM IBMX, a phosphodiesterase inhibitor, both cGMP and cAMP exhibited their respective effects at 1nM and became maximal at 1uM concentrations that are considered physiological. In the absence of IBMX, the cyclic nucleotides were only effective at 1mM a supraphysiological amount. These results suggest that phosphodiesterase activity is quite large even when assays were performed at 4C.

The generation of sites by micromolar additions of cGMP and the inactivation of sites by addition of cAMP occurred rapidly (Figure 22.) Levels of specifically bound 3H-E2

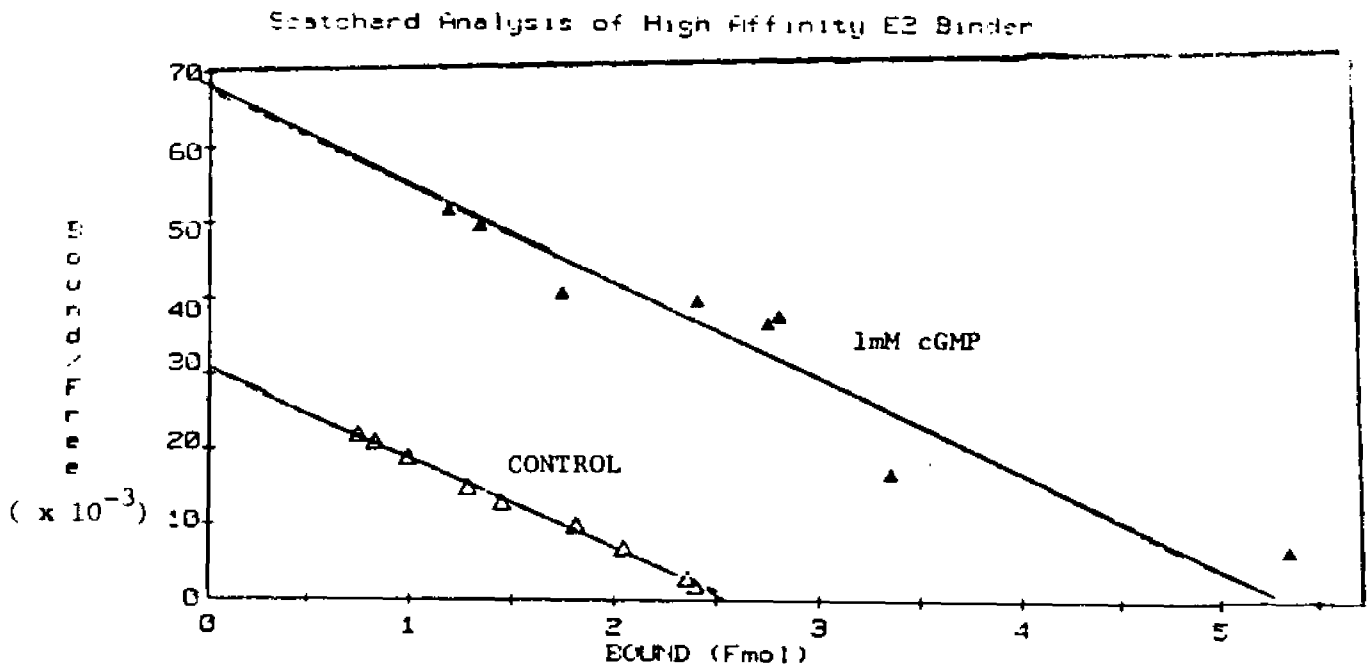


FIGURE 20 Scatchard analysis of the estrogen receptor in HEC-1 cells. Cells were homogenized in GMTD buffer and incubated with varying concentrations of 3H-E2 ± 100x DES (1nM to 15nM) for 2 hours at 4C in the presence or absence of cGMP. The incubation was stopped by rapid freezing and samples were processed as described in figure13.

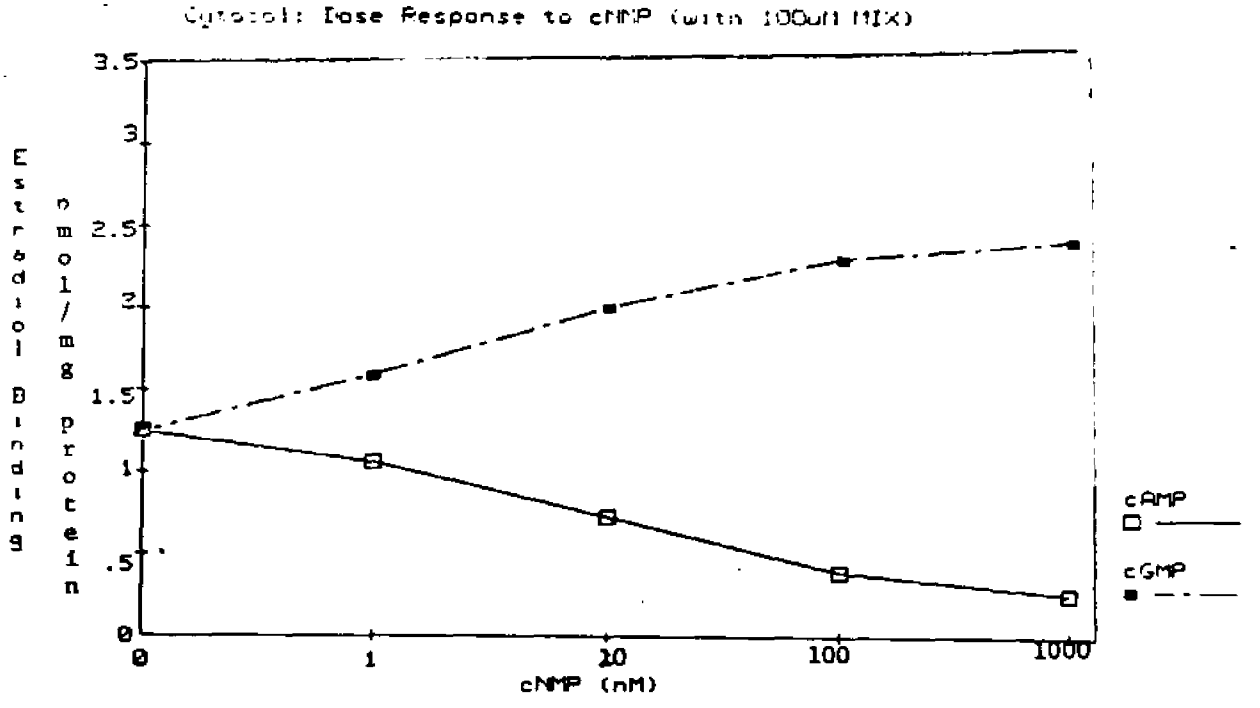


FIGURE 21: Effects of increasing concentrations of cGMP and cAMP on E2 biding in HEC-1 cell cytosol. HEC cells were homogenized in GMTD containing 250uM MIX. Cytosol was prepared and labled as in figure 18. The average of duplicate assays in a single experiment are shown, and this data are representative of 3 other experiments.

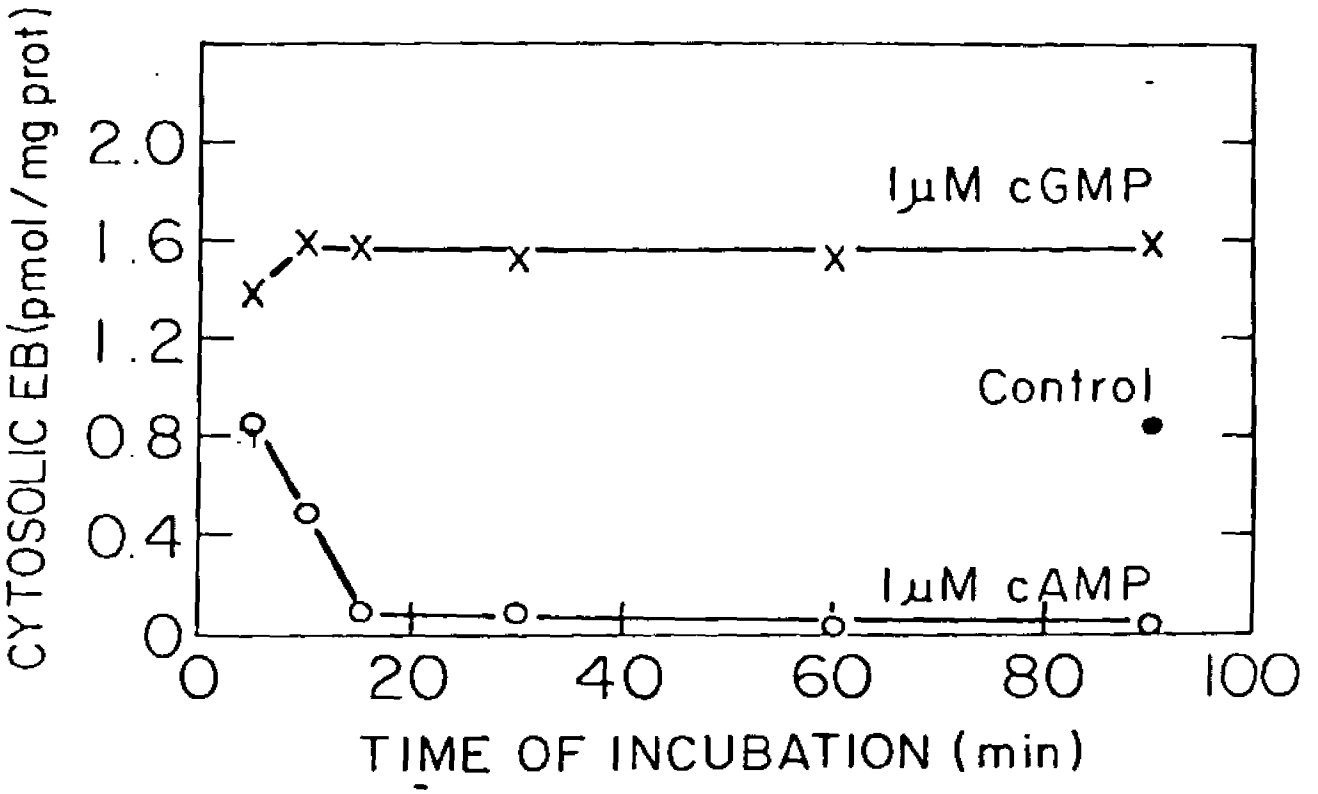


FIGURE 22: Effects of time of incubation on cGMP and cAMP-dependent changes in cytosolic EB levels. Cytosol from a specimen of proliferative endometrium was incubated at 4C with either cGMP or cAMP and 80nM 3H-E2 with or without DES for increasing periods of time. EB levels achieved during these incubations and during a 90 minute period in the absence of cyclic nucleotides was determined as in figure 13. The averages of duplicates in a single experiment are shown.

were determined after 90 minutes in the absence of cyclic nucleotides or after various times of incubation from 5-90 minutes in the presence of cyclic nucleotides. cGMP cGMP dependent activation of binding as well as binding of 3H-E2 to the newly generated sites reached steady state levels within 10 minutes. cAMP-dependent inactivation was complete within 15 minutes. The time needed to alter binding by cyclic nucleotides is quite comparable to the time required for molybdate to change the number of binding sites (Figure 8 and 13).

The observed change in binding occurred specifically in response to the purine nucleotide triphosphates and cyclic nucleotides. In Figure 23a, binding is not significantly affected by UTP and CTP, while ATP and GTP both triple the amount of E2 binding. Figure 23b shows that cCMP does not affect E2 binding while cUMP produces a slight decrease in binding and cIMP results in a decrease in binding that is comparable to the effect seen by cAMP. The mechanism of action of cIMP remains unexplained at this time.

Manipulations that have been reported to inhibit auto-phosphorylation of the cGMP dependent protein kinase and thus stimulate kinase activity (T.M. Lincoln et al., 1978) are seen to elevate EB (Figure 24). Butadione (20mM), an arginine specific blocking agent and limited proteolysis with trypsin (0.lug) both are known to activate cGMP depend-

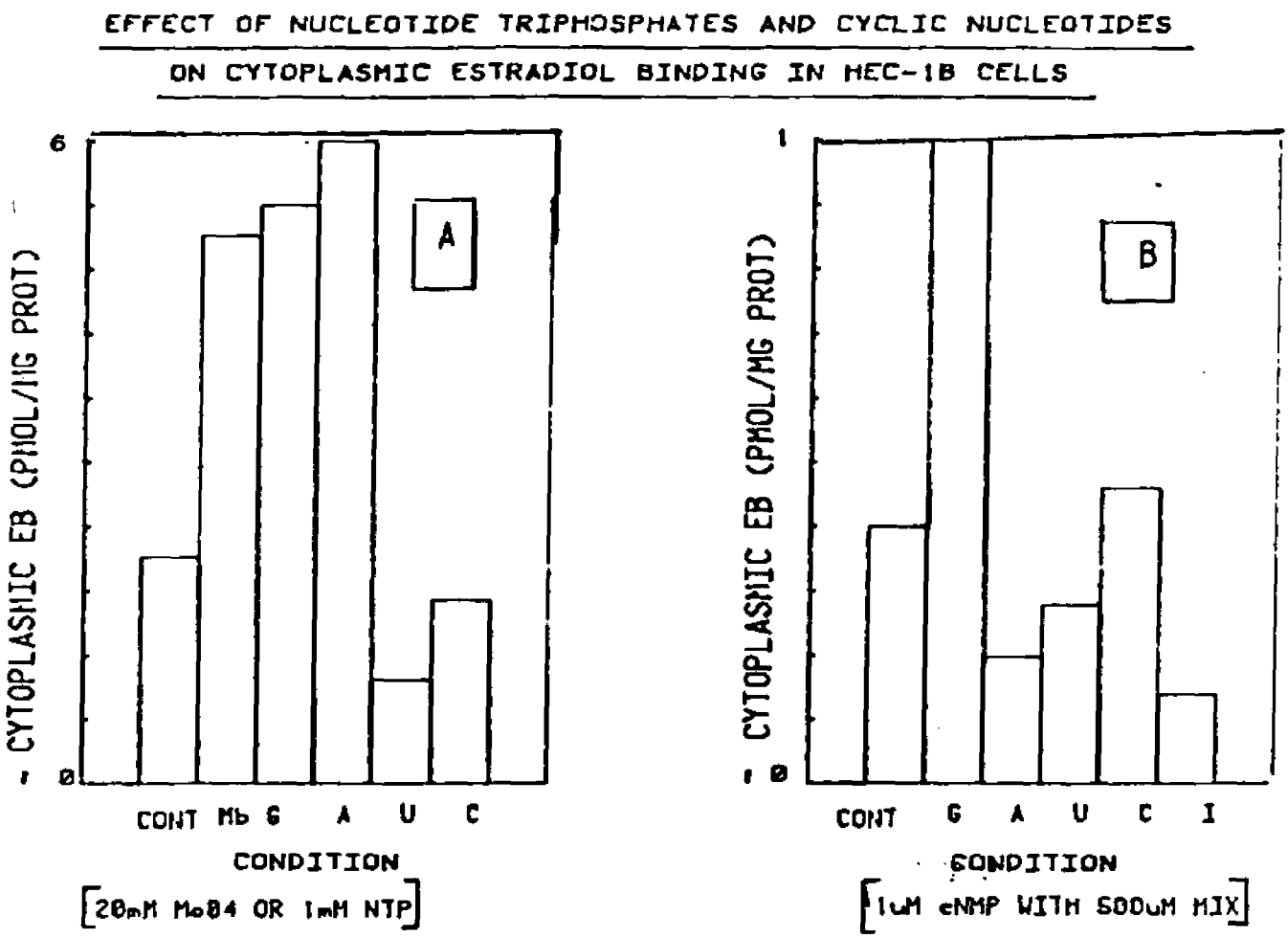


FIGURE 23: Effect of A) nucleotide triphosphates and B) cyclic nucleotides on estrogen binding in HEC cells. A) HEC cell homogenate was incubated with 80nM 3H-E2 ± 8uM DES without any additions or with 20mM $MoO_4^=$ or 1mM of either GTP ATP, UTP or CTP for 90 minutes at 4C. B) Aliquots of the homogenate were incubated with steroid in the absence of any cyclic nucleotide or in the presence of 1uM cGMP, cAMP, cUMP or cIMP. These samples all contained 500uM IBMX. Samples in A and B were processed as described in figure 12.

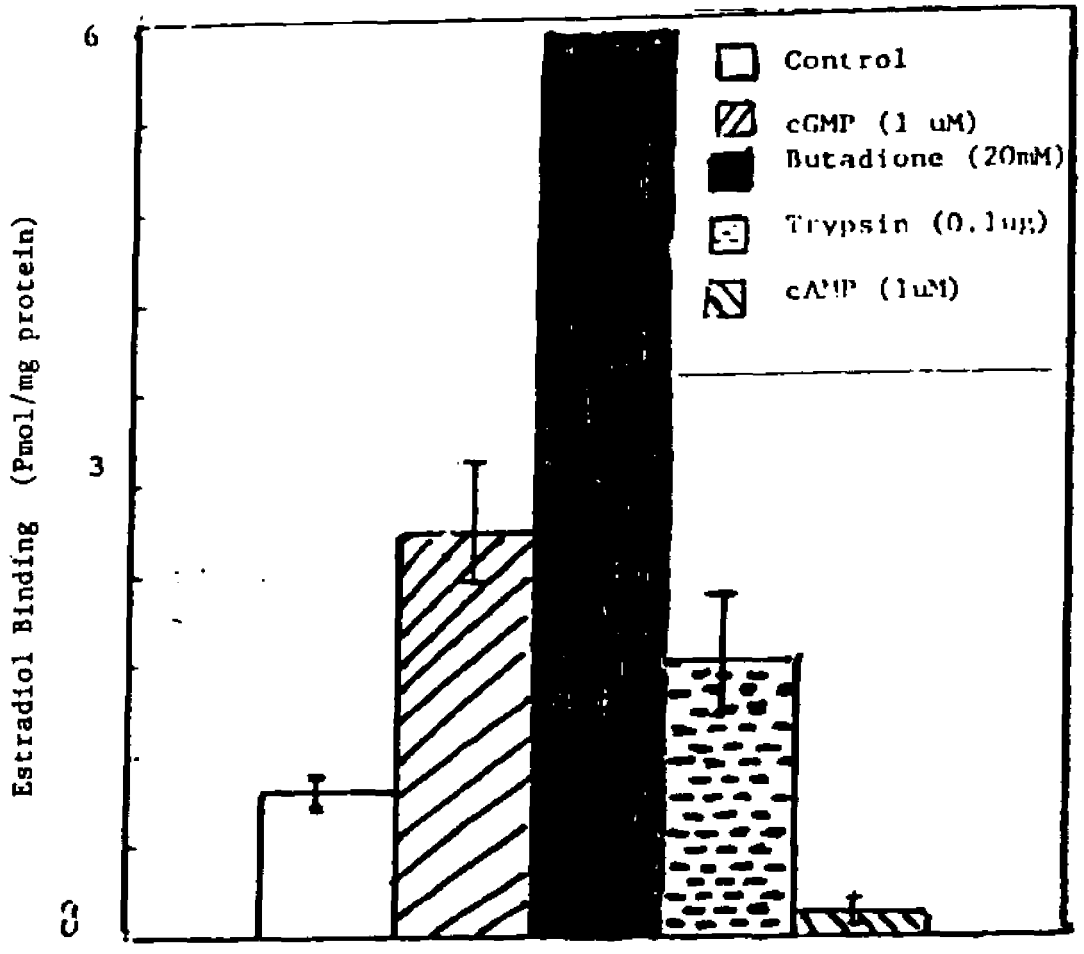


FIGURE 24: Effect of agents that modify cGMP-dependent protein kinase activity on estradiol binding. cGMP, butanedione, trypsin and cAMP were added to cytosol preparations of HEC cells in the concentrations listed. Estradiol binding was evaluated as previously described and the results are expressed as the mean ± SE for 5 experiments.

the kinase, though it should be emphasized that both of these compounds have rather nonspecific effects in the cell. cAMP has been reported to stimulate self phosphorylation of the kinase and thus decrease enzyme activity. This may in part explain how cAMP reduces estrogen binding. cAMP may shift the equilibrium towards the inactive form of the binding protein by inhibiting the mechanism that normally converts the nonbinding form of the protein to the binding form of the protein.

The regulation of binding observed by cGMP and cAMP has been seen in a number of other cancer cell lines besides the HEC-1 cell system. A second endometrial adenocarcinoma (HEC-50) and two breast tumor lines (CG5 and T47D) all show increases in E2 binding upon incubation with cGMP and decreases in binding often to negligable levels, upon addition of cAMP (Figure 25) whether labeling was done at 10nM E2 or 80nM E2.

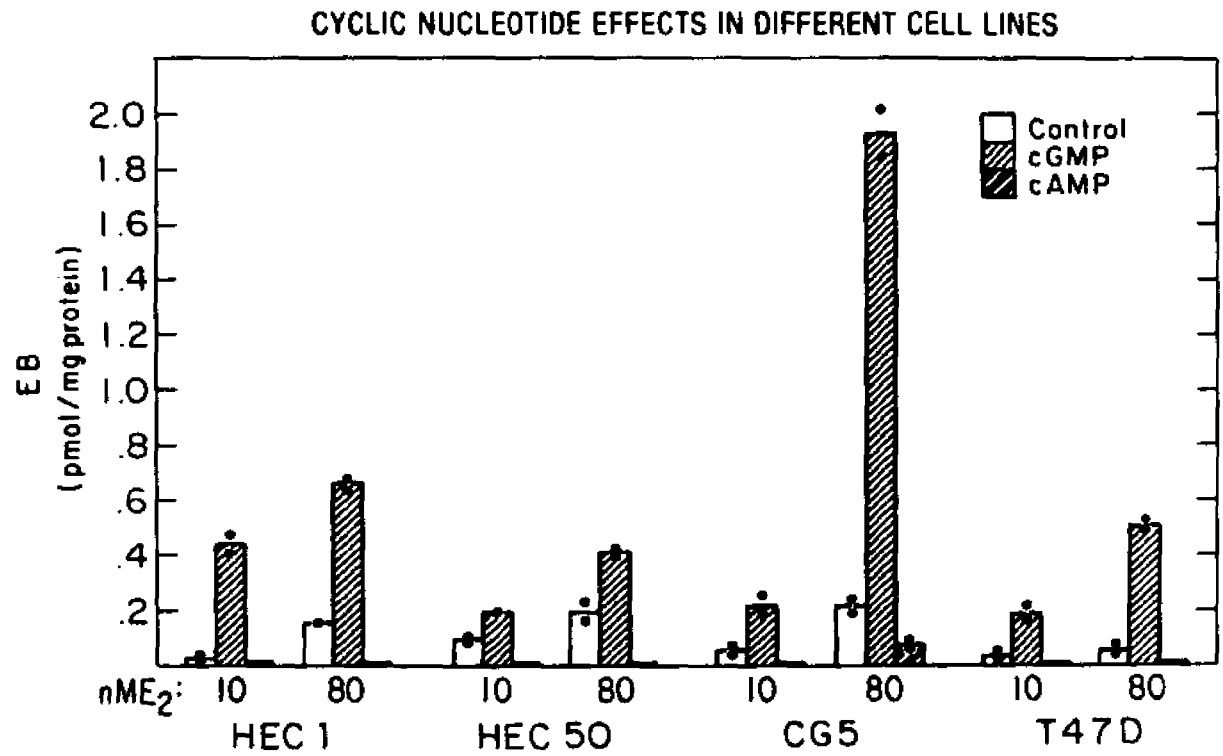


FIGURE 25: Effects of cyclic nucleotides on E2 binding in four different cell lines: HEC-1, HEC-50, CG5 and T47D. cells were cultured as described in methods section and homogenized in GMTD buffer with 20uM MIX and aliquots of each broken cell preparation were incubated with 10nM 3H-E2+ 1uM DES to saturate the high affinity binding protein or with 80nM 3H-E2± 8uM DES to saturate both the high and low affinity specific binders. Labelled ligand was dissolved in HBSS at 2.5 times the desired final concentration and 10ul of GMTD alone or GMTD with 5uM cGMP or cAMP and added to 20ul of the homogenate. The suspension was rapidly frozen following the 90 minute incubation at 4C. Specific binding was measured as described in figure 12.

The conditions for activation and inactivation of E2 binding by cGMP and cAMP respectively were studied. Both processes required the presence of divalent cations (an important finding since many laboratories attempting similar studies have been working in a Tris- EDTA buffer system). However, the reactions differ with regard to optimal cation concentration (Figure 26). The activation of E2 binders in response to luM cGMP continued to rise as the concentration of Mg++ or Mn++ was increased to 100mM (upper panel). In contrast inactivation of estrogen binding in response to luM cAMP was maximal at 25mM Mg++ or Mn++ and declined at greater concentrations of these cations. The maximal effects obtained with Ca++ were smaller than those achieved in the presence of Mg++ or Mn++. These results demonstrate that the effects of cGMP and cAMP on EB levels are mediated by proceses influenced differently by these cations. It is interesting to note that the dose dependence for cations by these two reactions is similar to those reported for both cyclic nucleotide dependent protein kinases (Takai et al., 1976). In addition in calf uterus, estrogen binding can be inactivated by a nuclear phosphatase and can be reactivated by a Mg++ and Ca++ dependent cytosolic phosphokinase (M. Migliacio et al., 1983).

Endogenous ATP was necessary to elicit changes in estrogen binding by both cyclic nucleotides (Figure 27). Under standard conditions when both cyclic nucleotide effects could be observed the concentration of ATP in HEC-1B cytosol

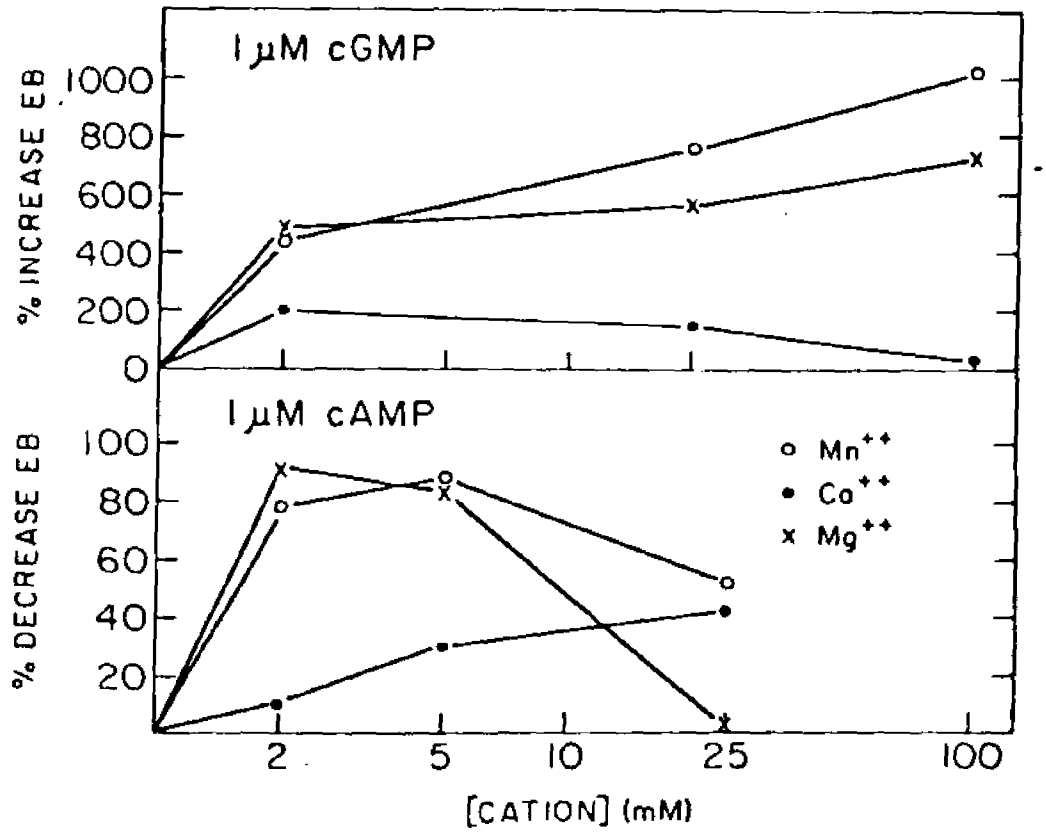


FIGURE 26: Effects of increasing concentration of MgCl2, MnCl2 or CaCl2 on EB levels in HEC-1 cell cytosol treated with cGMP or cAMP. HEC-1B cells were homogenized in GTD buffer (in the absence of MgCl2). Cytosol was prepared and labelled in the presence of cGMP (Upper panel) or cAMP (lower cAMP) and increasing concentrations of MgCl2 (X), MnCl2 (o) or CaCl2 (•). Labelling and measurement of levels of binding was carried out as described in figure 12. The average of duplicate assays in a single experiment are shown and these data are representative of results obtained in three similar experiments.

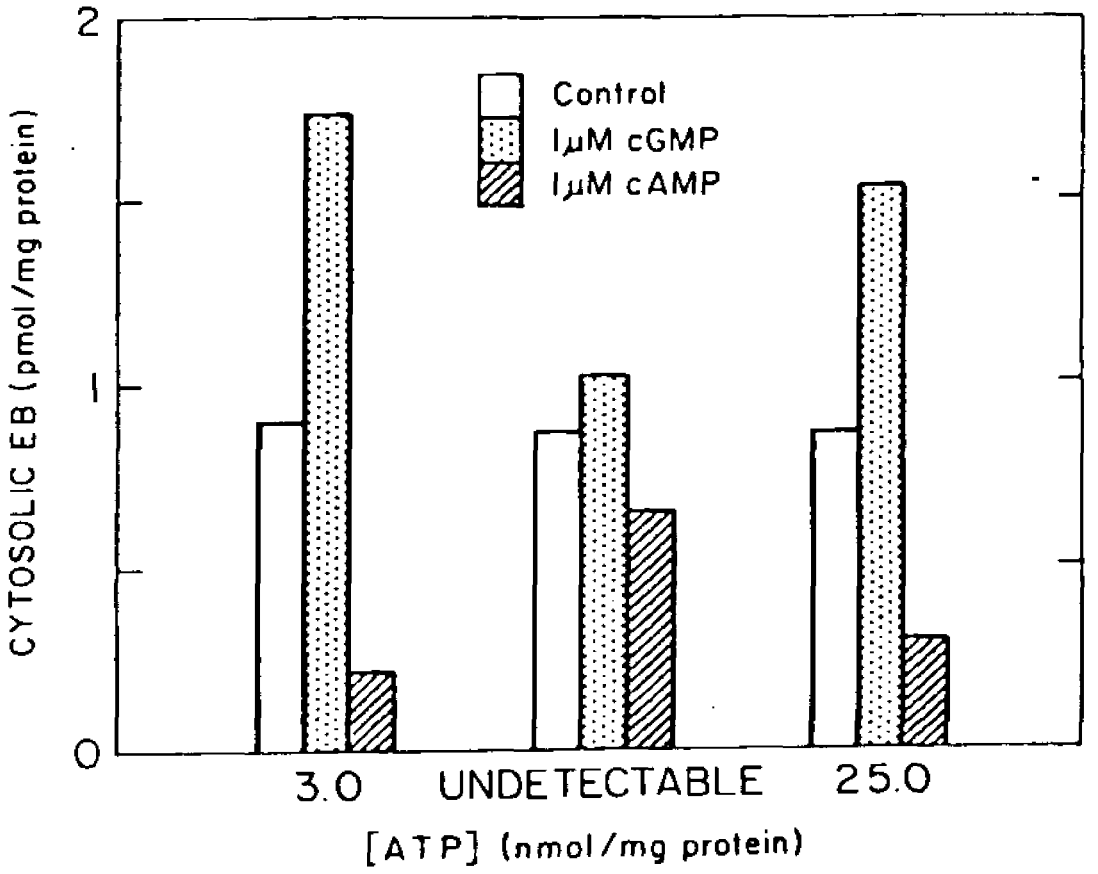


FIGURE 27: Effects of ATP on cGMP and cAMP-dependent changes in cytoplasmic EB levels. HEC-1 cells were homogenized as in figure 12 and one-third of the homogenate was immediately incubated at 4C for 90 minutes with 80nM 3H-E2 with or without 8uM DES in the presence of 0.1mM MIX and cGMP (1uM) cAMP (1uM) or no cyclic nucleotides. The remainder of the homogenate was stored at 4C for 3 hours to allow ATP levels to decline to undetectable levels and then was was incubated as described above with steroid+ cyclic nucleotide+ exogenous ATP. Concentrations of ATP were measured by the luciferin- luciferase method after extraction of the homogenate with 30% HClO4 and neytralizing with 1M KOH. EB levels were determined as in figure 13. The averages of duplicate assays in a single experiment are shown. Similar results were obtained in two other experiments.

approximated 3nmol/mg protein. If cytosol was stored at 4C for 3 hours, the amount of cytosolic ATP declined to undetectable levels and the response to both cyclic nucleotides was greatly diminished. When supraphysiological concentrations of ATP was added back to the ATP depleted cytosol, the responsiveness to the cyclic nucleotides was restored and the control and treated binding levels approximated those observed in the original cytosolic preparation.

In all estrogen binding studies, the labeled steroid was dissolved in HBSS because, as figure 28 shows, if HBSS is replaced by GMTD or by a Tris-EDTA buffer, the cyclic nucleotide effect was abolished. To examine this finding further, the factor(s) in HBSS required for cyclic nucleotide dependent alterations in E2 binding were identified. Each of the six components in HBSS were added separately and in their proper proportions to GMTD buffer containing 3H-E2 + DES and the effect of cGMP and cAMP on cytosolic EB was determined. The addition of 2mM KCl resulted in significant increases in EB by cGMP and significant decreases in binding by cAMP. There was also evidence of binder generation and inactivation by cGMP and cAMP, respectively, when 160uM KH_2PO_4 was present in the buffer system. It seems likely that the K^+ ion is responsible for these activities since both salts containing potassium had an effect which was greater at higher K^+ concentrations while NaH_2PO_4, NaCl and $NaHCO_3$ were all without effect. The function of potassium

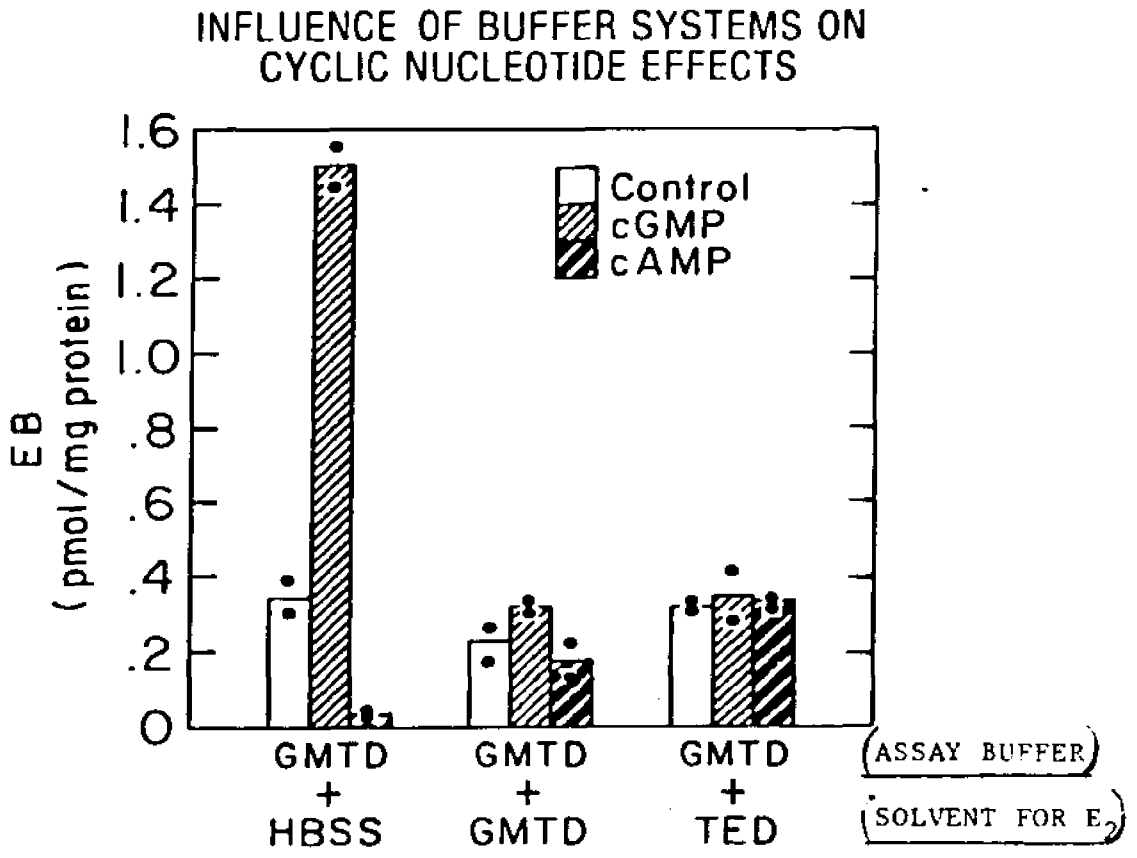


FIGURE 28: Influence of different buffer systems on the effects of cGMP and cAMP on specific cytoplasmic E2 binding levels. Estrogens were dissolved in HBSS, GMTD or Tris-EDTA buffer (0.01M Tris-HCl pH 7.4, 1mM EDTA and 1mM Dtt) to a final concentration of 200nM 3H-E2 + 20uM DES. To 20ul of HEC-1 cell homogenate, 10ul GMTD alone or containing 5uM cGMP or 5uM cAMP was added along with 20ul of steroid solutions. Incubations were done at 4C for 90 minutes and specific binding was determined as described in the legend to figure 12.

TABLE 6

INFLUENCE OF EACH COMPONENT OF HBSS ON CYCLIC NUCLEOTIDE EFFECTS IN HEC-1 CELL HOMOGENATES

	200μM IBMX control	200μM IBMX + 1μM cGMP	200μM IBMX + 1μM cAMP
	EB fmol/mg protein	EB fmol/mg protein	EB fmol/mg protein
GMTD buffer	200	250	300
+ 800μM glucose	250	1,700#	50#
+ 130μM NaH_2PO_4	250	250	250
+ 55mM NaCl	250	100	150
+ 2mM KCl	300	3,350†	50#
+ 160μM KH_2PO_4	50	750*	50
+ 1.7mM $NaHCO_3$	50	50	150

† $p < 0.01$
# $p < 0.02$
* $p < 0.05$

Two-way analysis of variance (SAS)
Dunnett Multiple Range Test

Figure 29 shows that dithiothreitol is also a necessary addition for both cyclic nucleotide effects to occur. The amount of binding in control preparations in the absence of cyclic nucleotide additions increased from 180 to 600 fmol/mg protein when Dtt was added to the homogenizing buffer. In the absence of Dtt, addition of cGMP resulted in some binder generation, however, the relative increase in binding due to cGMP was enhanced as the concentration of Dtt increased. The effect may be due to stabilization of binding sites as they are generated, or it may be due to a stimulation of the binder generating reaction. In the absence of Dtt, cAMP did not appear to have any activity, whereas its effect was noted in the presence of both 0.1 and 1.0mM Dtt.

All of the experiments described on cyclic nucleotide dependent regulation of E2 binding were performed at 4C. In figure 30 the effect of temperature on cyclic nucleotide changes in EB was evaluated. The results indicate that there is no difference in binder regulation at 4C and 30C. However at 37C control cGMP and cAMP regulated E2 binding levels were all reduced, perhaps due to some instability of the receptor at that temperature.

The regulatory role of cyclic nucleotides on steroid binding was extended by investigating the effects these substances have on progesterone binding in human endometrium.

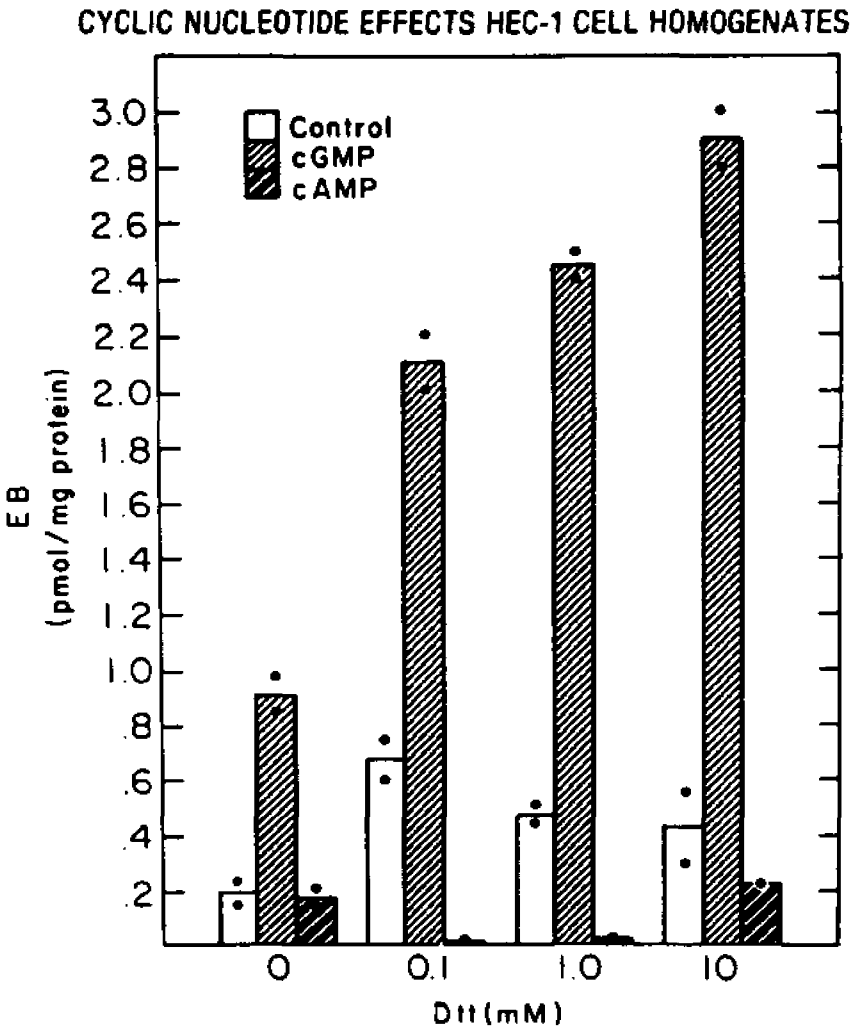


FIGURE 29: Influence of various concentrations of dithiothreitol on the cyclic nucleotide effect on cytoplasmic E2 binding levels. HEC-1 cells were homogenized in GMTD buffer in absence of Dtt or in the presence of 0.1, 1.0 or 10mM Dtt and aliquots of each preparation were incubated with 80nM 3H-E2 ± 8uM DES alone or in the presence of 1uM cGMP or 1uM cAMP. Radioactivity was determined as described in figure 13.

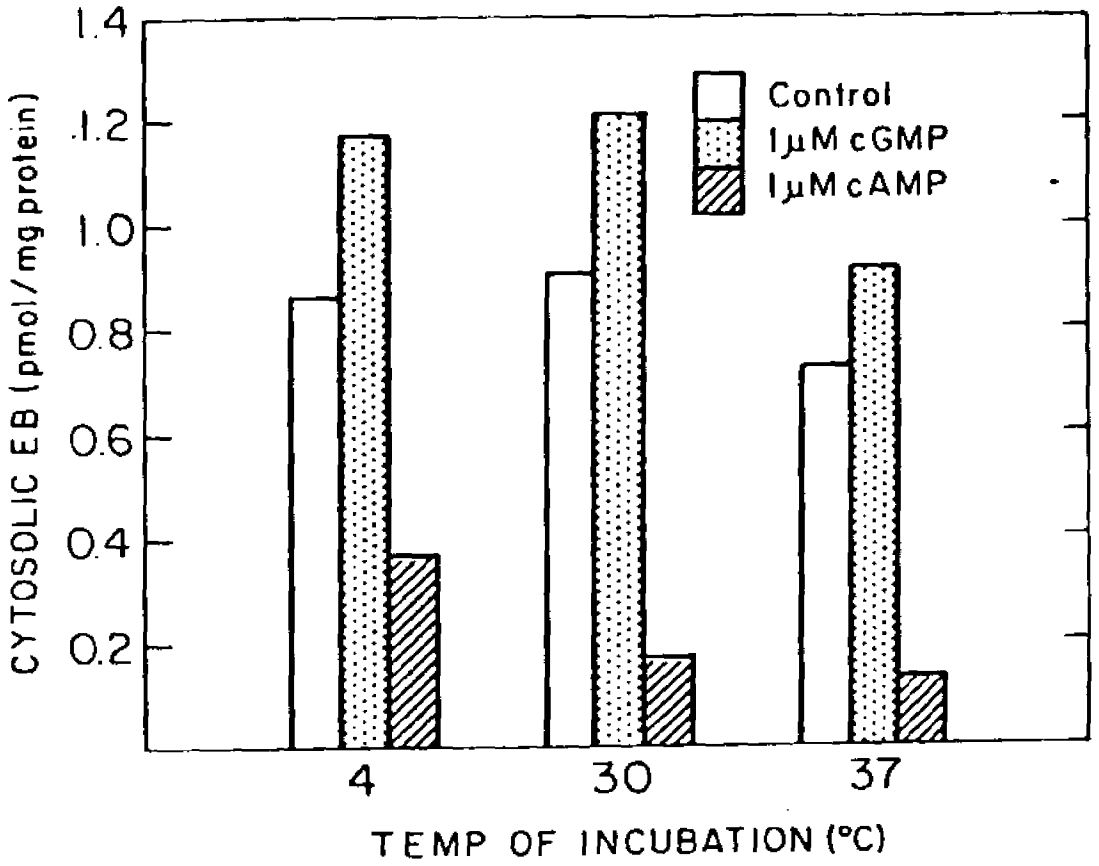


FIGURE 30: Effects of temperature of incubation on cGMP and cAMP dependent changes in cytosolic EB levels. HEC-1B cell cytosol was incubated with either cGMP or cAMP and 80nM 3H-E2 with or without 8uM DES for 90 minutes at 4C, 30C or 37C. EB levels were determined as in figure 12. The average of duplicates in a single experiment are shown.

The results are shown in Figure 31. Similar to the findings for estrogen binding cGMP elevated PgR and cAMP decreased PgR when the cyclic nucleotides were added at millimolar concentrations alone or at micromolar concentrations in the presence of 200uM IBMX. The buffer for the progesterone binding assay was identical to the one for the estrogen binding assay. If the assay was done in the presence of EDTA and in the absence of HBSS (without potassium or glucose), cyclic nucleotides were ineffective in causing changes in binding. It is unknown at this time whether other steroid binding (androgens, glucocorticoids) would be effected by the cyclic nucleotides as well.

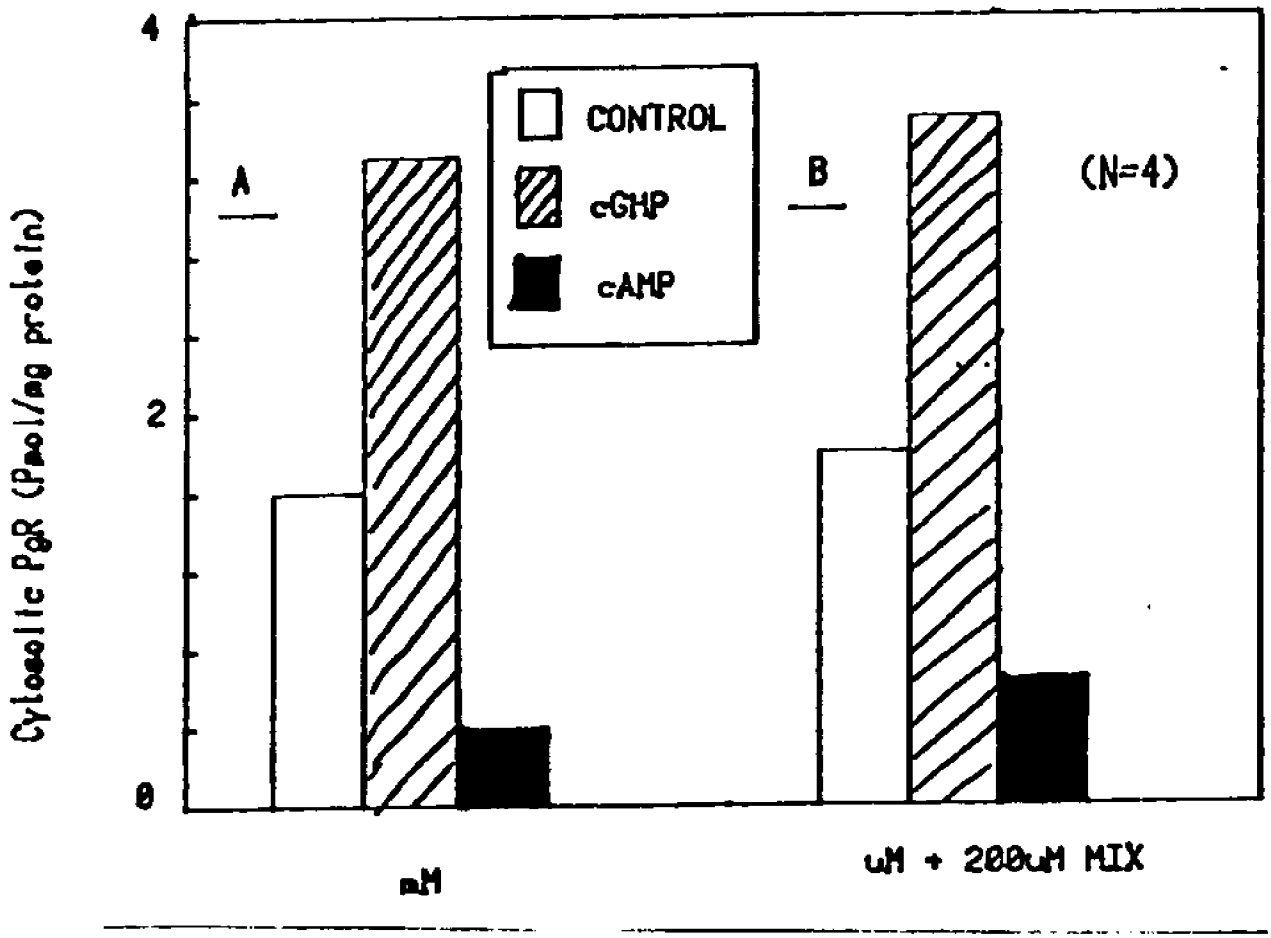


FIGURE 31: Effect of cyclic nucleotides on cytosolic progesterone binding in fresh endometrium. Endometrial samples (mid-proliferative and early secretory) were homogenized in GMTD buffer. The broken cell preparation was centrifuged at 105,000g for 60 minutes. ALiquots of the cytosolic supernatant were incubated with 2 pmol 3H-progesterone ± 200pmol cold progesterone for 3 hours at 4C. A) 1mM cGMP or cAMP were added to some samples. B) 1uM cGMP or cAMP were added in the presence of 500uM MIX. All sample were rapidly frozen, thawed and processed as described in figure 12 for the estrogen receptor assay. Results are the average of four experiments.

5 Regulation of Estradiol Binding in Cell Culture

The discovery that endometrial EB levels could be regulated by cyclic nucleotides IN VITRO raised the possibility that the hourly EB fluctuations seen in cell culture (Figure 3) might be explained by changes in endogenous levels of cyclic nucleotides. To investigate this possibility, the concentration of cAMP and cGMP together with the concentration of E2 binding sites were assayed in cultured endometrial cells every 60 minutes for a period of 9 hours as shown in Figure 32. Cell sampling began two hours after the addition of medium with serum to cells that had been cultured in serum free medium for several days prior to the start of the experiment. EB levels remained low for the first four hours following refeeding and then increased gradually over the next five hours (panel B). Increases in the concentration of EB sites paralleled increases in cellular cGMP and decreases in cellular cAMP (panel A).

When EB levels from this experiment were plotted as a function of the ratio of cAMP to cGMP at each time point, the results approximated a straight line with a correlation coefficient of 0.9 (Figure 33 open circles). These results are representative of six other experiments in which the correlation coefficient in each ranged from 0.85 to 0.99. In all experiments the correlation coefficient was highest when EB levels were regressed against the ratio of the

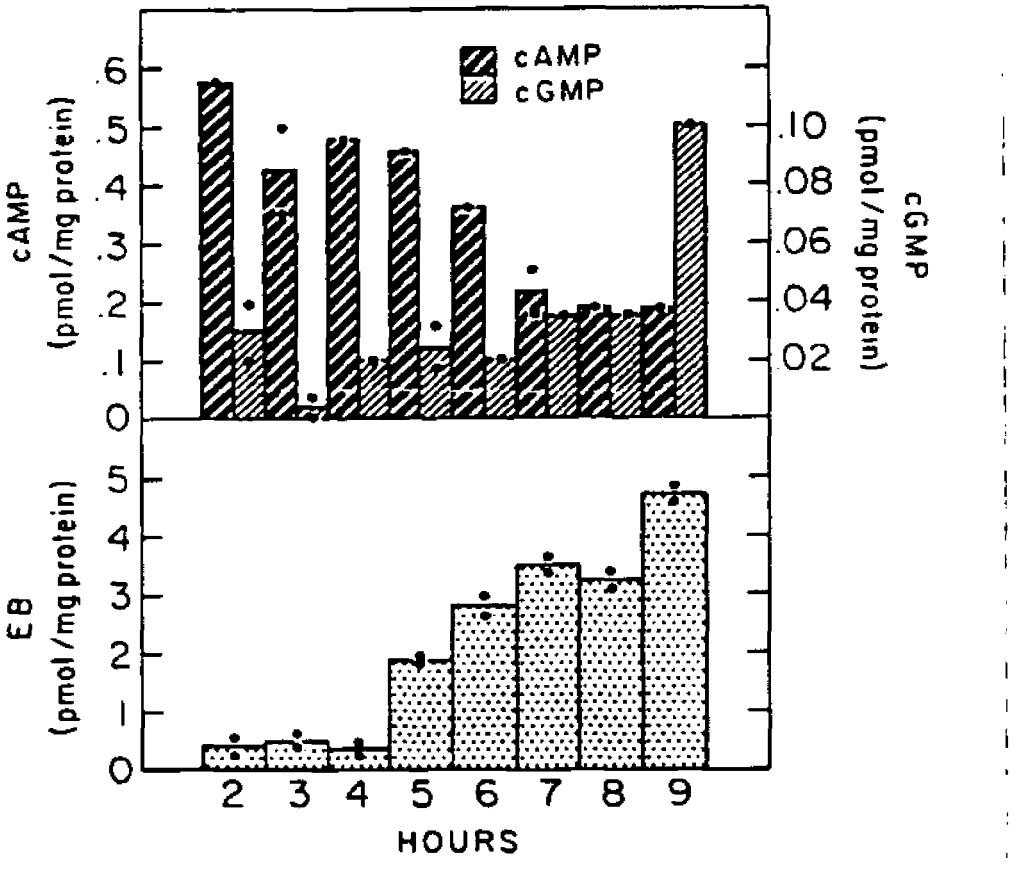


FIGURE 32: Hourly fluctuations of E2 binding, cellular cAMP and cellular cGMP in aliquots of HEC-1 homogenates. Cells were serum starved for 3-4 days prior to the start of the experiment. After two hours in fresh medium the first plates of cells were harvested. Sets of plates were harvested every hour after that time. Binding was measured in the homogenate as described in figure 12 (results in bottom panel). cAMP was measured by a competitive protein binding assay. Free cAMP was removed by charcoal treatment. cGMP was measured by a radioimmunoassay. Following the incubation, the bound cGMP was removed by ammonium sulfate treatment (results in top panel). The average of duplicate assays in a single experiment are shown and these data are representative of six other experiments.

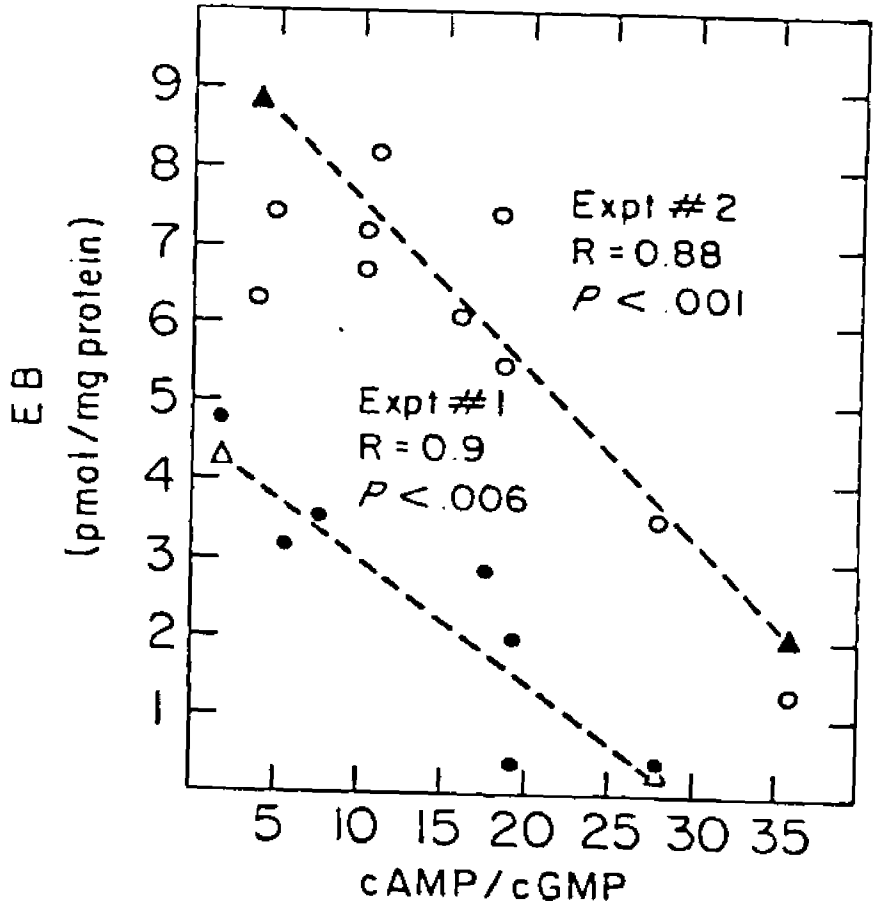


FIGURE 33: Correlation of cytoplasmic estradiol binding levels and whole cell cyclic nucleotide ratios in two different HEC-1 cultures following refeeding. The results of E2 binding in the experiment shown in figure 33 were regressed against the ratio of the cyclic nucleotides in that same experiment and the best fit line was plotted as "exp. #1". Results of a second experiment that was performed in the identical manner except that sampling was done for ten hours instead of seven hours are graphed as "exp #2".

cyclic nucleotides rather than either cAMP or cGMP alone. The slope and correlation coefficient for similarly plotted data from a second experiment is also shown in Figure 33 (closed circles). These results resemble those for the first experiment except that EB is approximately half of that in experiment #1. This difference could be explained by a difference in the amount of inactive binder available for activation.

The correlation of many metabolites change in cultured cells as a function of addition of fresh medium. An absolute correlation between cyclic nucleotide ratios and EB levels in cultured cells would not prove a cause and effect relationship since both parameters could be changing in response to a third variable. For this reason, the effect of changing cyclic nucleotide ratios on E2 binding was tested. Figure 34 shows the effect of adding different ratios of cyclic nucleotides to whole cell homogenates. The change in binding is expressed as a function of control binding levels. When the cAMP/cGMP ratio was high EB levels were low and the effect is gradually reversed as the concentration of cGMP/cAMP increases.

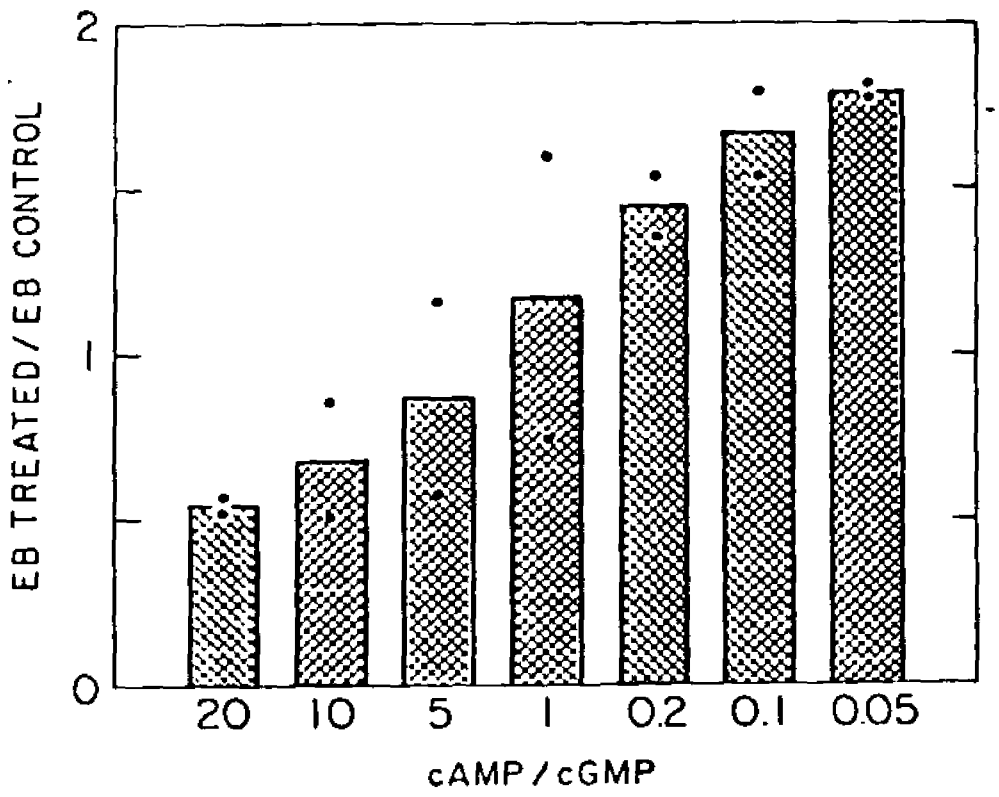


FIGURE 34: Addition of varying ratios of cAMP/cGMP: effect on cytoplasmic E2 binding. Estradiol binding in HEC-1 homogenates was measured as described in the legend to figure 12 in the absence of cyclic nucleotide additions or upon addition of varying ratios of exogenous cAMP and cGMP. The cNMPs were added in micromolar concentrations in the presence of 200uM MIX. The results are plotted as the amount of binding in cNMP treated cell aliquots divided by the binding in the absence of cyclic nucleotides against the ratio of the added cNMPs.

TABLE 7

MoO_4 (mM)	cAMP (fmol/mg protein)	cGMP	cAMP/cGMP	EB (fmol/mg protein)
0	0.831	0.121	6.87	1.30
5	0.620	0.145	4.28	1.85
10	0.743	0.160	4.64	2.31
15	0.668	0.245	2.73	3.14
20	0.549	0.247	2.22	2.55
30	1.532	0.153	9.03	0.31
40	1.651	0.155	10.65	0.72
50	2.109	0.136	15.51	0.66
60	1.838	0.145	13.02	0.13

TABLE 7: Effect of varying doses of molybdate on specific cytoplasmic estradiol binding and cellular levels of cAMP and cGMP.

6. Effect of Molybdate on Cyclic Nucleotide Levels

The effect of additions of molybdate to whole cell homogenates was reevaluated in order to compare the effect of $MoO_4^=$ on EB, cAMP and cGMP levels. Figure 35 shows a similar dose dependent effect of molybdate on EB as has been seen previously (Figure 10 and 12). As the concentration of molybdate is increased from 0 to 15mM, cGMP and EB levels rise, cAMP levels remain relatively constant. Larger amounts of molybdate cause a reduction in binding to levels below control values. At the same time, cellular cAMP increases and cGMP remains relatively constant. The preferential increase in cGMP at lower concentrations of $MoO_4^=$ and of cAMP at higher concentrations of molybdate not only helps to explain the unusual effect this substance has on estrogen binding but also provides some evidence for an additional action of $MoO_4^=$ besides the previously reported stimulation of adenylate cyclase activity (J.M. Richards and W.I. Swislocki, 1978); a possible stimulation of particulate guanylate cyclase as well. It is unlikely that molybdate is having its effects on cellular cyclic nucleotides by effecting phosphodiesterase activity, since these enzymes are soluble and molybdate has been shown to be ineffective in altering EB in cytosol preparations. Regression analysis of the data in Table 7 results in a linear plot with a correlation coefficient of 0.89 as seen in Figure 35. In this IN VITRO experimental design, we again see a close relationship between the ratio of cAMP/cGMP to cytoplasmic estrogen binding.

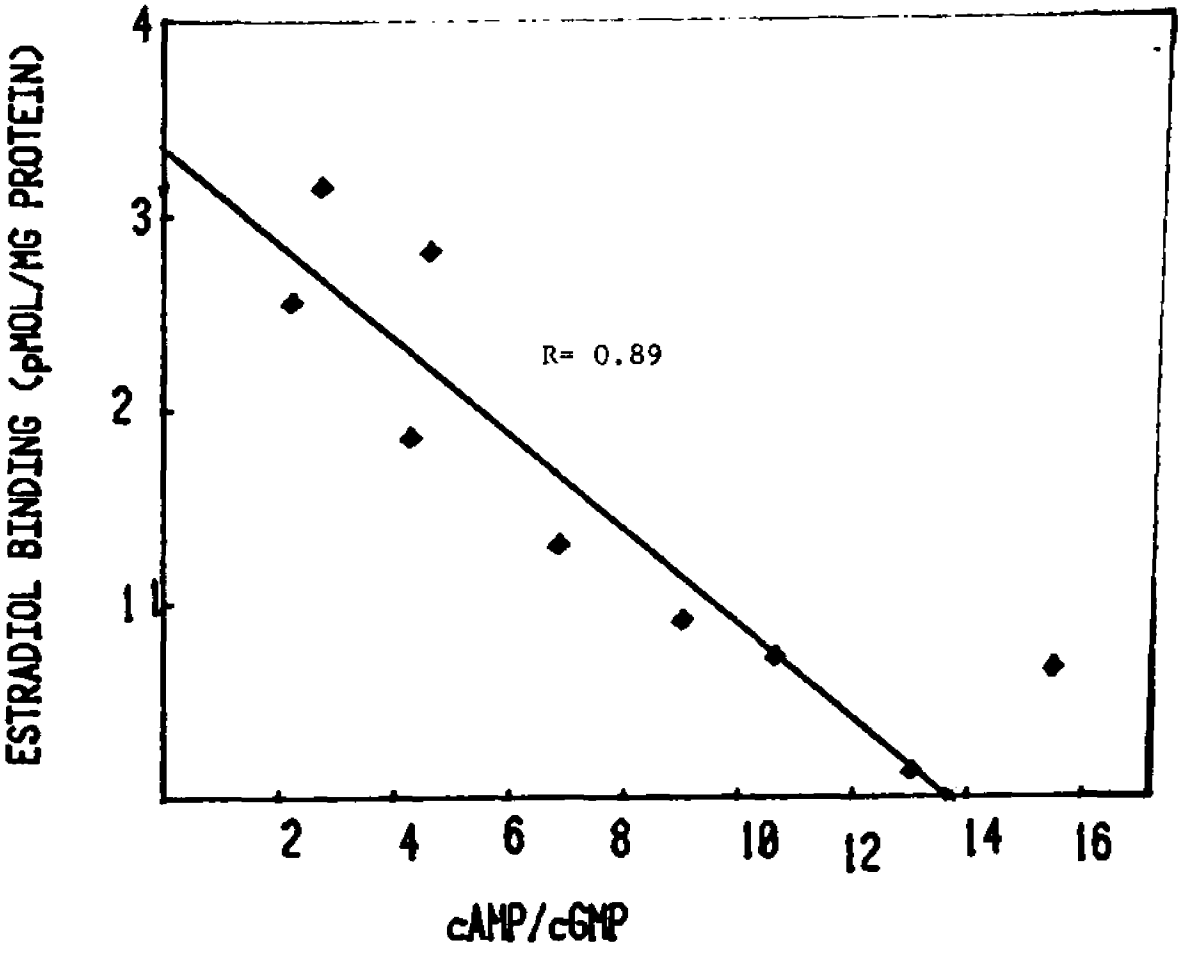
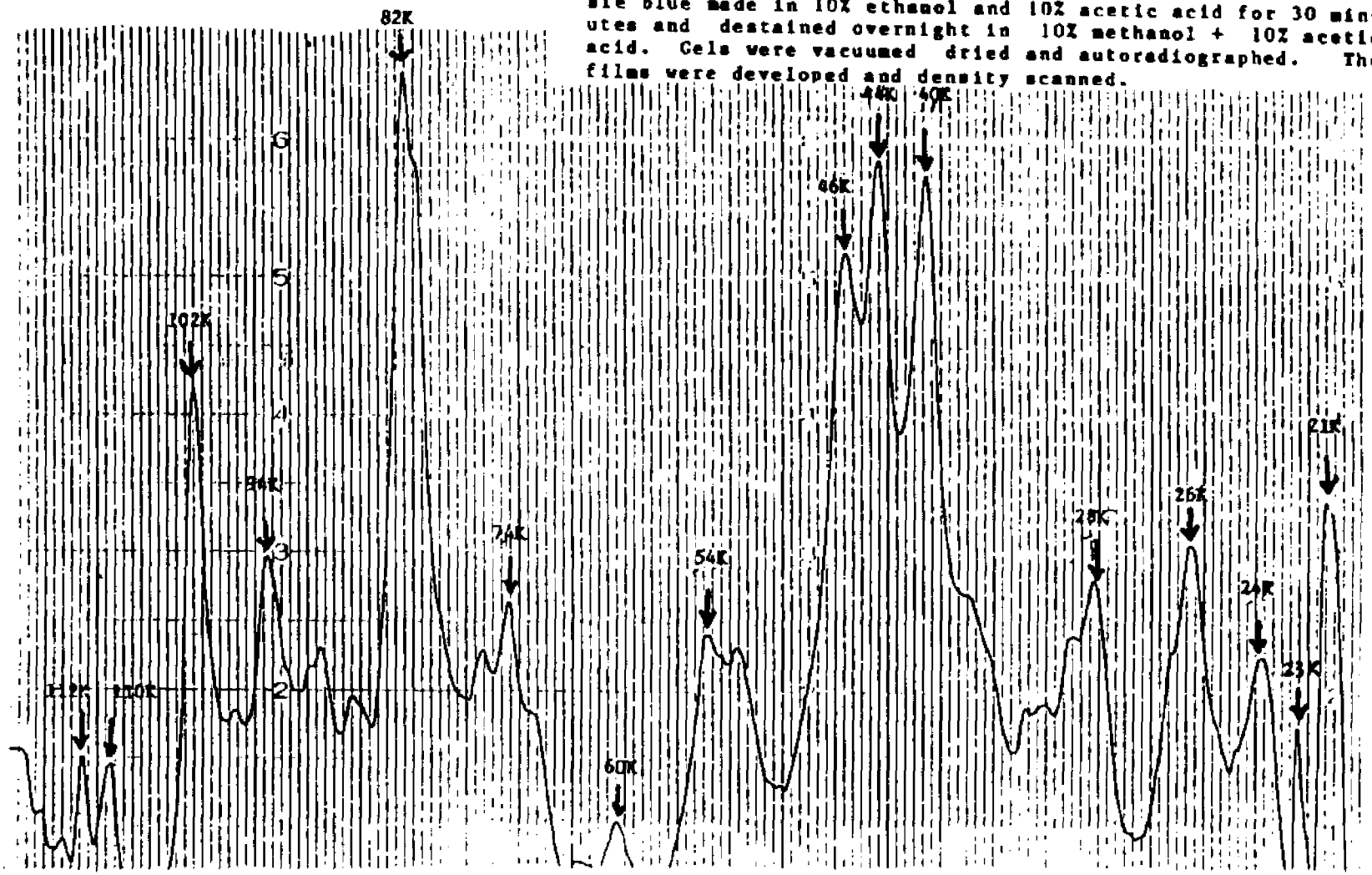


Figure 35. Correlation of cytoplasmic estradiol binding levels and the ratio of cAMP/cGMP in response to various concentrations of molybdate. The results of EB in HEC-1B homogenates that are listed in Table 7 were regressed against the ratio of the cyclic nucleotides in that same experiment and the best fit line was plotted.

IN VITRO Phosphorylation in HEC Cells

Since all asssays were performed at nonphysiological temperatures (0C), the question arose as to whether enzymatic processes such as phosphorylation, the suspected mechanism for cyclic nucleotide dependent changes in EB, could be responsible To answer this question HEC cell cytosol that had been radiolabelled with γ-32P-ATP was electrophoresed on polyacrylamide gels. Figure 36 represents a densitometric scan of an autoradiogram taken from one of these gels In this experiment, 16 proteins ranging in molecular weight from 21K to 112K were clearly labeled to varying degrees with 32P. In all experiments 10-16 proteins showed incorporation of the isotope thus proving that phosphorylation can occur in this system even at low temperatures.

FIGURE 36: Autoradiographic analysis of phosphorylation at OC in HEC cells. Cell homogenates were incubated in GMTD buffer with 10mM MgCl2, 10mM KCl 50uM cold ATP and 20,000 CPMof^{32}P-ATP/pmol of cold ATP for 1 hour. The reaction was stopped with sample buffer (3% SDS, 10% glycerol and 3% mercaptoethanol) and heated at 100C for 5 minutes. Samples were layered on 10% polyacrylamide gels. Following the run (20mA/slab), gels were fixed in 10% TCA, stained with coomasie blue made in 10% ethanol and 10% acetic acid for 30 minutes and destained overnight in 10% methanol + 10% acetic acid. Gels were vacuumed dried and autoradiographed. The films were developed and density scanned.



DISCUSSION

The desire of knowledge, like the thirst of riches, increases ever with the acquisition of it.

Sterne

DISCUSSION:

1. Model of Biochemical Regulation of Estradiol Binding

The regulation of estrogen receptor levels was previously thought to involve protein synthetic processes exclusively. The work presented here has demonstrated an additional form of regulation of these proteins; a metabolic "activation" and "inactivation" of estrogen binders in human endometrium. This mode of regulation of EB is characterized by rapid changes in specific 3H-E2 binding promoted by the addition of exogenous cyclic nucleotides, possibly as a result of phosphorylation processes.

A schematic representation of some of the basic experimental findings on which this "metabolic" level of receptor regulation is postulated is seen in Figure 37. Ri and Ra represent two forms of a specific estrogen binder; Ri denotes the "inactive" form, unable to bind E2 and Ra denotes the "active" form that is capable of binding estrogen.

Addition of millimolar concentrations of cGMP to endometrial cell cytosol during labelling with 3H-E2 results in a marked increase (approximately 3 fold) of specific binding. In contrast, addition of similar amounts of cAMP produces the opposite effect, often reducing binding capacity to undetectable levels. These effects can be observed at

labelling times as short as 15 minutes (Fig. 22), both at 4C and at 30C (Fig. 30). In the presence of IBMX, the respective effects of cGMP and cAMP are apparent at nanomolar concentrations and become maximal at micromolar concentrations (Fig. 21) suggesting that phosphodiesterase activity is quite high in this system even at 4C.

As indicated in Figure 37, GTP can increase specific estrogen binding perhaps by serving as a precursor to cGMP. The effect was clearly demonstrated by incubating cell homogenates with 3H-E2 and 1mM GTP. Increases in EB as high as those obtained with cGMP were observed under these conditions (Fig. 16). However, GTP was ineffective when added to cytosol, as would be expected if its conversion to cGMP required a membrane-bound guanylate cyclase.

ATP, also at 1mM concentrations increases specific E2 binding when added to cell homogenates. The effect of ATP cannot be explained solely by its ability to serve as a phosphate donor for phosphokinase activity since ATP has little or no effect in cytosol. ATP may increase EB through activation of the guanylate cyclase system, an effect that has been previously reported for the calf uterine particulate form of this enzyme (M.I. Siegel et al., 1976). Since ATP is a precursor of cAMP, it would be expected to decrease EB. In fact, a concentration dependence for ATP has been observed, concentrations of ATP in excess of 5mM cause a small decline in EB.

As indicated by the diagram in Figure 37, the presence of ATP is necessary to obtain the effects of both cyclic nucleotides on the estrogen binder. If the endogenous levels of ATP are allowed to fall to undetectable levels (Fig. 27) by keeping cytoplasmic preparations at 4C for a few hours, cGMP and cAMP have little or no effect of 3H-E2 binding; however, responsiveness is restored when ATP is added back to the ATP depleted cytoplasm demonstrating that storage of cytosol does not result in irreversible damage to the binding protein.

Both activation and inactivation processes require the presence of divalent cations (Fig. 26). The effect of cGMP continously increases as the concentration of Mg++ or Mn++ is increased up to 100mM. In contrast, the effect of cAMP is maximal at 2-5mM Mg++ or Mn++ and declines at greater concentrations of the cation. The concentration dependence for divalent cations for both effects is similar to those reported for cGMP and cAMP dependent phosphokinases (Y. Takai et al.,1976).

The ATP requirement and the divalent cation requirement suggest that activation involves a cGMP-dependent phosphorylation whereas inactivation involves a cAMP-dependent phosphorylation. Whether these phosphorylations occur on the receptor molecule itself or on some other molecule(s) that act as activators and/or inactivators of the binding protein once they are phosphorylated remains to be elucidated.

Metabolic inactivation of steroid binding proteins has been thought to involve a dephosphorylation reaction (see Introduction- IX-2G). This view has been supported by a number of findings which include: (1) using alkaline phosphatase to lower binding levels (C.J. Nielson et al., 1977; K.M. Yuh and P.L. Keyes, 1981; F.E.B. May and B.R. Westley, 1982) and (2) demonstrating the copurification of endogenous membrane associated receptor inactivating activity with endogenous dephosphorylating activity (C.J. Nielson et al.,1977). A putative dephosphorylation may remove the phosphate(s) that are attached in the presence of cGMP. This mechanism would represent one form of inactivation. The work presented here suggests a second mode of inactivation; a cAMP dependent decrease in binding, perhaps due to another phosphorylation on a different site of the binding protein. It is uncertain how cAMP has its effect. It may modify Ra, the binding form and shift it to Ri. Alternatively, it may affect Ri and prevent it from being activated to Ra. A third possibility is that it affects some other macromolecule, perhaps one that is responsible for the conversion of Ri to Ra. Further experimentation is required to elucidate this point.

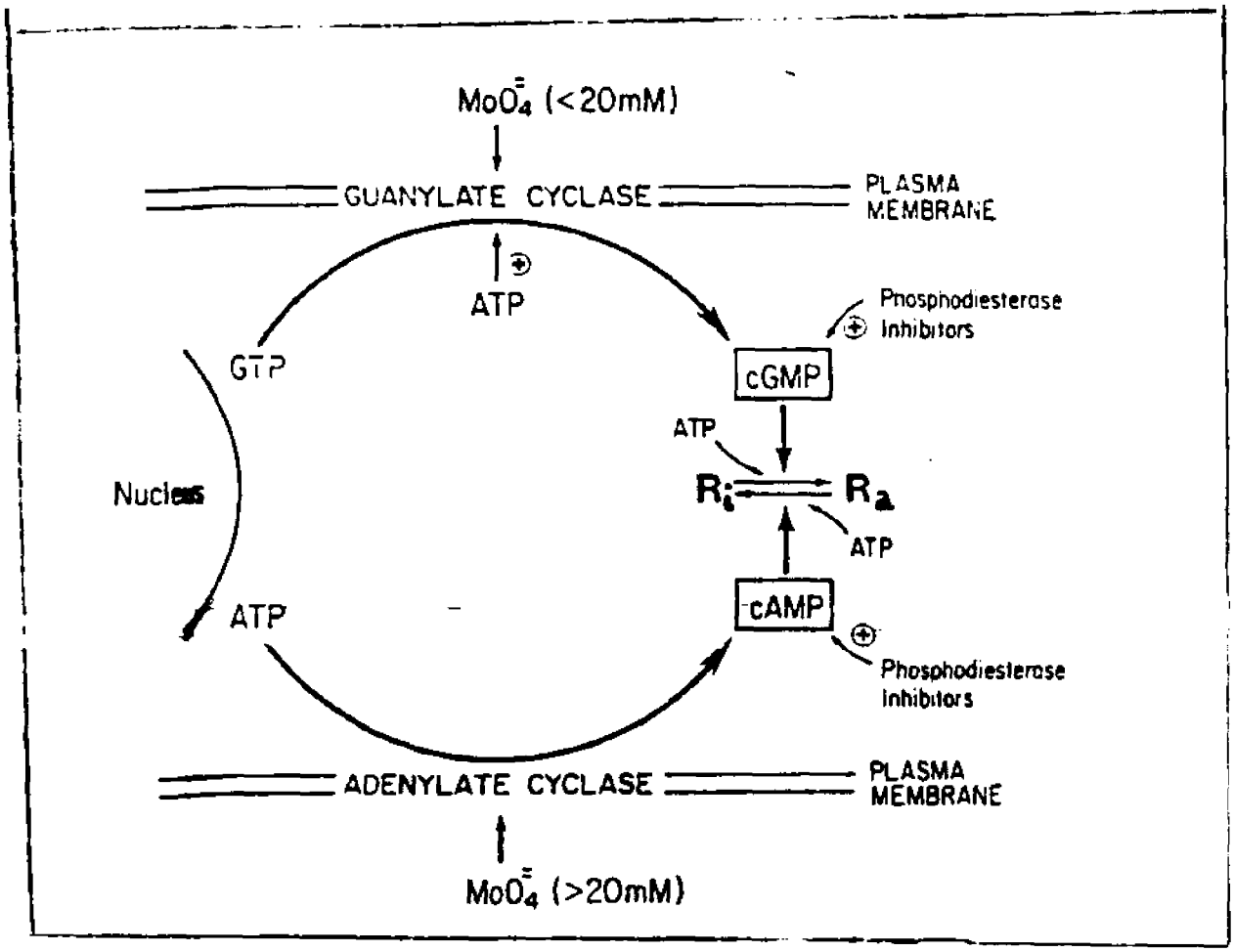


FIGURE 37: Model for cyclic nucleotide dependent regulation of estradiol binding.

2. THE ACTION OF MOLYBDATE:

As described for the two nucleotide triphosphates, ATP and GTP, addition of 10-20mM Na2MoO4 to endometrial homogenates enhances specific 3H-E2 binding to levels as high as those obtained with cGMP (Fig. 16) and has no effect when added to cytosol unless plasma membranes are included (Fig. 15). An inhibitory effect on estrogen binding is observed at molybdate concentrations that exceed 20mM. Interestingly, the hamster uterine cytosol progesterone receptor exists in greater quantities upon preincubation of cytosol with 1-10mM $MoO_4^=$ then in the absence of $MoO_4^=$. This effect appears to be dose dependent. The binding levels are lower with 20mM $MoO_4^=$ than with 5 or 10mM $MoO_4^=$ (and comparable to those observed at 1mM). This study did not determine the changes in binding at greater than 20mM $MoO_4^=$ concentrations (T.J. Chen et al., 1981).

Amongst the many biochemical activities associated with molybdate is it ability to stimulate adenylate cyclase activity (J.M. Richards and N.I. Swislocki, 1979). This $MoO_4^=$ dependent activation of enzyme activity is a general phenomenon found in many tissues and is apparent at concentrations as low as 5-10mM $MoO_4^=$. The effect is enhanced however, at concentrations that range from 20-60mM.

The results presented here suggest that $MoO_4^=$ can also function to stimulate the particulate form of guanylate cyc-

lase. The results shown in Table 7 demonstrates that in the endometrial cell system, low levels of $MoO_4^=$ (5-20mM) stimulate cGMP production while high levels of $MoO_4^=$ (20-60mM) stimulate cAMP production. It therefore seems possible that the bidirectional effect seen with the dose response to $MoO_4^=$ (Fig. 12) may be due to a preferential stimulation by low $MoO_4^=$ levels of guanylate cyclase and of adenylate cyclase at high levels of $MoO_4^=$. Though the actual enzymatic activities have not been measured, this remains the most reasonable explanation for the complex effects of $MoO_4^=$. The effect could not be due to an inhibition of cyclic nucleotide metabolism by inhibiting phosphodiesterase activity since these enzymes are soluble and $MoO_4^=$ requires the presence of membranes for any effect to be obtained. This then, would be the first demonstration of guanylate cyclase stimulation by $MoO_4^=$, though its mechanism of action remains to be elucidated.

All steroid receptor research to date has shown that larger quantities of cytosolic steroid binding could be detected when assays were performed in the presence of 10-20mM $MoO_4^=$ (K.M. Anderson et al., 1980, W.W. Grody et al.,1981; T.J. Chen et al., 1981). The explanation offered for this observation has rested on the known abilty of molybdate to inhibit phosphatase activity (K. Paigen,1958). It has been suggested that the receptor will only bind steroid when it is in the phosphorylated state and that steroid receptors are rapidly inactivated to a nonbinding form by a

dephosphorylation reaction. Therefore in the presence of molybdate, phosphatase activity is inhibited and the inactivation process is slowed or prevented entirely thus resulting in an enhancement of binding.

The work presented here demonstrates that aside from any stabilizing activity for steroid receptors that $MoO_4^=$ may possess, it is also able to rapidly generate new binding activity (Fig. 7), i.e. to shift the equilibrium from the inactive (nonbinding) to the active (binding) form of the receptor This may be the result of a stimulation of guanylate cyclase at these concentrations of $MoO_4^=$ which then results in cGMP dependent phosphorylation of the receptor protein itself or some associated molecule. One other laboratory has suggested that $MoO_4^=$ has an active involvement in receptor generation. Kidney glucocorticoid binding is enhanced approximately 100% by 10mM $MoO_4^=$ both at 4C and at 25C in th presence of 5mM dithiothreitol (J. Hubbard and M. Kalimi, 1982).

It is interesting to note that many of the researchers reporting that cytosolic binding is stabilized at 0C upon preincubation with $MoO_4^=$, add molybdate to tissue minces (that contain intact cell membranes) during the homogenization of the tissue rather than to the supernatant following the high speed ultracentrifugation (W.Y. Naritoku et al., 1982; C.M. Gaubert et al., 1980; E.F. Hawkins et al., 1981; W.W. Wright et al., 1981 and M. Toppila et al., 1982). It

is possible that part of the increase in binding thought to be due to 'stabilization' might well be due to an 'activation' phenomenon that requires particulate cell components; a mechanism that is similar to the type reported in this work.

In the introduction (section IX-2G), a number of observation were presented which questioned whether molybdate could be stabilizing binding by inhibiting a putative phosphatase. We can now reconsider some of these observations in light of the new findings our own postulated activity for molybdate. One observation addressed the problem that other phosphatase inhibitors (F, glucose-1- phosphate and tungstate) do not prevent inactivation (K.L. Leach et al., 1979). If molybdate functions to stimulate guanylate cyclase activity then other phsophatse inhibitors should not have an effect on the system while other guanylate cyclase stimulators, e.g. metcholine, nitroprusside and NaNO3 (F. Murad et al., 1978) should have an effect. These substances in physiological concentrations have been shown to stimulate EB levels. The second observation concerns the effect that $MoO_4^=$ has on steroid binder inactivation in response to high salt, ammonium sulfate precipitation, gel filtration or exhaustive dialysis (K.L. Leach et al.,1979; K. Noma et al., 1980; W.W. Grody et al., 1981). Each of these treatments could easily alter the conformation of the receptor protein (or associated protein) so that the site(s) that should be phosphorylated is buried within the molecule. The third

observation relates to the limited pH range (6.0-7.6) for $MoO_4^=$ activity (W.W. Wright et al., 1981). The effect of pH need not be on the putative phosphatase enzyme but could once again be on the conformation of the substrate (the receptor protein or associated molecule).

3.SIGNIFICANCE OF ESTROGEN RECEPTOR REGULATION

A. Responsiveness to Hormone

Although estrogen affects the function of many tissues in vertebrate organisms to some degree, the principal effect is considered to be stimulation of the growth and maturation of the female reproductive system and maintanence of its reproductive capability. Considerable attention has been directed towards determining the relationship between the uptake of the hormone by target cells and the primary effects on cell metabolism and macromolecular biosynthesis which culminates in the characteristic morphological and physiological responses (B.S. Katzenellenbogen and J. Gorski, 1979).

Morphological: At the beginning of the follicular or proliferative phase of the menstrual cycle the epithelial cells are low cuboidal in appearance with sparse cytoplasmic organelles and a few relatively short microvilli. In response to estrogen, mitotic figures are frequently seen in both the epithelium and compact stroma thus resulting in proliferation of the tissue. The epithelial cells increase in complexity; there is a marked increase in number and size of the mitochondria, development of the rough endoplasmic reticulum and golgi apparatus. In addition, these cells increase in size, first appearing columnar and then pseudostratified. At the start of the cycle, the glands are

straight with small circular cross-section. By the end of the proliferative phase the glands are much larger and exhibit slight tortuosity (C.A. Finn & D.G. Porter, 1975; R.M. Wynn, 1977).

Biochemical: The trophic action of estrogen on endometrium is mediated, at least in part, by specific increases in the synthesis of protein. Most of the early responses to estrogen are blocked by protein synthesis inhibitors. Electrophoretic profiles of (35S-) methionine labelled protein in human endometrium showed an increase of a 55,000 protein. On a two-dimensional gel, this protein was resolved into at least 2 proteins with isoelectric points of 5.3 and 5.9 (S. Iacobeli et al., 1981). It is not as yet possible to attribute a function to these specific estrogen induced proteins.

Changes in enzymatic activities of human endometrium during the menstrual cycle are regulated to a large extent by ovarian steroids. It is thought that enzymes which predominate during the follicular phase are stimulated by estrogens. Using both analytical and histochemical techniques of detection, the activity of the following enzymes have been reported to be primarily under estrogenic control: alkaline phosphatase, B-glucuronidase, glucose-6-phosphatase, triphospyridine nucleotide diaphorase and succinic dehydrogenase (C.F. Holinka and E. Gurpide, 1981).

Prostaglandin F2a is known to exist in both menstrual fluid and endometrium The level of PGF2a in endometrium shows cycle specific variations with the highest levels found in the secretory phase of the cycle. It has been suggested that this compound may have clinical significance, perhaps in the stimulation of the onset of bleeding via action on the spiral arterioles (G.S. Richardson and D.T. MacLaughlin, 1978). In a recent report (F. Schatz and E. Gurpide, 1983), the accumulation of PGF2a in the medium of primary monolayer cultures of epithelial cells derived from specimens of human endometrium has been shown to be markedly enhanced by physiological concentrations of estradiol. It is not certain at this time whether the synthesis or the metabolism of PGF2a is under the regulation of E2.

During the menstrual cycle, a preovulatory rise in progesterone receptor is observed. This increase occurs following the peak in plasma estrogen levels and when plasma progesterone is virtually absent (E.E. Baulieau et al. 1980). The human uterus has been shown to possess an estrogen-inducible progesterone binding protein (O. Janne et al. 1975). This represents an important example of one hormone regulating the tissue resonsiveness to another hormone.

Hormone receptor interactions are governed by the Law of Mass Action. This view was extensively developed in the 1920's and is based on the discrete relationships between chemical structure and biological activity and the competi-

tive interaction of chemically similar hormones. In the classical receptor theory developed 60 years ago, it was assumed that hormone effects are proportional to the fraction of occupied receptor and that maximal effects resulted when all receptors were occupied (A. Goldstein et al. 1974). While these assumptions are true in some cases, there are many exceptions. However, these assumptions serve as a useful model for quantifying hormonal effects. It would follow that both the concentration of hormone and the concentration of receptor protein are important in regulating responsiveness. Measurement of human endometrial steroid receptors has shown that during the preovulatory phase, total estrogen and progesterone receptor levels increase. This is followed by a decline of both receptors during the secretory phase of the cycle (C. Levy et al., 1980) The morphological and biochemical changes associated with these two hormones has been correlated with plasma levels of the hormones and with target tissue concentration of receptors for these hormones. As the concentration of receptor changes, the response to hormonal stimulation also changes. The use of cyclic nucleotides to modify estrogen binding levels may serve as an important tool for investigating changes in morphological and biochemical responsiveness to hormonal stimulation. Any of the above mentioned estrogenic effects can be used as an endpoint for such a study.

B. Translocatability of the Estrogen Receptor:

The mechanism of translocation has not been clearly elucidated. Several possible mechanism including association & dissociation of subunits, receptor proteolysis, and subtle chemical alterations such as phosphorylation of the receptor have been considered (W.W. Grody et al.,1982). For transformation to occur, a high energy barrier must be overcome. Large positive enthalpy and entropy values have been measured for the transformation process, thus suggesting that a number of bonds must be broken in order to expose the positively charged residues on the receptor's surface (E. Milgrom, 1981). In addition, the equilibrium between transformed and nontransformed receptor complexes can be modified by many exogenous compounds such as: (1) oxyanions of some of the transition metals (molybdate, tungstate and vanadate), (2) pyridoxal 5'-phosphate-- possibly through its ability to form Schiff bases with E-amino groups of lysine residues, (3) metal chelators such as o-phenanthroline, (4) rifamycin AF/013- an antibiotic derivitive that prevents the binding of polymerase to DNA, (5) sulfhydryl modifying reagents and (6) reagents that modify histidine residues such as ethoxyformic anhydride and Rose Bengal (W.W. Grody et al.,1982). It should be realized that while transformation can be modified IN VITRO by these various agents as well as by changes in pH and salt concentration, it is not known if any of these manipulations exerts an effect IN VIVO.

When suggesting that a change in cytoplasmic E2 binding could results in a change in responsiveness to estradiol, we make the assumption that the presence of excess cAMP or cGMP would not interfere with the transformation process of the steroid receptor and therefore, with its ability to translocate to the nucleus or with its ability to interact with the target cell genome. Since the process of transformation is not well understood, it would be difficult to speculate what effects if any the cyclic nucleotides might have. If, however, it can be shown that transformation and nuclear interaction are not affected by cyclic nucleotides, then one would expect that cNMP dependent changes in cytoplasmic binding could significantly alter responsiveness of a target

C. Endogenous Factors that Alter cNMPs:

The concentration of intracellular cyclic nucleotide levels are influenced by many factors; both by regulation of formation of the cNMP (cyclase activity) and by degradation of the cNMP (phosphodiesterase activity). Numerous review articles are available which provide partial listings of the polypeptide hormones, neurotransmitters and other physiological and nonphysiological compounds that have been shown to alter cellular cNMPs (C.A Kahn, 1976; K.J. Catt & M.L. Dufau, 1977; J.D. Baxter & J.W. Funder, 1979). Table 8 is a summary of the material found in these papers.

Estradiol binding levels in HEC cells are subject to dramatic changes over short periods of time (H. Fleming & E. Gurpide, 1981) In the work described here, these changes in E2 binding have been shown to correlate well with changes in the levels of intracellular cNMP (Fig. 31 and 32). Large oscillations in cellular cyclic nucleotides were found within the first few hours following refeeding of serum starved cells. Changes such as these have been reported for rat embryo fibroblast cultures as well (C. Rochette-Egly et al., 1979). Since serum contains many of the substances listed in Table 8, in specific concentrations, each with its own rate of degradation, changes in intracellular cNMP may be due to relative concentrations of these compounds within the bathing medium at any given time as a result of relative

Table 8:

Substances that Regulate the Intracellular Levels of Cyclic Nucleotides

Adenylate Cyclase	Guanylate Cyclase	Phosphodiesterase
ACTH	a-Adrenergics	Caffeine/xanthines
B-Adrenergics	Azides	Calcium-calmodulin
Calcitonin	Estrogen	Indomethacin
FSH	Melatonin	Phenothiazine
Glucagon	Metcholine	Spermine
GnRH	Nitric Oxide	Thiol reagents
LH	Nitroprusside	Thyroxine
Parathyroid Hormone	PGF2a	Tocopherol
PGE_1	Secretin	
Vasopressin	Triton X-100	
	Hemaglobin/Myoglobin (↓)	

activity of each of the enzymes regulating cNMP levels. In preliminary experiments, nitroprusside, nitric oxide, metcholine and melatonin; substances which stimulate guanylate cyclase activity all increase specific cytoplasmic estradiol binding in HEC cells.

It is interesting to note that substances like melatonin, which influences the growth of reproductive tissue IN VIVO by elevating the concentration of estrogen receptors (D. Dansforth et al.,1982) have also been shown to stimulate guanylate cyclase activity and thus elevate cellular cGMP concentrations (D.L. Vesley, 1980). It might be possible that some of the other reports on alteration of steroid receptors may be mediated by cyclic nucleotide changes rather than or in addition to changes in protein synthetic rates e.g. estradiol is known to increase the concentration of its own receptors (J. Gorski et al., 1971) as well as those for progesterone (O. Janne et al., 1975). This effect could be the result of estrogen's ability to increase the intracellular concentration of cGMP levels (L. Flandroy and P. Galant, 1982) followed by a cGMP stimulation of EB.

The importance of components within a buffer system for a steroid receptor assay should become increasingly obvious. The presence of divalent cations, sulfhydryl reagents, detergents (triton) and antimicrobial agents (azides) can all affect activation and inactivation phenomena and thereofore, can have an effect on the measurable amount of binding

This information should be taken into account when comparing interlaboratory reports.

Finally if the regulation of E2 binding IN VIVO is similar to the findings reported here on IN VITRO regulation then the IN VIVO concentration of E2 receptors within a target tissue might be influenced by the levels of many of the hormones found in the blood at that timeand not just be a function of circulating ovarian hormones. It would be interesting to determine if individuals with some unusual hormonal profile or individuals receiving specific drugs (e g. isoproterenol) that alter cellular cNMPs, demonstrate any significant change in the levels of their steroid receptors.

D.Steroid Hormone Receptors and Endocrine Therapy for Reproductive Cancers:

Mortality due to carcinoma of organs such as breast uterus and prostate which are known targets for steroid hormones, represents over 30% of total cancer incidences. Much of our current knowledge on the relationship between steroid hormones and reproductive cancer has developed from work on the breast. The information gained from this work could probably now be used to study hormonal dependent effects in endometrial cancer.

Growth and regression of breast tumors has been shown to be modulated by appropriate endocrine manipulations. Ablative proceedures such as ovariectomy, adrenalectomy and hypophysectomy to remove sources of circulating hormones which typically stimulate or support breast tumor growth have resulted in regression of metastatic tumors and objective remission for 5-10 years. Alternatively, breast cancer regression can be achieved by administering large pharmacological doses of estrogen androgen, progestin, glucocorticoid or antiestrogen (O.H. Pearson et al., 1955; M.A. Younes et al., 1982). Unfortunately, endocrine responsive tumors constitute only 30% of all cases. The ability to predict which patients would most likely benefit from hormonal therapy and exclude those with little probability of improvement would be of great value The choice to administer endocrine

therapy has been in large part empirical, guided by the receptor status (i.e. whether or not the tissue shows hormonal dependence) of a sample of biopsied tissue. Following malignant transformation, if a population of cells retains all or part of their normal receptor sites, its growth and function is potentially capable of being regulated by its hormonal environment.

The success rate of hormonal therapy can be improved from 30% to about 55% by selecting those patients whose tumors contain estrogen receptors (ER). However, 40% of ER+ patients demonstrate endocrine resistance; the reason for this is uncertain.

A hypothesis was then extended suggesting that since binding to receptors was only an early step in steroid hormone action, the absence of response to endocrine manipulation in some patients was due to a lesion at a later step. Therefore, a measurable product of estrogen action rather than the initial binding step should be an ideal marker of endocrine responsiveness (K.B. Horwitz et al., 1975). Synthesis of the progesterone receptor (PgR) is dependent upon estrogen. It was then predicted that tumors containing PgR and ER would indicate that the tumor was capable of synthesizing at least one end product under estrogenic stimulation and should therefore be endocrine responsive. Tumors with ER but no PgR would be resistant to endocrine therapy. Many investigators have tested this hypothesis (W.L

McGuire, 1980 K.B. Horwitz, 1981). The results are summarized in Table 8. The inclusion of PgR assays improve the selection of hormone responsive candidates to 70%, however 30% of patients with "functional" estrogen receptors are still unresponsive to endocrine manipulations. Interestingly, 34% of ER+/PgR- patients (or 12% of the breast cancer population) with "nonfunctional" ER are also responsive to hormonal manipulation. A small group of patients (4%) have been found, whose tumors contain PgR but no ER. Thirty two percent of the members in this group exhibit tumor regression upon endocrine manipulation. The last group of patients (ER-/PgR-) comprised 27% of the population being studied. They exhibited a 9% responsiveness, a rather surprising result since it contradicts the basic premise that hormonal dependence is directly related to receptor status. However, it is clear that the hypothesis put forth by Horwitz has been useful in improving the selection of endocrine responsive tumors.

If endocrine therapy is only offered to patients that are ER+/ PgR+, then a large percentage of individuals will show improvement and fewer people will experience the trauma of such therapy without any benefit. However, limiting therapy to this group exclusively would prevent 16% of breast cancer patients from benefiting from endocrine therapy (13% who are ER+/PgR-, 1% who are ER-/PgR+ and 2% who are ER-/PgR-) while effectively selecting out the 56% who would not respond. I would therefore suggest that these 16%

Table 9 :

Response to Endocrine Therapy as a Function of ER and PgR

LAB:	ER+/PgR+	ER+/PgR-	ER-/PgR+	ER-/PgR-	Total
Osbourne	16/20	15/45	-----	3/20	85
Nomura	20/30	12/37	1/4	4/34	105
Bloom	26/33	3/14	1/1	0/14	62
Maillot	21/28	18/32	1/8	1/23	91
Brooks	4/6	2/7	-----	-----	13
Nemato	10/13	18/31	-----	2/28	73
King	10/11	3/15	0/2	2/9	37
Manni	15/24	3/5	-----	0/2	31
Minner	9/12	2/6	2/3	3/30	51
Leung	20/29	3/14	1/2	2/9	54
Allegra	11/14	8/14	0/4	0/12	44
McGuire	13/16	7/17	-----	0/11	44
Degenshein	13/16	1/9	1/1	0/7	32
Le Clercq	1/3	1/3	0/1	-----	7
Singhakowinta	4/5	0/3	-----	-----	8
Desombre	3/4	1/3	-----	1/7	14
Young	13/13	1/7	1/1	1/3	29
Totals:	191/282 (68%)	98/262 (37%)	8/27 (30%)	19/209 (9%)	780
% of patients:	36%	34%	4%	27%	

might have been incorrectly placed into their respective groups and if categorizing patients could be further refined, these patients could be selected for as well. I wish to offer the hypothesis that some of the receptor negative measurements that were made are inaccurate, i.e. these tumors were not negative for one or both of the receptors measured but should be considered ER+/PgR+. There are two approaches that can be used to test this hypothesis based on the information presented here. The first approach would be to mince the tumor sample, place it in organ culture and then to perform the receptor assay on the tumor a number of times after different times in culture (e.g. 0 2,4 hours). Perhaps a single measurement results in observation of the receptor in a masked or "inactive" state while at a later time a metabolic unmasking might have occurred and the receptor status would appear positive. This suggestion is based on the rapid fluctuations of E2 binding that have been observed in endometrial cell cultures (H. Fleming & E Gurpide, 1981). A single binding assay in these cells would not be sufficient to state whether the cells were positive or negative for E2 binding. The second approach would be to assay the tumor once under standard conditions as well as in the presence of cGMP (or any other stimulating agent e.g. MoO4=, ATP, GTP). Some of the negative measurements might be "masked" receptors that could be activated. If certain tumors show increases in specific estrogen binding in the presence of cGMP then administration of cGMP or physiologi-

cal substances that increase cGMP may be useful in promoting a response of tumor to estrogenic therapy (estrogen or tamoxifen therapy). Conversely, reduction of estrogen receptor levels of estrogen sensitive cancers by administration of cAMP or substances that elevate cellular cAMP may inhibit tumor growth promoted by endogenous estrogens. Recently, a report appeared demonstrating that cholera toxin caused a reduction in estrogen recptors and a decline in the hormone-dependent growth of DMBA induced mammary carcinoma in rats (Y. Cho-Chung et al. 1983). If either one or both of these approaches result in a change from receptor negative to receptor positive status, then the tumor should be treated with some form of endocrine therapy and the percent that show regression should be established.

In addition to information on responsiveness to endocrine therapy, receptor status has been used to select a subset of patient, those that were receptor negative, who will recur earlier and have a lower survival rate (W.L. McGuire, 1980). Similar manipulations of binding assays as has been described above might be useful for these purposes as well.

The use of steroid receptor assays as a tool to select patients for endocrine therapy has not been limited to breast cancer. Reports on hormone dependency of prostatic cancer (P. Ekman et al., 1979), renal cancer (G. Concolino et al., 1979) and leukemia (M. Lippman et al., 1979) are available. A limited amount of research has been done on

endometrial cancer. Since our results have shown an effect of cyclic nucleotides on endometrial cancer cells as well as breast cancer cells (CG5 & T47D- Figure 25), it would be interesting to learn whether the IN VIVO hormonal dependency of endometrial cancer is similar to that of breaast cancer and whether the information gained from the latter could be applied to the former.

The possibility that steroid receptors could be modulated with drugs and hormones affecting cAMP or cGMP levels opens a promising new line of investigation. Much remains to be learned about the biochemical changes resulting in generation and inactivation of binding proteins.

E. Estrogen-linked Cytotoxic Agents·

Screening programs are currently underway to assess the potential therapeutic value of newly synthesized estrogen-cytotoxic molecules for the treatment of hormone-dependent cancer. Five substituted estrogens have been prepared which include an aziridine derivitive of estrone and four nitrogen mustard derivitives of hexestrol, diethylstibestrol and estrone (G. LeClercq et al. 1980). The process of tagging a cytotoxic compound with an estrogenic molecule could potentially allow for controlled distribution of the cytotoxic compound to areas of specific interest and eliminate the unnecessary side effects that these compounds have.

The IN VITRO binding affinities of these drugs for the cytoplasmic estrogen receptor have been established. Each of these compounds in a concentration range of 0.1 to 1.0 micromolar inhibits the growth of MCF-7 cells (an ER+ cell line) but has no effect on EVSA-T cells (an ER- cell line) (G. LeClercq et al. 1980). With these preliminary results, research is currently underway to investigate the toxicology of these compounds and the IN VIVO drug-antitumor effectiveness using the DMBA induced rat mammary tumor and the hormone dependent MXT mouse mammary tumor systems. If these additional studies show an effct of these compounds IN VIVO, then the ability to modify the amount of estrogen bindng within a target tissue will be crucial for maximal effec-

tiveness of these drugs Exogenous substances that elevate intracellular cGMP could potentially change an estrogen receptor negative tumor to one with receptor proteins and therefore, to one that will respond to cytotoxic drugs that are conjugated to estrogens.

SUMMARY AND CONCLUSIONS

SUMMARY AND CONCLUSIONS:

Recent experimental results from this work has revealed the following facts:

- Addition of cGMP to homogenates or cytosol prepared from endometrial tissue or cultured endometrial adenocarcinoma cells during the assay for specific estrogen binders markedly increases specific binding levels. The effect is completed in about 15 minutes at 4C. Cyclic AMP has the opposite effect and in many cases lowers the number of binding sites to undetectable levels.
- ATP, a nucleotide that stimulates a particulate form of guanylate cyclase, $MoO_4^=$, a compound that can elevate cGMP levels and GTP, a metabolic precursor of cGMP, increase specific estradiol binding protein in the presence of plasma membranes and soluble factors.
- Cyclic AMP reduces the levels of estrogen binding when added to cell homogenates or to cytosol and counteracts the effects of cGMP, $MoO_4^=$, ATP and GTP.
- ATP and divalent cations are required for the expression of cGMP and cAMP effects on estradiol binding. It is therefore likely that phosphorylations are involved in the generation and inactivation of estrogen binding sites.
- The reported effects of nucleotides and molybdate have been observed in specimens of histologically normal endome-

trium, in specimens of endometrial carcinoma, in two endometrial adenocarcinoma cell lines, HEC-1 and HEC-50 and in two breast cancer cell lines, CG5 a variant of MCF-7 and in T47D cells.

- Rapid changes in the levels of estrogen binding capacity observed in endometrial cells in culture can be associated with changes in cAMP/cGMP ratios which have been shown to vary in cells in culture.
- Rapid changes in the levels of estrogen binding capacity observed in response to varying concentrations of molybdate can be correlated with changes in the cAMP/cGMP ratios in cell homogenates.
- Approximately 12 cytosolic proteins can act as substrates for phosphorylation in HEC cells at nonphysiological temperatures (46).

At this time it is not yet known whether any of the 12 proteins is the estrogen receptor. In one report (B.S. Katzenellenbogen et al., 1980), the molecular weight of the human endometrial cytoplasmic etrogen receptor was estimated by Ferguson plots to be between 40-50K. In a second report (A.C. Notides et al., 1976) the molecular weight of the human myometrial estrogen receptor has been estimated from sedimentation gradient analysis to be between 30-40K. In the experiments described in our work, three proteins, (40K, 44K and 46K) are phosphorylated. It is possible that one of

these three proteins corresponds to the cytoplasmic E2 binding protein. It would be interesting to determine whether the phosphorylation pattern for HEC cell cytosol is altered in the presence of cAMP or cGMP and if so, if they phosphorylate the same or different proteins.

Preliminary evidence exists favoring regulation of E2 binding by cNMP dependent phosphorylation of the binding protein (or an associated protein). This mode of regulation raises a number of interesting questions, e.g. Is the receptor directly phosphorylated ? Are there two distinct phosphorylatable sites on the same protein (one for cGMP to activate and one for cAMP to inactivate) ? Is it possible for this protein not to be phosphorylated at all ? Would the protein have any activity in this nonphoshorylated state ? If you treat a cytosolic preparation with a phosphatase, would one or both phosphates be removed ? Would the protein have activity under these conditions ?

Since the evidence for phosphorylation is still preliminary, we can speculate that the mechanism by which cNMPs alter E2 binding levels is through an allosteric modification of the receptor protein (or an associated protein) rather than through covalent modification. The use of radiolabelled cyclic nucleotides and 32P-ATP could be helpful in further exploring this problem. Dual labelling a cytosolic preparation with 3H-E2 and either labelled cNMP or 32P-ATP and separation of cytosolic proteins by some method such as

isoelectric focussing should provide information on whether the receptor protein (or associated protein) is directly or indirectly modified and whether this modification is through a phosphorylation reaction or through an allosteric change.

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Who is wise ?
He who learns from all men
as it is said:
"From all my teachers
I gained wisdom"

Ethics of our Fathers (4:1)

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