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CYCLIC AMP AND MAMMARY CANCER GROWTH

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cAMP AND MAMMARY CANCER GROWTH

by

ROBERT M. MAYER

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

1980

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This manuscript has been read and accepted for the Executive Committee in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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ABSTRACT

There is solid experimental evidence that adenosine cyclic 3',5'-monophosphate (cAMP) is involved in the regulation of cellular proliferation. Other investigators have shown that: (1) administration of substances which increase cAMP concentrations in cells frequently affect cell growth, and (2) changes in the growth pattern of cells are often associated with concomitant changes in cAMP concentrations and in the cellular constituents controlling cAMP metabolism and action. In the present study, several aspects of cAMP metabolism and action were compared in selected rat mammary tumors to determine whether substantive changes in sensitivity to cAMP and in cAMP metabolism occur concomitantly with progression to hormone independence and with regression stimulated by endocrine ablation.

MTW9 is a hormone-dependent rat mammary adenocarcinoma which requires the presence of a mammosomatotropic tumor coimplant (MtTW10) for growth. MTW9B is an autonomous subline of MTW9. The direct effects of various sera, hormones, and substances which elevate cellular concentrations of cAMP on tumor DNA synthesis were compared in MTW9 and MTW9B using organ culture methods. Significant results were extended in studies using an established human breast cancer

cell line (MCF-7). Since aggregation of these cells prevented direct counting, a technique was developed to assess growth by estimating the number of nuclei after mild sonication of experimental cultures.

Sera taken from MtTW10-bearing rats (MtT serum) was found to promote both (^{3}H)thymidine incorporation into MTW9 explants and MCF-7 cell growth. These effects could not be duplicated by normal female rat serum alone or in combination with hormones simulating the known endocrine constituents of MtT serum. Insulin was stimulatory only at very high concentrations. 1 mM cAMP inhibited MCF-7 cell growth, but did not significantly affect (^{3}H)thymidine incorporation into MTW9 explants. Whereas _in vivo_ administration of 10 mg DBcAMP (dibutyryl cAMP) suppressed tumor DNA synthesis in MTW9, 1 mM DBcAMP failed to directly alter DNA synthesis in organ-cultured MTW9 or MCF-7 cell growth. Several cAMP phosphodiesterase inhibitors, including 1-methyl-3-isobutylxanthine (MIX), markedly suppressed DNA synthesis in MTW9 and MCF-7 cell proliferation. Though neither 1 mM DBcAMP nor a low concentration of MIX (0.1 mM) was inhibitory alone, the combination of these components was effective in suppressing cell proliferation. Addition of MtT serum to cultures prevented the inhibitory effects of MIX, but not those of cAMP or DBcAMP plus MIX. These actions of MtT serum could not be duplicated by selected combinations of hormones and sera.

MTW9B was observed to be markedly less sensitive than MTW9 to the inhibitory action of MIX on DNA synthesis. Although substantial high-Km phosphodiesterase activity was evident in MTW9, this activity was not detectable in the autonomous tumor, MTW9B. Low-Km activity was also significantly diminished in MTW9B as compared with MTW9. Both cAMP-binding and cAMP-dependent protein kinase activities were enhanced in MTW9B cytosols. Anion-exchange chromatography of tumor cytosols showed that Type II protein kinase activity was 2-fold greater in MTW9B than in MTW9. However, exposure of each of these tumors to MIX in organ culture elicited a similar dose-dependent activation pattern of cAMP-dependent protein kinase activity. Addition of MtT serum failed to influence the pattern of activation seen with MTW9 explants.

MTW9 regresses rapidly when the supportive MtTW10 is surgically removed. cAMP phosphodiesterase, cAMP-binding, and protein kinase activities were found to be significantly reduced in regressing tumor cytosols. When purified particulate fractions were isolated by methods used by other investigators to obtain cAMP phosphodiesterase activities that are optimally responsive to hormonal control, activity

in these fractions from regressing tumors was substantially reduced from values found in fractions from growing tumors.

These data are consistent with, but do not prove, the concept that hormone-dependent tumor growth may be at least partially controlled by opposing interactions between the cAMP system and growth-stimulating hormones. It is further possible that sera from MtTW10-bearing animals contains an unidentified substance capable of antagonizing the inhibitory actions of the cAMP system on the growth of some mammary cancers.

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This thesis is dedicated to

Trish

Whose courage and understanding helped
make these challenging years joyful and
fulfilling.

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ABBREVIATIONS AND SYMBOLS

cAMP, adenosine cyclic 3',5'-monophosphate

cGMP, guanosine cyclic 3',5'-monophosphate

cIMP, inosine cyclic 3',5'-monophosphate

cCMP, cytidine cyclic 3',5'-monophosphate

cUMP, uridine cyclic 3',5'-monophosphate

DBcAMP, dibutyryl cAMP

GMP, guanosine monophosphate

AMP or 5'-AMP, adenosine monophosphate

ADP, adenosine diphosphate

ATP, adenosine triphosphate

I, insulin

ACTH, adrenocorticotropic hormone

AR, activation ratio

DTT, dithiothreitol

PMSF, phenylmethylsulfonylfluoride

8-Br-cAMP, 8-bromo-cAMP

♀, female rat serum

♂, male rat serum

MTW9, a transplantable mammary adenocarcinoma grown in female W/Fu rats

MtTW10, a mammosomatotropic tumor

MtT serum, serum from MtTW10-bearing rats

MHS, MtT hormone simulant

MEM, Eagle's Minimum Essential Medium

SMEM, MEM supplemented as described

FCS, fetal calf serum

HBSS, Hanks' Balanced Salt Solution

MIX, 1-methyl-3-isobutylxanthine

PDE, phosphodiesterase
M199, Medium 199
PCT, perchloric acid
rPRL, rat prolactin
rGH, rat growth hormone

INTRODUCTION

Objectives

Following the discovery of adenosine cyclic 3',5'-monophosphate (cAMP) in mammalian cells by Rall and Sutherland (1958), the biological role of this ubiquitous nucleotide as a major intracellular regulator has been firmly established. In sharp contrast to the advanced state of investigations of cAMP mechanisms mediating specific endocrine effects, our knowledge of the possible role of cAMP in the control of cell growth and differentiation is fragmentary. Experimental evidence supporting the concept that cAMP is involved in the regulation of cellular proliferation include the following:

(1) changes in the intracellular concentrations of cAMP and the activation of cAMP-dependent protein kinases are often correlated with changes in the growth status of tissues (proliferating, static, regressing);

(2) agents which increase intracellular concentrations of cAMP or which activate protein kinases often elicit subsequent changes in the growth status of tissues; and

(3) alterations in the growth status of cells during developmental and neoplastic processes are frequently associated with aberrations in the proteins governing cAMP

metabolism.

However, there are wide variations in the responses of diverse cell types to changes in cAMP levels, and the information available on mechanisms whereby the nucleotide exerts its influences on growth is limited.

In the present study, experimental models for human breast cancer were utilized to explore the purported growth-regulatory role of cAMP. Important characteristics of cAMP action and metabolism were compared in the hormone-dependent rat mammary adenocarcinoma, MTW9, and its hormone-autonomous subline, MTW9B. Attempts were made to determine whether:

(1) substances which elevate cellular concentrations of cAMP directly inhibit DNA synthesis in MTW9;

(2) progression to hormone autonomy in MTW9 is associated with decreased sensitivity to growth control by cAMP;

(3) progression to hormone autonomy is also associated with aberrations in cAMP metabolism;

(4) alterations in cAMP metabolism accompany mammary tumor regression induced by endocrine ablation; and

(5) growth-stimulating hormones directly oppose the inhibitory action of cAMP on hormone-dependent tumors.

In the course of this study, the following key cellular components controlling cAMP metabolism in MTW9 were partially characterized: cAMP-dependent protein kinases, cAMP-binding proteins, and cAMP phosphodiesterases. Significant results obtained using organ-cultured rat mammary tumors were applied to the examination of human mammary cancer cells in culture.

The cAMP System: cAMP Metabolism and Protein Phosphorylation

Sutherland's discovery of cAMP and subsequent formulation of the second messenger hypothesis were important achievements in molecular endocrinology. cAMP has since been implicated in the regulation of numerous cellular processes, including transport, permeability, secretion, transcription, growth, and differentiation (Robison, 1971). The second messenger hypothesis provided a conceptual framework for explaining some of the mechanisms whereby membrane-acting effectors can elicit a variety of specific metabolic changes within cells. Early research explored endocrine modulation of adenylate cyclase activities in the control of intracellular cyclic nucleotide concentrations. Although cAMP mediation of endocrine effects was extensively documented by the end of the 1960's, the mechanisms of its intervention remained unclear until the discovery by Walsh et al. (1968) of a cAMP-dependent protein kinase in skeletal muscle and its role in the regulation of glycogenolysis. It has subsequently become generally recognized that protein kinases mediate most, if not all, of the effects of cAMP.

The state of phosphorylation has been shown to exert considerable influence over the structure and function of protein molecules (Kleinsmith, 1975). Control is exerted via the regulation of kinases that promote the attachment of phosphate and of protein phosphatases that catalyze its removal. Hormones play important roles in the regulation of the synthesis, degradation, and activities of these enzymes.

Cellular components which regulate the metabolism and effects of cAMP are collectively referred to as the cAMP system. A simplified representation of the underlying mechanisms is illustrated in Figure 1. Membrane-acting effectors regulate cAMP synthesis by binding to specific receptor proteins which are functionally linked to adenylate cyclases within cell membranes. Besides extrusion from the cell, the subsequent fate of cAMP depends upon its interaction with either of two molecular entities: phosphodiesterases or cAMP-binding proteins. cAMP phosphodiesterases irreversibly hydrolyze the nucleotide to

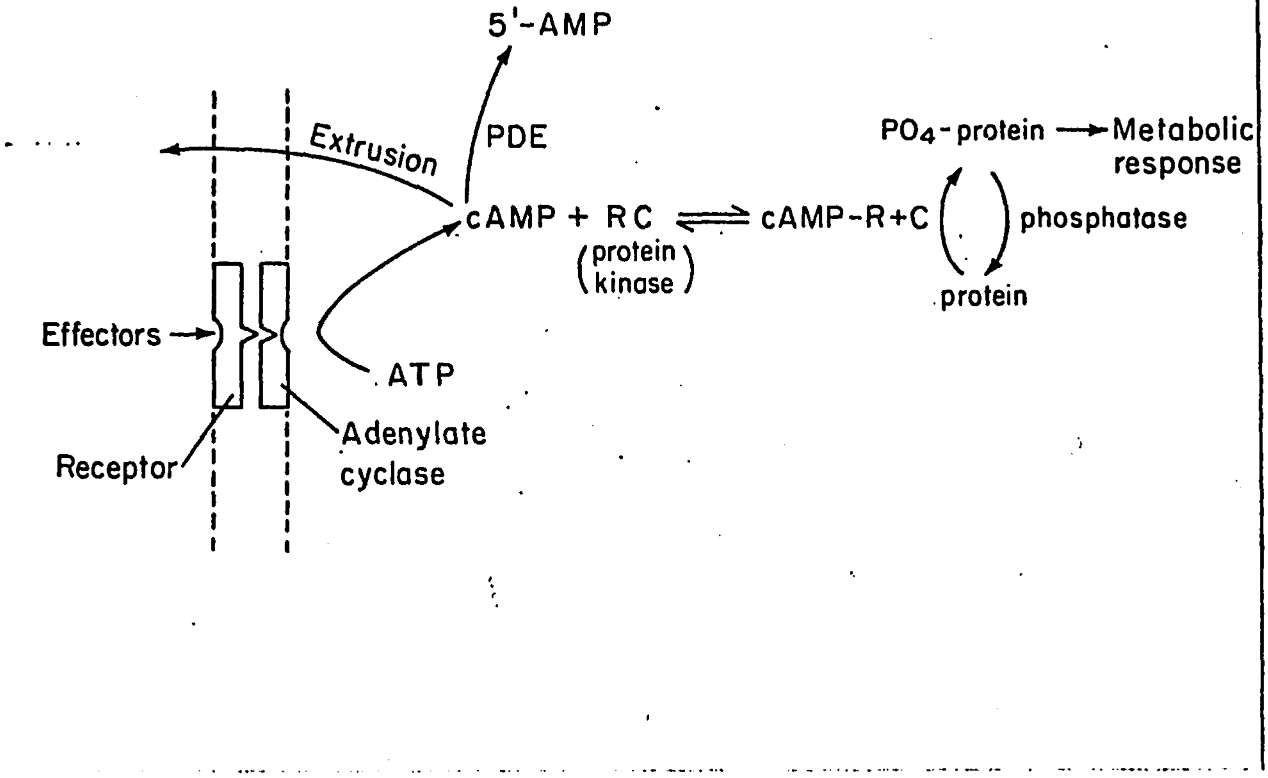

Figure 1. Schematic representation of the major components of the cAMP system, which is thought to mediate the biological effects of numerous humoral substances upon cells. PDE, cAMP phosphodiesterase; RC, c-AMP-dependent protein kinase holoenzyme; R, regulatory subunit of RC; C, catalytic subunit of RC.

5'-AMP. Biological effects of cAMP are thought to follow its binding to the regulatory subunit of cAMP-dependent protein kinases. The cAMP-dependent enzyme exists as a relatively inactive dimer (RC) or tetramer (R_2C_2) in which the enzymic activity of the catalytic subunit (C) is restrained by the associated regulatory subunit (R). cAMP frees the catalytic subunit by binding with high affinity and specificity to the regulatory subunit. In cAMP-dependent protein kinases, the nucleotide promotes the dissociation of the inactive RC complex and thereby generates catalytically-active C subunits.

Protein kinases are commonly classified on the basis of two characteristics: substrate specificity and cyclic nucleotide responsiveness. Nucleotide-unresponsive kinases are designated as phosphoprotein kinases (Rubin, 1975), and usually act on nutritive proteins, e.g., phosvitin or casein. Nucleotide-responsive protein kinases are usually studied with histone substrates. Two major forms of cytoplasmic cAMP-dependent protein kinases have been found in most tissues, Types I and II (Corbin, 1975). Type II differs from Type I in its requirement for autophosphorylation of the regulatory subunit for full responsiveness to cAMP, and its lower salt dissociability (Rubin, 1975). Hofmann _et al_. (1975) determined that the holoenzyme of

Type II in bovine cardiac muscle is a tetramer consisting of dimeric regulatory and catalytic subunits, with the following apparent molecular weights: R_2C_2, 174,000 daltons; R_2-$cAMP_2$, 98,000 daltons; and C, 38,000 daltons. cAMP-dependent protein kinases have been characterized in a variety of tissues. In most cases the catalytic subunits appear to be identical; differences in molecular behavior are usually attributable to dissimilar regulatory subunits. It is possible that kinase isozymes are regulated primarily through changes in the availability of different regulatory subunits. Activated catalytic subunits can alter the function of specific proteins by controlling their states of phosphorylation. Ionic conditions and certain enzyme substrates have been shown to modify the dependence of kinases upon cAMP for activation (Walsh, 1979). Additionally, evidence has been presented for the translocation of cytoplasmic catalytic subunits to the nucleus with subsequent increases in nuclear protein phosphorylation (Turkington, 1973). Cho-Chung _et_ _al_. (1978) have recently shown that the latter process may be an important mechanism in the induction of rat mammary tumor regression by DBcAMP (dibutyryl cAMP).

Hormones can influence cAMP-dependent phosphorylation systems at several levels:

(1) adjustment of cyclic nucleotide concentrations by direct influences on the activities of adenylate cyclases and phosphodiesterases;

(2) induction and degradation of enzymic and binding activities involved;

(3) control of cytosol ions, modulator proteins, and other modifiers; and

(4) regulation of substrate availability.

In addition to cAMP, several other substances participate in the regulation of protein phosphorylation. Analogous to cAMP, cGMP (guanosine cyclic 3',5'-monophosphate) levels are elevated by several hormones and neurotransmitters; and specific cGMP-dependent protein kinases and substrate proteins have been identified (Schlichter, 1978). It has been proposed that cGMP acts as a positive stimulant for cell growth and that it may function antagonistically to cAMP. However, this simplistic model has been seriously challenged (Pardee, 1978). Changes in calcium transport and compartmentation have been associated with specific protein phosphorylations, and these may be catalyzed by calcium-dependent protein kinases (Krueger, 1977). Calmodulin, a putative intracellular calcium-binding protein, may mediate many of the effects of calcium on protein phosphorylation via regulation of phosphodiesterases, adenylate cyclases, and protein kinases (Chafouleas, 1979). Many steroid hormone effects are either synergistic with or antagonistic to the actions of cAMP. There is evidence that steroid hormones influence protein phosphorylations both directly by altering the level of cAMP (Ross, 1977), and indirectly by modifying the autophosphorylation of protein kinases via processes dependent upon protein synthesis (Liu, 1976). Insulin often acts in opposition to hormones whose effects are mediated by cAMP. It has been observed to inhibit specific protein phosphorylations and lipolysis without decreasing intracellular levels of cAMP in fat cells stimulated by ACTH (adrenocorticotropic hormone) (Forn, 1976). Insulin may decrease the sensitivity of protein kinases to cAMP-stimulated subunit dissociation in the rat diaphragm (Walsh, 1979). Other stimuli associated with alterations in the phosphorylation of specific proteins include light, interferon, hemin, thyroid hormone, and viruses.

Effects of cAMP on Growth

Many physiological effects thought to be mediated by cAMP can be mimicked or potentiated by the administration of cAMP, cAMP analogs, adenylate cyclase stimulators, or phosphodiesterase inhibitors. Conventional methods for eliciting cAMP effects in tissues involve the administration of such agents either singly or in combination. However, widely differing effects on growth have been obtained, and these vary with the specific tissue examined and the experimental conditions employed. In most studies to date, cAMP has been shown to suppress cell growth and promote differentiation. Burk (1968) was first to report the direct inhibition of cell proliferation by cAMP. The nucleotide was added in combination with theophylline to cultures of BHK hamster cells. Other investigators have observed reversible and dose-dependent inhibitions by cAMP, DBcAMP, and phosphodiesterase inhibitors of a variety of normal and neoplastic cells in culture, including HeLa (Ryan, 1968), 3T3 (Sheppard, 1971), human lymphocytes (Gallo, 1971), mouse embryo fibroblasts (Johnson, 1972), and mouse lymphosarcoma (Daniel, 1973). Naseem and Hollander (1973) demonstrated a concentration dependent and readily reversible inhibition of growth in plasma cell tumors in culture by cAMP and prostaglandin E_1 (PGE_1). cAMP has also been found to be effective in reversing other tumor cell characteristics, e.g., loss of density inhibition, random organization, infinite lifespan, agglutination by plant lectins, and tumorigenicity (Johnson, 1971b). Subcutaneous administration of DBcAMP has been shown to inhibit the growth of dimethylbenzanthracene (DMBA)-induced skin tumors in mice (Posternak, 1976). Both cAMP and aminophylline have been used to suppress the proliferation of Ehrlich tumor cells in ascitic fluid and in solid tumors (Seller, 1973). Keller (1972) inhibited the growth of Walker 256 carcinomas in rats using DBcAMP both alone and in combination with theophylline. PGE_1, together with DBcAMP or a phosphodiesterase inhibitor, suppresses the proliferation of neuroblastoma cells (Prasad, 1972). Theophylline administration has been used to promote the regression of a human desmoid tumor (Waddell, 1975). Papaverine has caused temporary regression of disseminated neuroblastomas in patients (Helson, 1975), and has been effectively used in clinical trials with metastatic neuroblastomas (Helson, 1976). Cho-Chung _et al_. (1974) observed that _in vivo_ administration of DBcAMP, 8-thiomethyl cAMP, or 8-bromo cAMP suppressed the growth, and in some cases stimulated the regression, of a variety of rat mammary tumors, including MTW9 and tumors induced by N-nitrosomethylurea and DMBA. In the latter study, 1 mg/day/200 g body weight proved to be an effective dose of DBcAMP. No toxic effects of the drug at this dosage were evident, and 2',3'-AMP, 5'-AMP, and sodium butyrate failed to affect tumor growth. DBcAMP injections have been reported to enhance the growth of the hormone-responsive rat mammary adenocarcinoma R3230AC; however, this effect was attributed to an increase in cell size and differentiation rather than a change in cell number (Klein, 1977).

In contrast, there is evidence that cAMP can promote the growth of some cells. Green (1978) demonstrated the stimulation of epidermal cell proliferation by several substances known to increase cAMP concentrations, e.g., cholera toxin, DBcAMP, 1-methyl-3-isobutylxanthine, and isoproterenol. The growth of thymocytes and rat fibroblasts is enhanced by cAMP; however, this effect can be reversed by elevated concentrations of calcium (Whitfield, 1973). Proliferation of chick embryo fibroblasts (Hori, 1973) and 3T3 cells (Schor, 1974) has been promoted by low concentrations of cAMP (10^{-5} M). <u>In vivo</u> administration of cAMP (20 μg/day) stimulates the growth of methylcholanthrene-induced tumors in mice, and also influences other abnormal morphological characteristics (Johnson, 1971a). cAMP administration also increases the incidence of DMBA-induced skin tumors in mice (Curtis, 1974).

A biphasic response to cAMP has been seen in some cells, with growth stimulation by low concentrations of cAMP or DBcAMP and growth suppression by elevated concentrations. Such observations have been made with lymphoblasts (Whitfield, 1973) and BHK cells (Furmanski, 1973). However, this phenomenon has not been generally found. Byus et al. (1977) have suggested that growth responses to cAMP may be at least partially due to the activity levels of different protein kinases within a tissue.

DBcAMP administration has been observed to inhibit the growth of MTW9 *in vivo* (Cho-Chung, 1974), and recent evidence provides support for the concept that mammary tumor regression in response to ovariectomy shares common mechanisms with the regression achieved by administration of DBcAMP (Cho-Chung, 1978). It has been proposed that growth control in some tumors may involve antagonism between cAMP and stimulatory hormones (Shafie, 1979).

In the present study, I examined the effects of cAMP, DBcAMP, and phosphodiesterase inhibitors on DNA synthesis in MTW9 in organ culture. In attempts to avoid the extreme complexities of whole animal studies, *in vitro* models were used for the study of factors affecting tumor growth. Organ culture techniques have been widely used to investigate endocrine mechanisms in mammary tissue (Welsch, 1977; Ceriani, 1972; Lewis, 1974), and advantages over cell culture methods include the preservation of tissue organization, known to be important for some hormonal effects (Heuson, 1975). Results from these experiments were further evaluated by a similar investigation using human breast cancer cells in culture (MCF-7). While optimizing the *in vitro* growth conditions of these tissues, I also studied the direct effect of several hormones and sera on (^{3}H)thymidine incorporation in MTW9 and on MCF-7 cell growth.

Alterations in the cAMP System in Neoplasia

A growing body of evidence supports the concept that the protein phosphorylation system participates in the regulation of growth, and that the principal effectors of this system, e.g., cyclic nucleotides and calcium ions, play important roles in controlling cell proliferation (Rubin, 1975; Greengard, 1978). Therefore, one might expect major changes in the growth status of tissues to be associated with substantial alterations in the cAMP system. Indeed, neoplastic transformation is often associated with aberrations in both the cellular concentration of cAMP and the major components controlling cAMP metabolism and protein phosphorylation: adenylate cyclases, phosphodiesterases, cAMP-binding proteins, and cAMP-dependent protein kinases.

In general, transformed cells in culture have been reported to have lower concentrations of cAMP than their normal counterparts (Ryan, 1973). Tisdale and Phillips (1974) found cAMP levels to be inversely correlated with malignancy in somatic cell hybrids. However, investigations have shown that cAMP levels need not be correlated with transformation (Peytremann, 1973). Similarly, cAMP levels within tumors _in vivo_ do not follow any apparent pattern. Cohen _et al_. (1975) observed that cAMP concentrations in mammary adenocarcinoma cells in culture were substantially reduced from normal values, though cAMP levels were significantly elevated in DMBA-induced rat mammary tumors. Other researchers have found elevated levels of cAMP in a variety of human breast cancers, including breast carcinomas (Minton, 1976). An inverse relationship has been reported for cAMP levels and meiotic activity in mouse epidermis (Marks, 1972) and DMBA-induced rat mammary tumors (Matusik, 1976). Minton _et al_. (1974) found substantially elevated levels of cAMP in human breast cancers. However, these authors have postulated that defects in cAMP-binding or protein kinase activity may account for the failure of cAMP to inhibit growth in such cancers.

Defects in cAMP metabolism may be associated with neoplasia which have low endogenous concentrations of cAMP or whose transformed characteristics are reversed by stimulated increases in cAMP. Adenylate cyclases in some neoplastic tissues are less sensitive to physiological activators, e.g., Morris hepatoma cells to glucagon (Makman, 1971), transformed 3T3 cells to PGE_1 (Peery, 1971), and leukemia cells to epinephrine (Polgar, 1973). Ney et al. (1969) have observed that although cAMP levels are elevated in adrenocortical carcinomas when compared with normal tissues, these tumors are unresponsive to the action of ACTH (which raises cAMP levels 20-50-fold in normal tissues). Alterations in plasma membrane characteristics may influence hormone-receptor and adenylate cyclase systems (Bentwich, 1972). Anderson et al. (1973) have suggested that kinetic analysis may be necessary for revealing certain defects in adenylate cyclase activity.

Positive correlations have been found between adenylate cyclase activities and growth rates in rat hepatomas (Brown, 1970). The same investigators reported increased adenylate cyclase activity in dimethylaminobiphenyl-induced rat mammary carcinomas (Brown, 1969). Adenylate cyclases in hepatocarcinomas have been shown to be refractory to hormone stimulation (Christoffersen, 1972). Only DBcAMP-sensitive Walker 256 rat mammary carcinomas are responsive to PGE_1-stimulated adenylate cyclase activity in vitro and to PGE_1-promoted regression in vivo (Clair, 1974). Although alterations in adenylate cyclase activities may be related to aberrant growth patterns in some neoplastic tissues, no simple correlation has been found causally linking adenylate cyclase activity to the control of proliferation.

cAMP phosphodiesterases play a strategic role in regulating cellular levels of cAMP by irreversibly hydrolyzing the nucleotide to 5'-AMP. Cells exert fine control over cAMP hydrolysis by altering the number, distribution, and molecular forms of cAMP phosphodiesterases, and also through mediation by small molecules and regulatory proteins (Richman, 1978; Katz, 1978). Generally, most phosphodiesterase activity is associated with cytosol fractions and can be resolved using electrophoresis and gel filtration into two major molecular forms: low- and high-Km (Michaelis constant) enzymes (Thompson, 1978). Investigations of tumor phosphodiesterase activity have revealed a variety of enzyme alterations in neoplastic tissues. However, changes in phosphodiesterase activity do not adhere to any apparent pattern. Cohen _et al._ (1976) reported phosphodiesterase activity to be diminished in transformed rat mammary cells in culture. Phosphodiesterase activity is drastically reduced in some transformed cells, e.g., SV40-transformed 3T3 cells (D'Armiento, 1972) and Novikoff rat hepatoma cells (Schroder, 1972). Chatterjee _et al._

(1975) observed that diminished phosphodiesterase levels and elevated cAMP concentrations were associated with metastasizing capacity in rat mammary tumors. Low-Km phosphodiesterase activity was increased in human breast tumors and correlated positively with malignancy in one study (Singer, 1976). Cho-Chung et al. (1977a) identified two major forms of cAMP phosphodiesterase activity in Walker 256 mammary carcinoma cells. Whereas both DBcAMP-responsive and unresponsive cell lines had similar basal levels of phosphodiesterases, PGE_1 induction of low-Km activity in organ cultures occurred only in those cells which were growth-inhibited by DBcAMP. cAMP is known to induce phosphodiesterases in a wide variety of cells (Raska, 1973), and a defect in this process is suggested in DBcAMP-unresponsive mammary carcinoma cells (Cho-Chung, 1977a).

Transformed mouse fibroblasts have been observed to contain enhanced levels of cAMP phosphodiesterase and unusually low levels of cGMP phosphodiesterase (Lynch, 1975). In normal rat mammary gland, Sapag-Hagar et al. (1974) have reported that high-Km phosphodiesterase increases significantly during lactation; the authors suggest that this enzyme may play a role in the growth and differentiation of the mammary gland.

In many tumors, there is a molecular defect in the cAMP system beyond the level of cAMP generation and degradation. Alterations in cAMP-binding activities have been observed in several neoplastic tissues. Only responsive strains of Walker 256 mammary carcinoma regress in response to exposure to DBcAMP. Unresponsive strains continue to grow and have been shown to contain very low concentrations of cAMP-binding protein (Cho-Chung, 1977b). A hepatoma cell line with low levels of cAMP and adenylate cyclase has been further characterized as unresponsive to cAMP because of a deficiency in binding protein (Granner, 1974). Deficient cAMP-binding has been reported in C3H mouse mammary carcinomas (Majumder, 1977a). Using somatic cell genetics, Daniel _et_ _al_. (1973) observed that decreased sensitivity to the lympholytic effects of DBcAMP were correlated with defective regulatory subunits of protein kinases in mutant lines of S49 lymphoma cells. Furthermore, Tisdale _et_ _al_. (1976) observed that diminished cAMP-binding activity parallels the loss of sensitivity to the cytotoxic action of both DBcAMP and alkylating agents thought to act through cAMP in cultured mammary cancer cells. Decreased cAMP-binding affinity was associated with human adrenocortical tumors (Riou, 1977). In very interesting work, Prasad (1975) has used DBcAMP and PGE_1

to achieve irreversible differentiation in cultured neuroblastoma cells. These agents augment cAMP-binding protein levels, and this may provide a permanent mechanism for the protection of cAMP. The authors suggest that such autoregulation of binding protein should be considered in designing therapy for patients with neuroblastoma. However, related mechanisms do not seem operative in non-neural tumors, e.g., sarcoma and glial. Yet, enhancing cAMP-binding protein for the treatment of cancers may prove useful. Further study could provide insights into processes involved in the spontaneous remissions that occur in patients with other cancers.

Several important observations have been made regarding protein kinase activities in normal and neoplastic tissues. Distinct molecular forms of cytoplasmic (Majumder, 1971) and nuclear (Desjardins, 1975) protein kinases have been characterized in the rat mammary gland. Turkington further demonstrated prolactin induction of kinase and cAMP-binding activities, followed by extensive phosphorylations in mouse mammary explants (Turkington, 1973). The same authors also described significant differences between normal and neoplastic rat mammary tissue in nonhistone chromosomal protein (NHCP) phosphorylation patterns. Eppenberger et al. (1976) recently reported enhanced levels

(3-fold) of cAMP and cAMP-dependent protein kinases in human primary carcinomas. Diminished sensitivity to activation of protein kinases was observed in DBcAMP-unresponsive Walker 256 tumors (Cho-Chung, 1977c). In urethane-induced lung tumors, cytosolic cAMP-dependent protein kinase activity is enhanced 2-fold over the normal tissue (Malkinson, 1977).

In the present study, I explored the possibility that substantive changes in cAMP metabolism might be meaningfully associated with (1) the progression of hormone-dependent tumors to a hormone-autonomous state, and (2) the induction of regression in hormone-dependent tumors by endocrine ablation. Several key components in the cAMP system were compared in growing MTW9 tumors, regressing MTW9 tumors, and autonomous tumors (MTW9B). Numerous differences among these tumors were observed, and results are interpreted in view of previously reported investigations.

MATERIALS AND METHODS

Rat Mammary Tumors

MTW9, a transplantable rat mammary adenocarcinoma, is an excellent research tool for investigation of human mammary cancer because of the stability of its endocrine dependence and ease of propagation. The maintenance and important characteristics of this tumor line have been described in detail elsewhere (MacLeod, 1964; Kim, 1975; Hollander, 1978). Briefly, MTW9 is maintained by coimplantation with a mammosomatotropic tumor, MtTW10, at different sites in syngeneic Wistar-Furth female rats. After a latent period of about 3 weeks, which presumably permits establishment of the supportive MtTW10, MTW9 becomes palpable, and then grows steadily to a diameter of 2 to 3 cm in about 6-8 weeks. Ovariectomy during this growth phase results in the cessation of further growth, but does not stimulate regression. However, resection of the MtTW10 during this period causes the rapid regression of the mammary tumor within 2 weeks. Growth of the mammary tumor can be prevented by ovariectomy at the time of implantation.

MTW9B is an autonomous subline of MTW9 which grows rapidly in male, female, and ovariectomized rats. Measurements in Dr. V. Hollander's laboratory have determined that prolactin and estradiol receptors are undetectable in the MTW9B tumor used in this study (unpublished observation). Evidently, the tumor used in this study is a variant of tumors used in other laboratories, since substantial levels of estradiol receptor have been found in another MTW9B subline (Ip, 1978).

Only small, rapidly growing tumors were used for biochemical study since these are most likely to be free of necrosis and to be maximally responsive to hormones (Lewis, 1974).

Organ Culture Technique

Animals were sacrificed by cervical dislocation and submerged in 95% ethanol for 2-3 minutes. Tumors were excised under aseptic conditions, trimmed of surrounding connective tissue, and cut with a scalpel blade into pieces measuring approximately 1 mm^3. All tissue was kept moist with M199 supplemented with penicillin (1000 U/ml) and streptomycin (1000 μg/ml). Explants were randomly distributed into plastic multiwell tissue culture dishes (9.6 cm^2, Flow Laboratories, Rockville, Md.) containing 2 ml of M199. In each well ten explants were supported at the surface of the culture medium on a stainless steel grid. Cultures were incubated in a humidified atmosphere of 95% O_2/5% CO_2 at 37°C. Preliminary experiments established these conditions as optimal for explant viability and (^{3}H)thymidine incorporation.

Representative tissue explants were fixed in 9% formalin and processed for histological examination using hematoxylin-eosin staining. Subsequent examination indicated that there was good maintenance of tissue organization with little evidence of necrosis for at least 96 hours in culture.

Measurement of DNA Synthesis

Four hours prior to the end of each incubation period, 1 µCi of (methyl-^{3}H)thymidine (40-60 Ci/mmol, New England Nuclear, Boston, Mass.) was added to each culture to determine the rate of (^{3}H)thymidine incorporation into DNA. Cultures were terminated by freezing at -90°C, and all subsequent procedures were performed at 2-4°C unless otherwise stated. The tissue from each dish was homogenized in 2.0 ml of 0.5 N PCA using a Polytron PT-10 tissue disruptor (Brinkmann Instruments, Inc., Westbury, N. Y.) at its maximum setting for 5 seconds. The homogenate was centrifuged at 10,000 x g and the pellet was washed with 1.5 ml of 0.5 N PCA and again centrifuged. The washed pellet was resuspended in 1 ml of 0.5 N PCA and maintained at 90°C for 20 minutes. This preparation was chilled at 2-4°C for 15 minutes and centrifuged as before. To measure the incorporation of (^{3}H)thymidine into tumor DNA, the resulting supernatant fraction was analyzed for DNA using a diphenylamine colorimetric method (Burton, 1956), and for radioactivity by liquid scintillation counting in a Packard Tri-Carb Liquid Scintillation Spectrometer. Appropriate corrections were made for quenching. The results are expressed as dpm (^{3}H)thymidine/μg DNA. A single tumor was used in each experiment and every condition was performed in duplicate.

Control studies established the above procedures as optimal for the extraction of DNA from tumor explants. Prior extraction with organic solvents did not influence the results, presumably because tumor fat content is very low. The incorporation of radioactivity was completely inhibited by the addition of 1 mM hydroxyurea, an inhibitor of DNA synthesis, 10 minutes before the (^{3}H)thymidine pulse.

Substantial changes in the incorporation of (^{3}H)thymidine into DNA are generally interpreted as indicative of changes in DNA synthesis (Lewis, 1974; Hallowes, 1977; Takizawa, 1970a). However, it should be noted that changes in the uptake and metabolism of (^{3}H)thymidine can influence incorporation rates where there are no net changes in DNA synthesis.

Using representative samples under the various experimental conditions employed in this study, the acid-soluble pool of radioactivity was measured and found not to be significantly altered by hormone, serum, or methylxanthine treatment. Therefore, the substantial changes in (^{3}H)thymidine incorporation into DNA found in this study were most probably due to alterations in DNA synthesis. Changes in cellular uptake or metabolism of (^{3}H)thymidine have, at most, a minor effect. Because of the limitations inherent in estimating growth by (^{3}H)thymidine incorporation, observations made using organ culture were extended in studies using an established breast cancer cell line.

Human Breast Cancer Cells

The human breast cancer cell line, MCF-7, was originally derived from a pleural effusion (Soule, 1973) and has been extensively characterized (Lippman, 1975; Lippman, 1976a; Linebaugh, 1977). Our line was generously donated by Dr. M. Lippman (NCI, NIH, Bethesda, Md.) and has been maintained by weekly passage in Falcon plastic tissue flasks (75 cm^2, Becton, Dickinson and Co., Oxnard, Calif.) containing 10% FCS in 20 ml of MEM supplemented with 2 mM L-glutamine, 0.1 mM MEM non-essential amino acids, penicillin (1000 U/ml), and streptomycin (1000 μg/ml) (SMEM). Cultures were incubated in a humidified atmosphere of 5% CO_2 in air at 37°C. For subculturing and initiating experiments, cells were detached using 1 mM EDTA in Hanks' Balanced Salt Solution (HBSS) without Ca^{++} or Mg^{++}. MCF-7 cells were cultured according to the standards of biological safety set by the National Cancer Institute (Bethesda, Md.).

Measurement of MCF-7 Cell Growth

In all experiments, cells in logarithmic growth phase were plated at a density of 10^4 cells/well in plastic multiwell tissue culture dishes (2 cm^2, Flow Laboratories, Hamden, Conn.) containing 1 ml SMEM with 3% FCS. After an initial 24 hour period for cell attachment, the medium was removed, each well was rinsed with 1 ml MEM, and 1 ml of the experimental medium was added. After 4 days the medium was replaced with fresh medium and after 8 days, experiments were terminated by rinsing each well with HBSS, detaching the cells with 1 ml HBSS - 1 mM EDTA, and sonicating for 5 seconds using a Kontes tissue disruptor (Kontes, Vineland, N. J.) set at its lowest speed. Cell number was determined by counting nuclei with a cytograf (Model 6300A, Ortho, Mahopac, N. Y.) previously calibrated using hemocytometer counts. Data are expressed as means ± S.E. for triplicate determinations. All experiments were repeated two or more times with similar results.

Extreme aggregation of MCF-7 cells prompted the development of the above technique for determining cell number. The percentage of cells having multiple nuclei was insignificant. Phase contrast microscopy with aceto-orcein nuclear staining revealed that mild sonication resulted in complete disruption of cells without significant nuclear disruption or aggregation.

Cell growth was not limited in this study by vessel size since comparable growth kinetics were observed when cells were plated at either higher (2×10^4 cells/well) or lower (0.3×10^4 cells/well) density. There was an exponential increase in cell number to a final density of at least 2.5×10^5 cells/well.

To eliminate the possible influence of differential cell attachment on subsequent growth kinetics, cultures were permitted to attach under uniform conditions prior to the addition of experimental media. Experimental conditions used in this study did not cause significant cell detachment from the plating surface.

Preparation of Hormone Solutions and Sera

Porcine crystalline insulin (25.4 U/mg, Lilly Research Laboratories, Indianapolis, Ind.) was dissolved in 0.005 N HCl at a concentration of 1 mg/ml and stored at 2-4°C.

Ovine (NIH-P-S-12) and rat (NISMDD-I-2) prolactins and rat growth hormone (NIAMDD GH-I-2) were dissolved directly into culture media at concentrations 50-fold greater than the desired final concentrations.

Corticosterone, progesterone, and 17-β estradiol (Steraloids, Wilton, N. H.) were dissolved in absolute ethanol and diluted with medium so that the final ethanol concentration after addition to cultures was less than 0.1%. The concentration of ethanol used did not influence the experimental results.

To simulate the major endocrine constituents in the sera of MtTW10-bearing animals, several hormones were combined in a mixture referred to here as MHS (Table 1).

Blood was collected following decapitation from groups of normal male and female Wistar-Furth rats and from MtTW10-bearing rats. After overnight clot retraction at 2-4°C, serum was prepared by centrifugation. Prolactin was measured by radioimmunoassay and concentrations in MtT sera consistently exceeded 1000 ng/ml, while those from

TABLE 1

Serum hormone concentrations

Hormone	Normal Female Serum	MtT Serum	MtT Hormone Simulant	Reference
Estradiol (pg/ml)[a]	5 - 45	11 ± 4[b]	11	
Progesterone (ng/ml)[a]	12 ± 1	34 ± 3	33	
Corticosterone (μg/100 ml)[a]	20 ± 1	33 ± 4	33	
Prolactin (ng/ml)	10 - 250	500 - 10,000	10,000	Diamond, 1976
Growth Hormone (ng/ml)	5 - 10	5400 ± 670	5,000	Ito, 1971
Insulin (μU/ml)	27 ± 8	142 ± 43	142	Martin, 1968

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[a] Measured by RIA in the laboratory of Dr. Neena Schwartz (Biological Sciences Department, Northwestern University, Evanston, Ill.).

[b] Mean ± S.E.

control animals were always below 50 ng/ml.

Subcellular Fractionation of Mammary Tumors

Animals were sacrificed by cervical dislocation and mammary tumors were quickly excised and submerged in ice-cold 0.9% saline. All subsequent procedures were performed at 2-4°C. Tumor tissue was trimmed free of connective tissue, finely minced, and homogenized with a Polytron PT-10 set at 1/2 maximum speed for 20 seconds in 10 volumes (w/v) of homogenization buffer (HB) containing 10 mM potassium phosphate buffer, pH 7.2, 0.5 M sucrose, 5 mM $MgCl_2$, and 0.1 mM phenylmethylsulfonylfluoride (PMSF). Cytosols were obtained by direct centrifugation of the homogenate in a Beckman Model L ultra-centrifuge for 1 hour at 35,000 rpm using an SW-36 rotor. Centrifugation of the homogenate in a Sorvall RC2-B centrifuge for 10 minutes at 3,000 rpm using an SS-34 rotor produced a post-nuclear supernatant fraction and a crude nuclear pellet. A crude particulate fraction was obtained by ultracentrifuging the post-nuclear supernatant fraction as described above, followed by rehomogenizing and washing the pellet in HB, and resuspending the washed pellet in 10 volumes of HB. The Triton-extracted particulate fraction was prepared by treating the washed pellet with 0.2% Triton X-100 and ultracentrifuging as before. A purified particulate fraction resulted from layering the post-nuclear supernatant fraction onto a discontinuous sucrose gradient (0.5M/1M/2M), ultracentrifuging as described above, and collecting the particulate material settling at the 1M/2M interface. A purified nuclear fraction was produced by resuspending the crude nuclear pellet in 10 volumes of HB, filtering through 4 layers of cheesecloth, and ultracentrifuging as described above through a 2.0 M sucrose cushion. Nuclei were washed with 5 volumes of nuclear buffer (NB) containing 10 mM potassium phosphate buffer, pH 7.2 and 5 mM $MgCl_2$. Extraction of nuclei using 10 mM potassium phosphate buffer, pH 7.2 with 2 mM EDTA and 150 mM NaCl for 1 hour and centrifugation for 10 minutes at 10,000 rpm in a Sorvall RC2-B produced a saline nuclear extract. The resulting nuclear pellet was resuspended in 2.33 volumes of HB and slowly brought to 0.6 M NaCl by the dropwise addition of 1 volume of NB containing 2 M NaCl. The resultant chromatin preparation was sheared with the Polytron PT-10 set at 1/2 maximum speed for 20 seconds, stirred for an additional hour and ultracentrifuged as described above. The supernatant fraction was dialyzed against 50 volumes of HB changed 3 times during a 36 hour period and was centrifuged for 10 minutes in a Sorvall RC2-B at 10,000 rpm with a

SS-34 rotor. This final supernatant fraction constituted the 0.6 M NaCl chromatin extract.

Anion-Exchange Chromatography of Tumor Cytosols

All procedures were carried out in a preparative cold room at 2-4°C. Tissue cytosols were desalted by Sephadex G-25 gel filtration using the following procedure. Ten ml of cytosol were applied to a 1.6 x 38 cm column previously equilibrated with .01 M potassium phosphate buffer, pH 7.0 containing 0.1 mM dithiothreitol (DTT). The column was eluted with the same buffer at a hydrostatic pressure of 100 cm and 4-ml fractions were collected with an LKB fraction collector. Effluent protein concentration was monitored by absorbance at 206 nm and 280 nm using an LKB Uvicord III UV absorptiometer and salt concentration was estimated from effluent conductivity using an LKB conductolyzer (Model 5300A). Protein and salt effluent peaks were effectively separated, with virtually all of the protein kinase activity associated with the protein peak (Figure 2).

The desalted protein peak was pooled and slowly applied to a DE-52 (Whatman) anion-exchange column (1.6 x 24 cm) previously equilibrated with the above buffer. The column was eluted over a 16-hour period with a linear

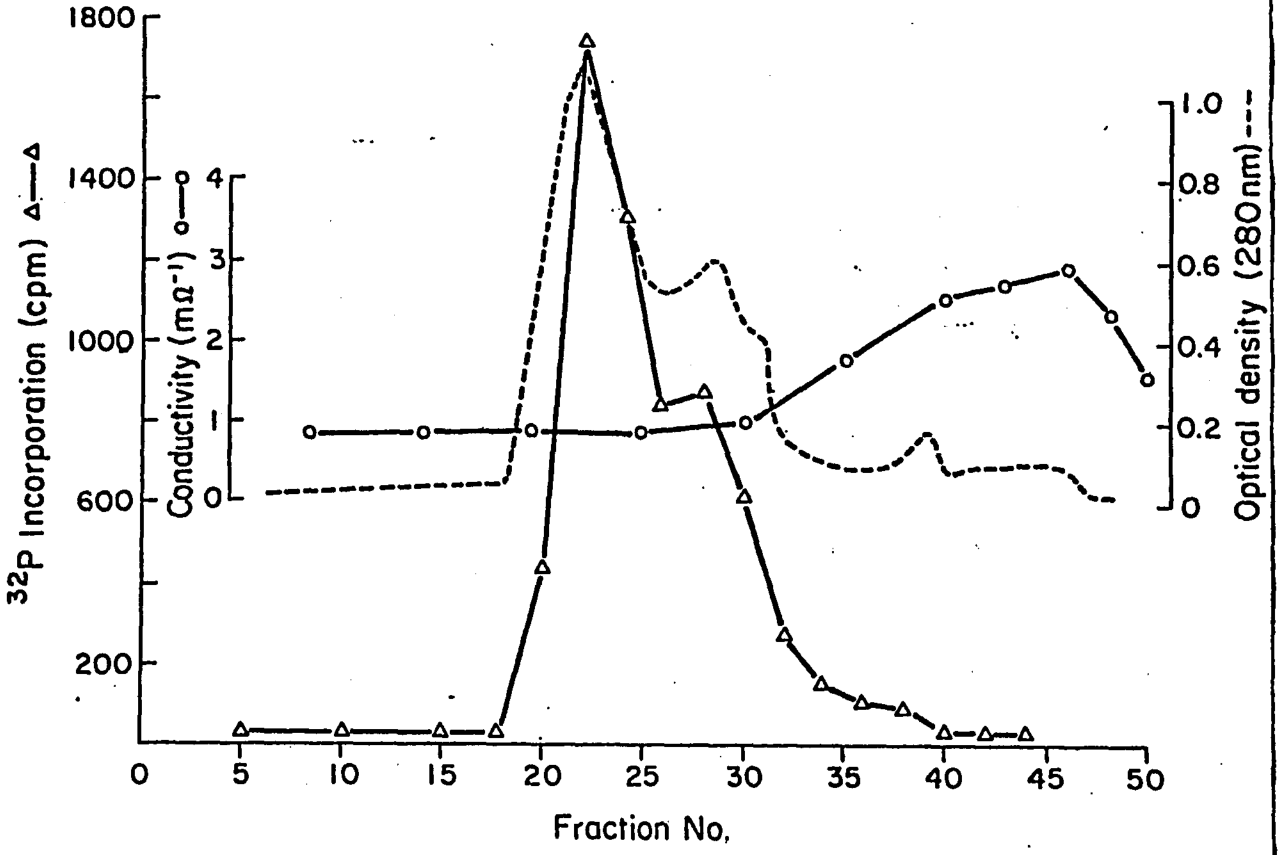

Figure 2. Desalting of MTW9 cytosol by Sephadex G-25 chromatography. All procedures described in Materials and Methods.

gradient consisting of .01 - 0.4 M potassium phosphate buffer, pH 7.0 with 0.5 mM DTT, and 4-ml fractions were collected.

Control experiments established that chromatography did not influence the association of catalytic and regulatory subunits of cAMP-dependent protein kinase; and the activation ratio (AR: -cAMP/+cAMP) remained constant throughout these procedures using either activated or unactivated cytosol preparations. Preliminary experiments indicated the importance of reducing the cytosol salt concentration prior to application onto the anion-exchange column in order to prevent flow-through of cAMP-binding and protein kinase activities.

Animal sacrifice, subcellular fractionation, and column chromatography were performed on the same day, and fractions collected from the DE-52 column were assayed immediately for cAMP-binding and protein kinase activities. The recovery of these activities from the anion-exchange column was greater than 80% of the cytosolic activity.

cAMP-Binding Assay

Binding of (^{3}H)cAMP to protein was determined using a modification of the membrane filtration method of Gill and Garren (1969) under cAMP exchange conditions (Wilchek, 1971; DoKhac, 1973). In a total volume of 0.2 ml the assay mixture contained: 66 mM Tris-HCl buffer, pH 7.3, 6.8 mM aminophylline, 12.5 mM $MgCl_2$, 100 μM ATP, 50 nM (^3H)cAMP (37.7 Ci/mmol) (New England Nuclear, Boston, Mass.), and 20-50 μg protein. Following incubation at 24°C for 1 hour, reactions were filtered through Millipore filters (0.45 μm HAWP) prewetted with ice-cold 0.25 mM Tris-HCl buffer, pH 7.3, containing 10 mM $MgCl_2$. Filters were rapidly washed 3 times with 5 ml of ice-cold buffer, dried, and dissolved in 5 ml of scintillation fluid. Radioactivity was counted in a Packard Tri-Carb liquid scintillation spectrometer.

Binding of (^3H)cAMP to MTW9 cytosol was linear up to 50 μg protein. Under these conditions more than 90% of the prebound sites were exchanged within 30 minutes (Figure 3). Competitive binding studies suggested that this assay was highly specific for cAMP (Table 2). Only non-radioactive cAMP and 8-Br-cAMP competed effectively with (^3H)cAMP at equimolar concentrations. At 100-fold molar excess, cIMP and DBcAMP were significantly competitive and GMP and cGMP were only weakly competitive. A 2,000-fold molar excess of adenine, adenosine, AMP, ADP, or ATP did not decrease (^3H)cAMP binding. Adenine and hypoxanthine enhanced cAMP binding at 100-fold molar excess.

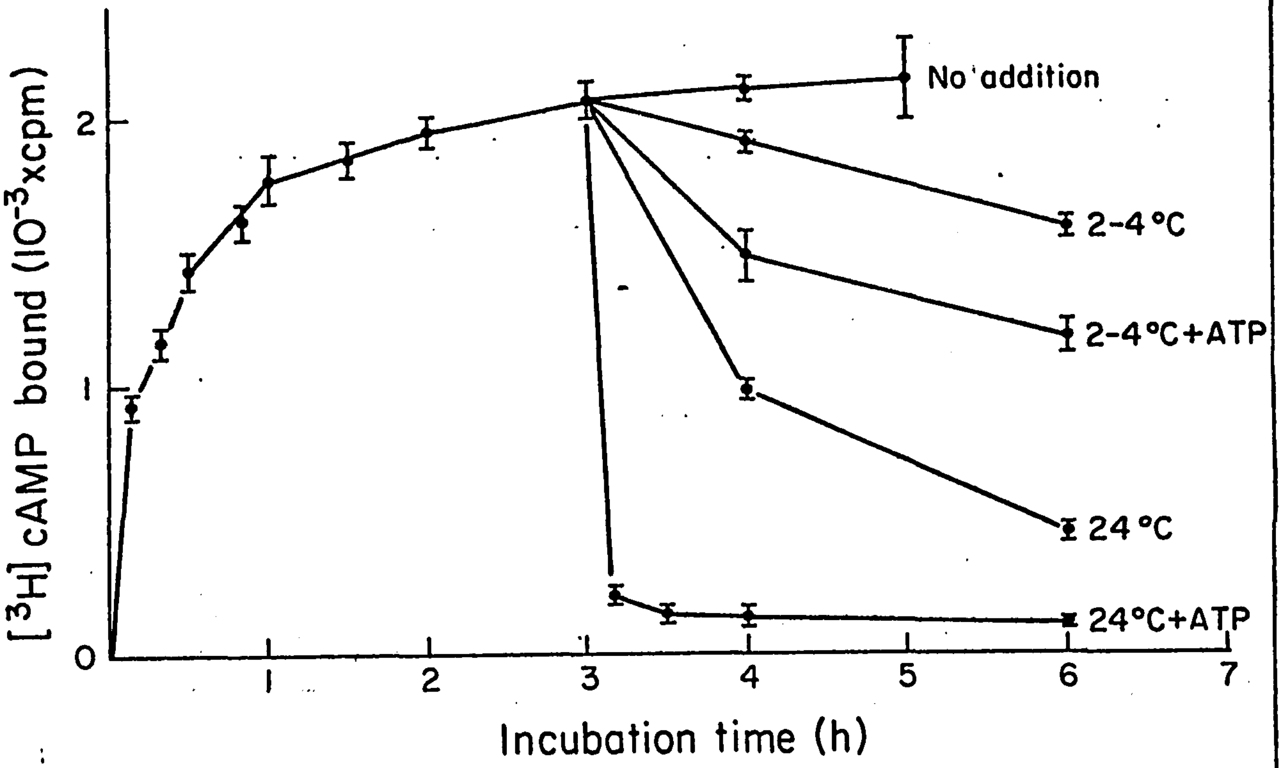

Figure 3. Exchange of prebound cAMP. cAMP-binding sites in MTW9 cytosol were saturated by incubation with 50 nM (^{3}H)cAMP as described in Materials and Methods except that incubation was carried out for 3 hours at 2-4°C without the addition of ATP. Effective conditions for the exchange of prebound cAMP were determined by measuring the amount of bound radioactivity which was released following the addition of a 100-fold molar excess of nonradioactive cAMP under the conditions indicated. Data shown are the means of duplicate determinations from one experiment, and equivalent results were obtained in a second experiment.

TABLE 2

Competition study of cAMP-binding activity in MTW9

cAMP-binding was determined in MTW9 cytosols using 50 nM (^{3}H) cAMP as described in Materials and Methods in the presence of various potential competitors at the indicated concentrations (molar excess of competitor in the parentheses). Results are expressed as the percentage of (^{3}H) cAMP bound in the presence of competitor/(^{3}H) cAMP bound in the absence of competitor. The results are from a single study and have been repeated. -, Not determined.

	% cAMP-binding		
Competitor	50 nM (1X)	5 μM (100X)	100 μM (2,000X)
None	100	100	100
cAMP	41	16	3
cGMP	98	91	32
cIMP	97	61	-
cUMP	103	96	-
8-Br-cAMP	68	21	-
DBcAMP	89	55	30
Adenine	-	126	116
Adenosine	-	107	117
AMP	-	-	97
ADP	-	-	100
ATP	-	-	104
GMP	-	91	-
Hypoxanthine	-	135	137

Determination of Protein Kinase Activity

(γ-^{32}P) ATP was prepared from (^{32}P)H_3PO_4 (New England Nuclear, Boston, Mass.) using the glycolytic exchange reaction of Glynn and Chappell (1964). The product was verified by polyethyleneimide thin layer chromatography using 2 solvent systems and strip counting with a Packard radiochromatogram scanner (Model 7201).

A protein kinase assay was developed from the method described by Witt (1975). The reaction was in a total volume of 50 µl containing: 50 mM potassium phosphate buffer, pH 7.0, 10 mM NaF, 0.2 µM EGTA, 1 mM aminophylline, 1 mM $MgCl_2$, 200 µg histone (Sigma Type II-A), 1-10 nmol (γ-^{32}P) ATP (0.1 - 0.4 µCi), and in some cases 2.5 µM cAMP. Reactions were initiated by the addition of 10-50 µg of protein and were incubated for 20 minutes at 30°C. Assays were terminated by pipetting 40 µl of the mixture onto a 2.5 cm phosphocellulose paper disc (Whatman P81). Discs were batch-washed in running tap water, rinsed with acetone and petroleum ether, dried, and counted in 3 ml of scintillation fluid using an Intertechnique SL-30 liquid scintillation spectrometer.

Agreement between replicate samples was consistently excellent and isotope incorporation was linear during the assay time for the enzyme concentrations used. The incorporated isotope was not labile to mild acid treatment (0.1 N HCl, 80°C, 10 minutes). Blank values, which were obtained by either using boiled enzyme or assaying in the absence of exogenous substrate, were always very low.

Determination of cAMP Phosphodiesterase Activity

cAMP phosphodiesterase activity in various subcellular fractions was measured by the quantitative separation of (^{3}H)cAMP metabolites by ion-exchange thin layer chromatography (TLC) (Rangel-Aldao, 1978). Assay tubes contained the following components in a total volume of 15 μl: 40 mM Tris-HCl buffer, pH 8.1, 10 mM $MgCl_2$, 2 mM DTT, 25 μg BSA, 1-100 μM (^{3}H)cAMP (34 Ci/mmol), and 1-10 μg protein. After incubation for 5 minutes at 32°C, the reaction was terminated by the addition of 1.5 μl of a stopping solution which included 0.2 M EDTA, pH 7.0, 12.5 mM 5'-AMP, and 12.5 mM cAMP. Five μl of the final mixture were chromatographed as described by Rangel-Aldao _et al_. (1978), except that optimum metabolite separation was obtained using 15 mM $MgCl_2$ for elution. The radioactivity in each metabolite band was measured in 3 ml of scintillation fluid using an Intertechnique SL-30 liquid scintillation spectrometer. Blank values were determined by the addition of stopping solution prior to incubation, and they were consistently very low. The production of (^3H)inosine was found not to be significant in these studies.

Although (^3H)cAMP hydrolysis was linear under the present assay conditions for at least 10 minutes, the appearance of the two major degradative products, (^3H)5'-AMP and (^3H)adenosine, followed separate time courses. The importance of fully quantitating both products is illustrated in Figure 4. This became especially important when measuring phosphodiesterase activity in MTW9B homogenates and in all particulate fractions, since 5'-nucleotidase activity was substantially elevated in these preparations. (^3H)cAMP hydrolysis was linear with the addition of up to 10 μg of cytosol protein.

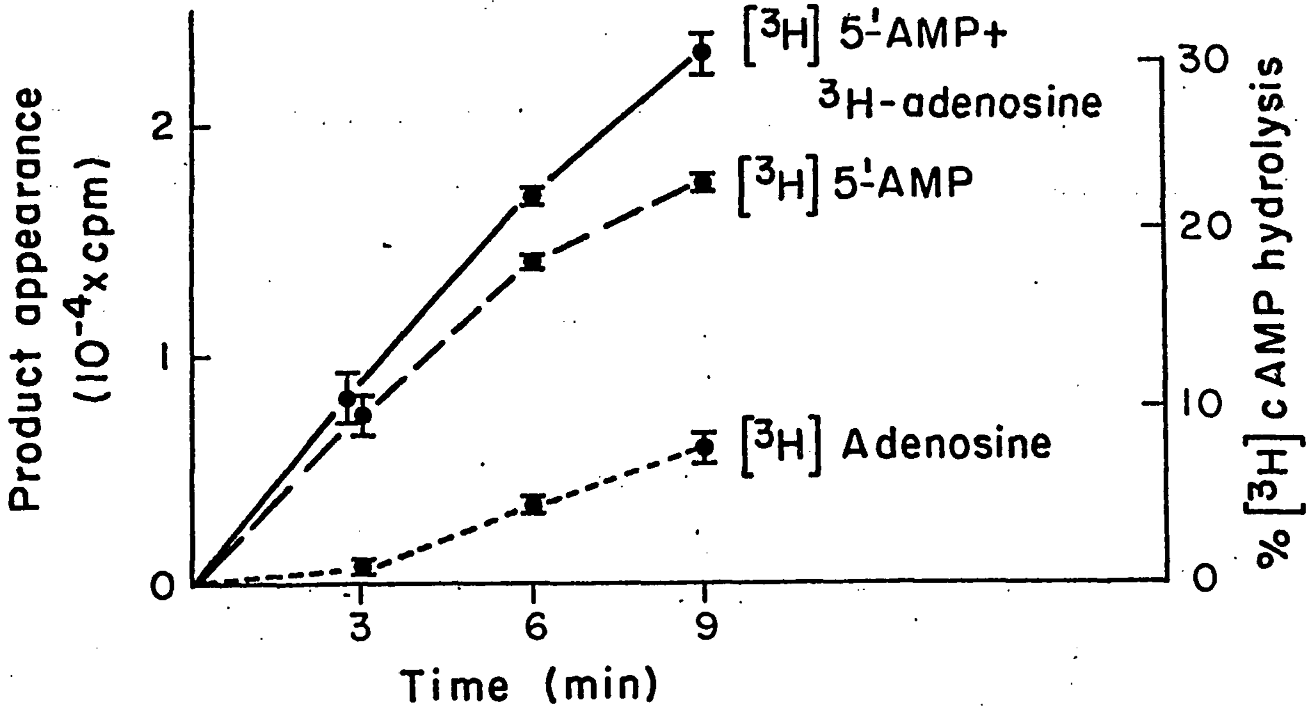

Figure 4. Time-course for the appearance of the major metabolic products of (^{3}H)cAMP hydrolysis. (^{3}H)cAMP was hydrolyzed by MTW9 cytosol under the conditions described in Materials and Methods. Results are the means ± S.E. for one experiment and equivalent results were obtained in a second experiment.

RESULTS

Effects of Sera and Hormones on Tumor DNA Synthesis

An organ culture technique was developed to assess the direct effects of various hormones and sera on tumor DNA synthesis under well-defined conditions. The rate of DNA synthesis in MTW9 explants was substantial when compared to synthetic rates observed in other rapidly-growing mammary tumors in culture (Iturri, 1976; Wang, 1971). Following an initial 25% decrement during the first 24 hours in culture, tumor DNA synthesis was maintained at a steady rate in medium alone for at least 96 hours (Figure 5). Addition of 10% MtT serum to the incubation medium produced a peak of activity at 48 hours. Hence, this time-point was selected for examining the effect of various hormones, sera, and cAMP phosphodiesterase inhibitors on DNA synthesis.

Insulin and MtT serum were observed to stimulate thymidine incorporation 4-fold and 2-fold, respectively (Figure 6). Normal female rat serum, rat prolactin, and rat growth hormone each increased the incorporation of thymidine into DNA by approximately 30%. Neither MHS, a combination of hormones simulating the known endocrine constituents of MtT serum, nor male rat serum influenced DNA synthesis under the experimental conditions. Supplementing female rat serum

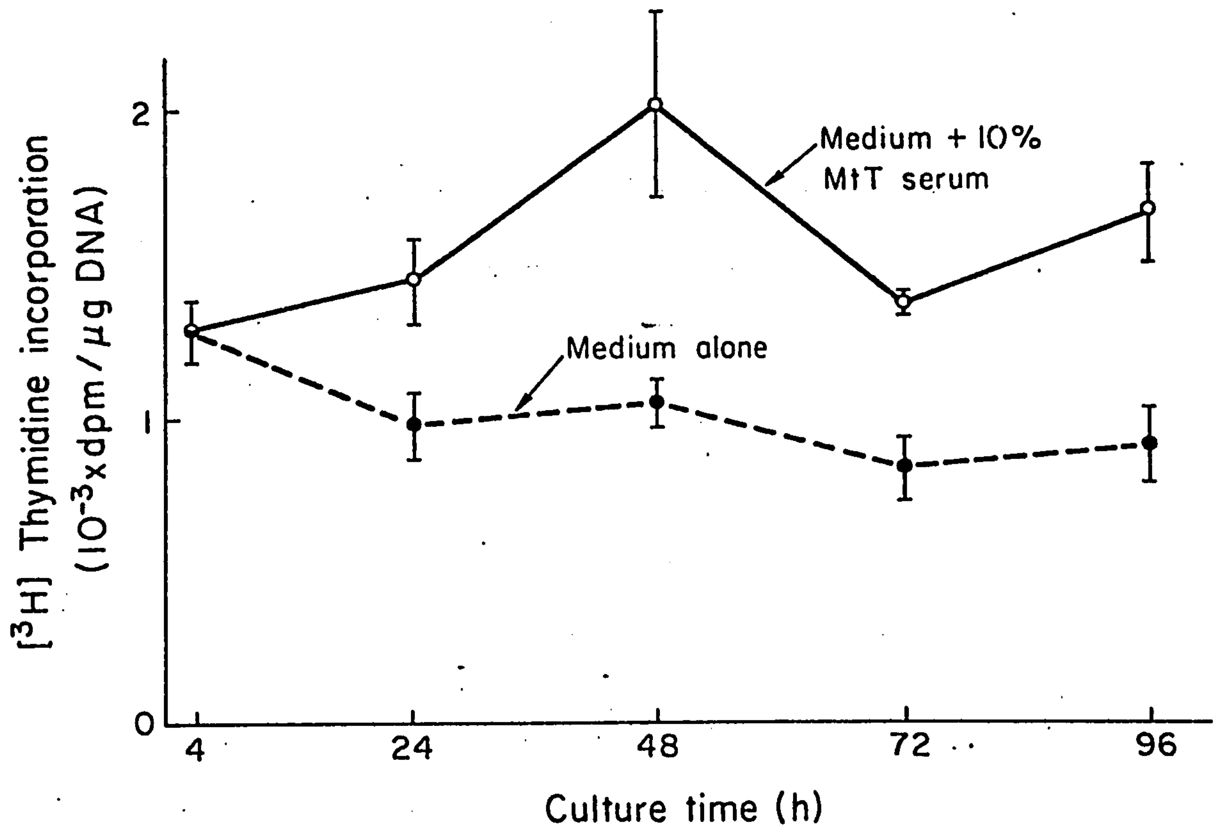

Figure 5. Time-course of DNA synthesis in MTW9 explants during the initial 96 hours in culture. DNA synthesis was measured by the incorporation of (^{3}H)thymidine into DNA as described in Materials and Methods. Cultures were incubated in culture medium alone or in culture medium containing 10% MtT serum. Each point represents the mean ± S.E. (n=10).

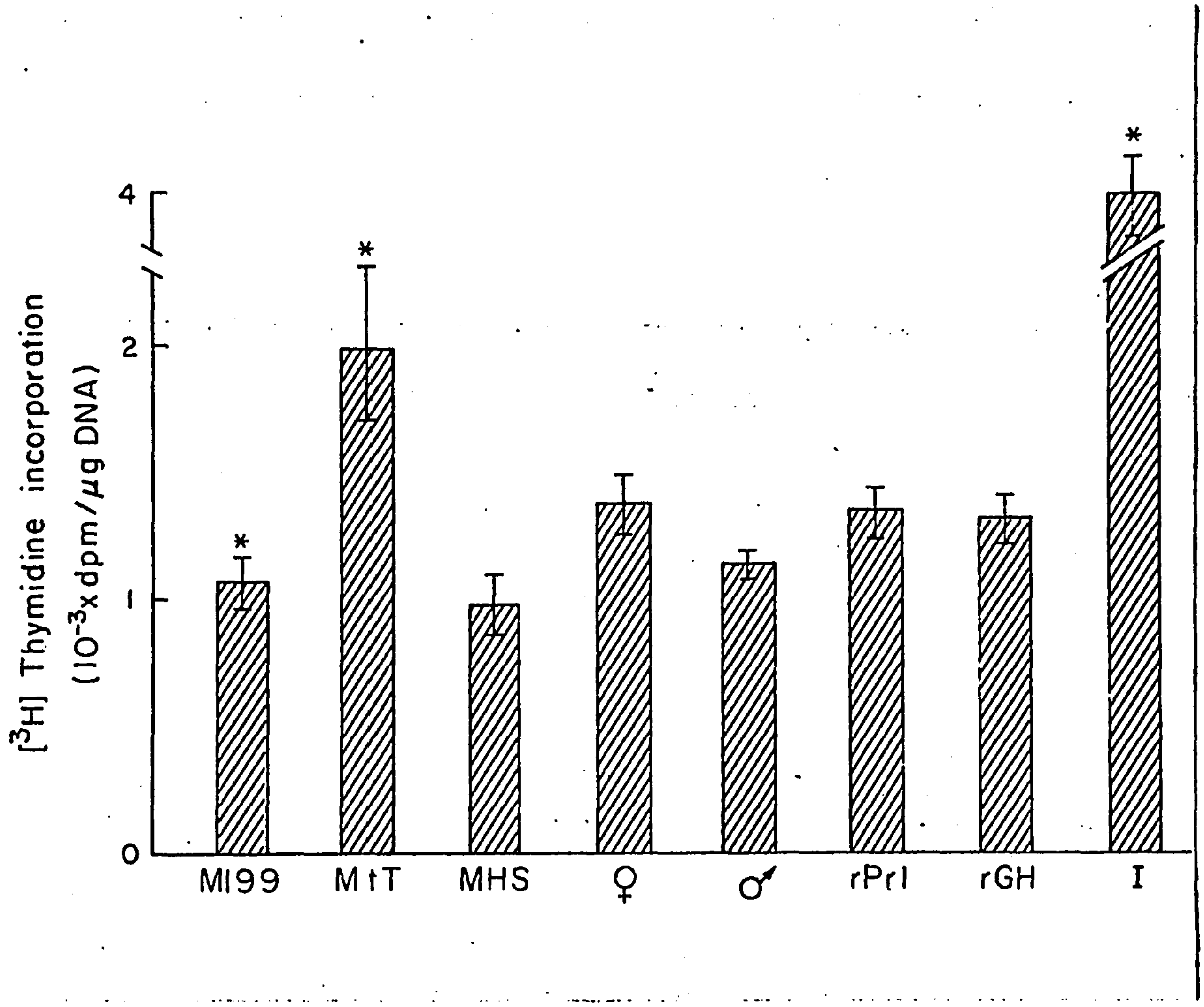

Figure 6. Effects of various hormones and sera on DNA synthesis in organ-cultured MTW9. DNA synthesis was measured by the incorporation of (^{3}H)thymidine into DNA during 44-48 hours of incubation as described in Materials and Methods. The indicated components were present in the medium from the beginning of the incubation period. M199, Medium 199; MtT, serum from MtTW10-bearing animals (10%); MHS, MtT hormone simulant (10%); ♀ , female rat serum (10%); ♂ , male rat serum (10%); rPrl, rat prolactin (5 µg/ml); rGH, rat growth hormone (5 µg/ml); I, insulin (10 µg/ml). Statistical analysis was performed using Student's t test. *, significantly different from control value ($p < 0.01$). Each value is the mean ± S.E. (n=5).

with either prolactin, ovine prolactin, rat growth hormone, or MHS also failed to significantly affect the rate of DNA synthesis observed with serum alone.

Effect of MtT Serum on the Growth of MCF-7 Cells

For this study I decided that the optimum method for assessing growth would be to measure increases in cell number over several generations. However, clumping of MCF-7 cells after detachment from culture dishes prevented direct cell counting. Hence, I developed the technique of counting nuclei described in Materials and Methods. This method proved to be very reliable, and a comparison study showed that attempts at counting cells using a hemocytometer significantly underestimated the number of cells as determined by counting nuclei.

In view of the unique endocrine composition and growth-promoting activity of MtT serum (Hollander, 1978), I explored possible effects of MtT serum on the growth of human mammary cancer cells. Since rat serum concentrations greater than 5% caused considerable variability in growth rates, only concentrations below 5% were employed in this study.

Figure 7 shows that MCF-7 cells were especially sensitive to low serum concentration, and that maximal increases

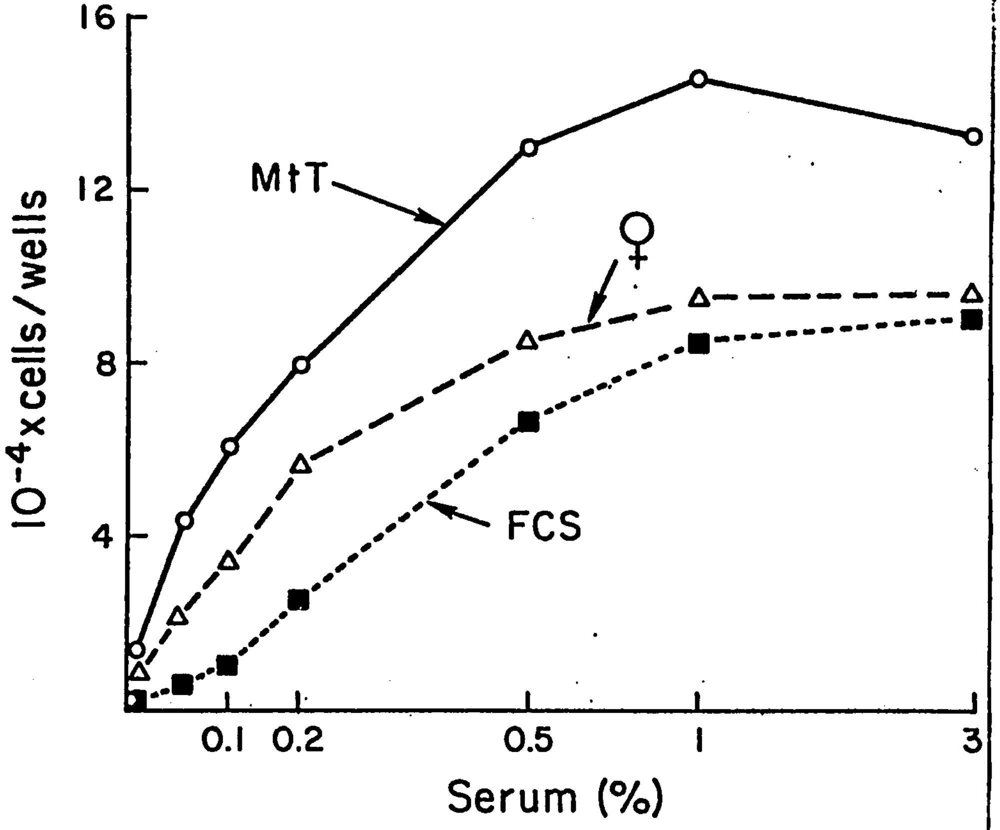

Figure 7. Effects of various sera on the growth of MCF-7 cells. Cells were cultured and cell proliferation was measured as described in Materials and Methods. For each experimental condition the medium consisted of SMEM containing the indicated amount of serum. MtT, serum from MtTW10-bearing rats; ♀ , serum from normal female rats; FCS, fetal calf serum. Each point represents the mean of triplicate determinations and S.E.'s were less than 15%.

in cell number were produced at a concentration of 1%. MtT serum was significantly more effective than FCS in promoting cell proliferation, and this effect was most pronounced at very low concentrations in the range of 0.01 - 0.20%. MtT serum was generally 50% more effective than normal female rat serum in stimulating cell growth.

Both prolactin (Shafie, 1977) and estrogen (Lippman, 1976b) have been reported to stimulate MCF-7 cell growth. However, in this study neither prolactin alone nor MHS (a combination of hormones known to be present in MtT serum, which included 11 pg/ml estradiol) augmented cell growth when combined with 0.2% FCS (Figure 8).

Effects of cAMP Phosphodiesterase Inhibitors on Tumor DNA Synthesis

Addition of the potent cAMP phosphodiesterase inhibitor, MIX, to organ cultures after 24 hours of incubation produced a marked suppression of thymidine incorporation into MTW9 explants (Figure 9). This effect was dose-dependent with 50% inhibition occurring at approximately 0.08 mM MIX. The maximal effect of 1 mM MIX was observed after 24 hours.

While less active than MIX, other methylxanthine phosphodiesterase inhibitors, oxtriphylline, aminophylline, and

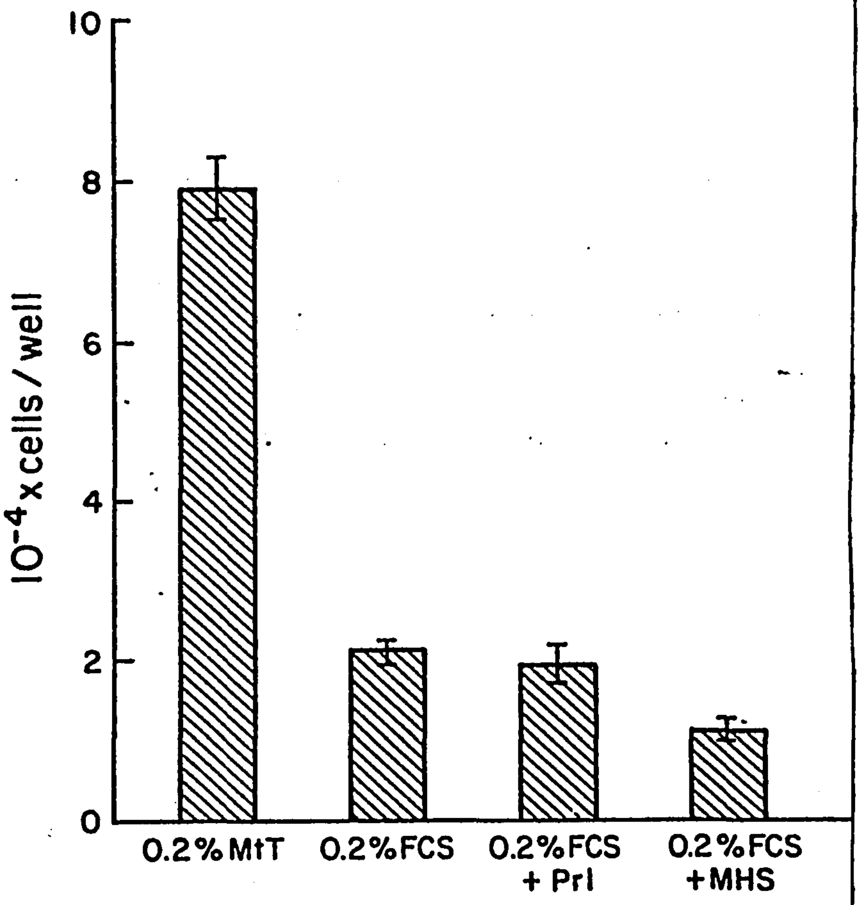

Figure 8. Comparison of the effects of MtT serum, FCS, prolactin, and MHS on MCF-7 cell growth. Cells were cultured and cell proliferation was measured as described in Materials and Methods. For each experimental condition the medium consisted of SMEM containing the indicated amount of serum and hormones. MtT, serum from MtTW10-bearing female rats; FCS, fetal calf serum; Prl, rat prolactin (1 μg/ml); MHS, MtT hormone simulant (10%). Each value is the mean ± S.E. of triplicate determinations.

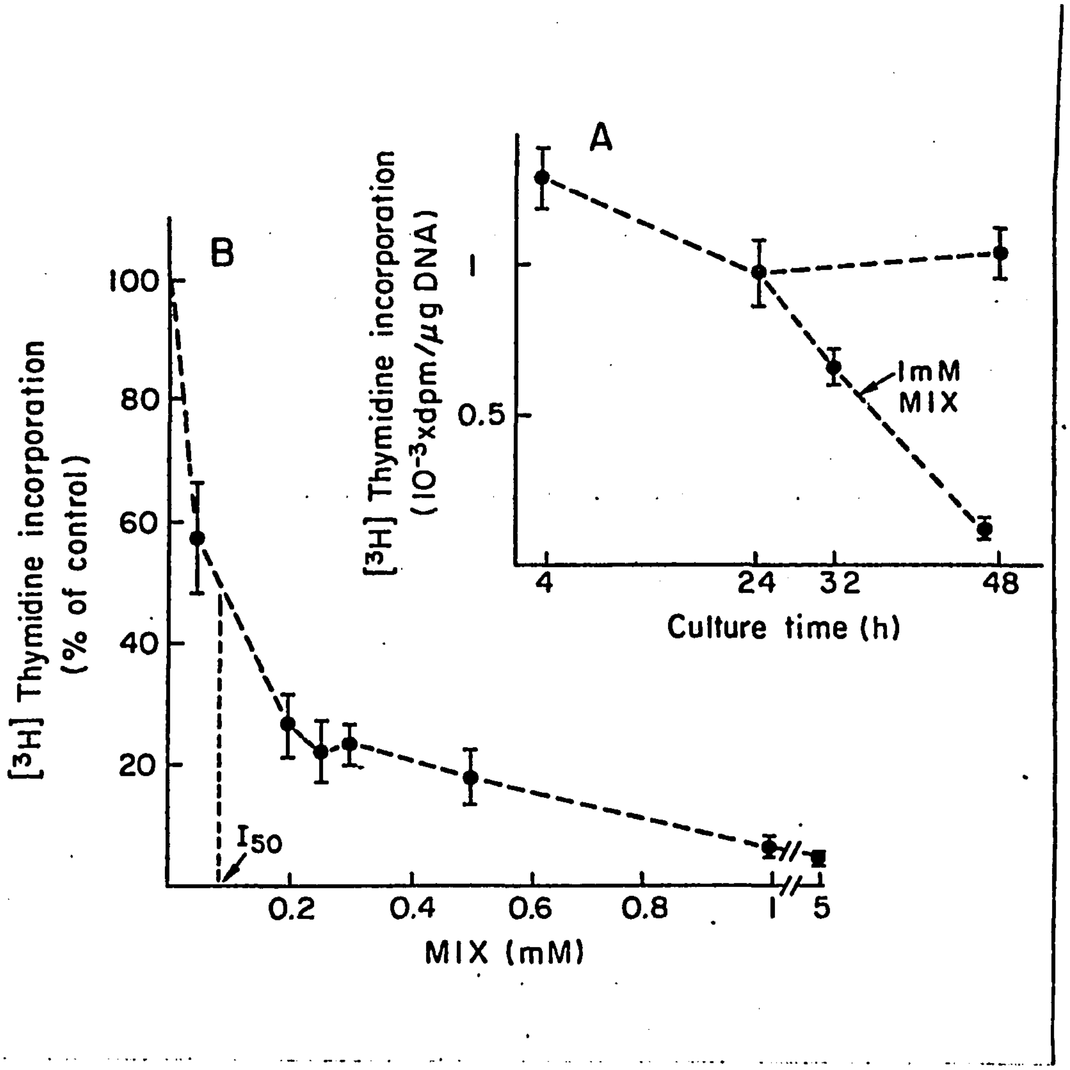

Figure 9. (A). Time-course of the inhibition of DNA synthesis in MTW9 explants by 1-methyl-3-isobutylxanthine (MIX). Tumor explants were incubated for 24 hours in Medium 199 alone. After this initial period, 1 mM MIX was added to the culture medium. DNA synthesis was measured by the incorporation of (^{3}H)thymidine into DNA at the indicated time points as described in Materials and Methods. Each point represents the mean ± S.E. (n=3). (B). Dose-response curve of the inhibition of DNA synthesis in MTW9 explants by MIX. After an initial incubation for 24 hours in Medium 199 alone, cultures were exposed to the indicated concentration of MIX for an additional 24-hour period. DNA synthesis was measured by the incorporation of (^{3}H)thymidine into DNA during 44-48 hours of incubation as described in Materials and Methods. I50, concentration of MIX causing 50% inhibition of (^{3}H)thymidine incorporation. Each point represents the mean ± S.E. (n=4).

theophylline, gave qualitatively similar results (Figure 10). The non-methylxanthine cAMP phosphodiesterase inhibitor, papaverine, had greater potency in suppressing thymidine incorporation. However, direct addition of 1 mM cAMP or of 1 mM DBcAMP (a lipid-soluble analog of cAMP) caused only modest decreases in DNA synthesis.

Inhibition of MCF-7 Cell Growth by cAMP and cAMP Phosphodiesterase Inhibitors

The effects of cAMP phosphodiesterase inhibitors observed in the previous experiments were further evaluated using human mammary cancer cells in culture. These agents were also found to be effective inhibitors of growth in MCF-7 cells. MIX suppressed cell proliferation in a dose-dependent manner, with 50% inhibition occurring at a concentration of approximately 0.5 mM (Figure 11). Other methylxanthine cAMP phosphodiesterase inhibitors, oxtriphylline, aminophylline, and theophylline, and the non-methylxanthine cAMP phosphodiesterase inhibitor, papaverine, were also effective in suppressing cell growth (Figure 12). Although the addition of 1 mM cAMP to the culture medium prevented cell growth, 1 mM DBcAMP did not significantly influence the growth rate when added alone. However, the combination of 1 mM DBcAMP with a very low concentration of

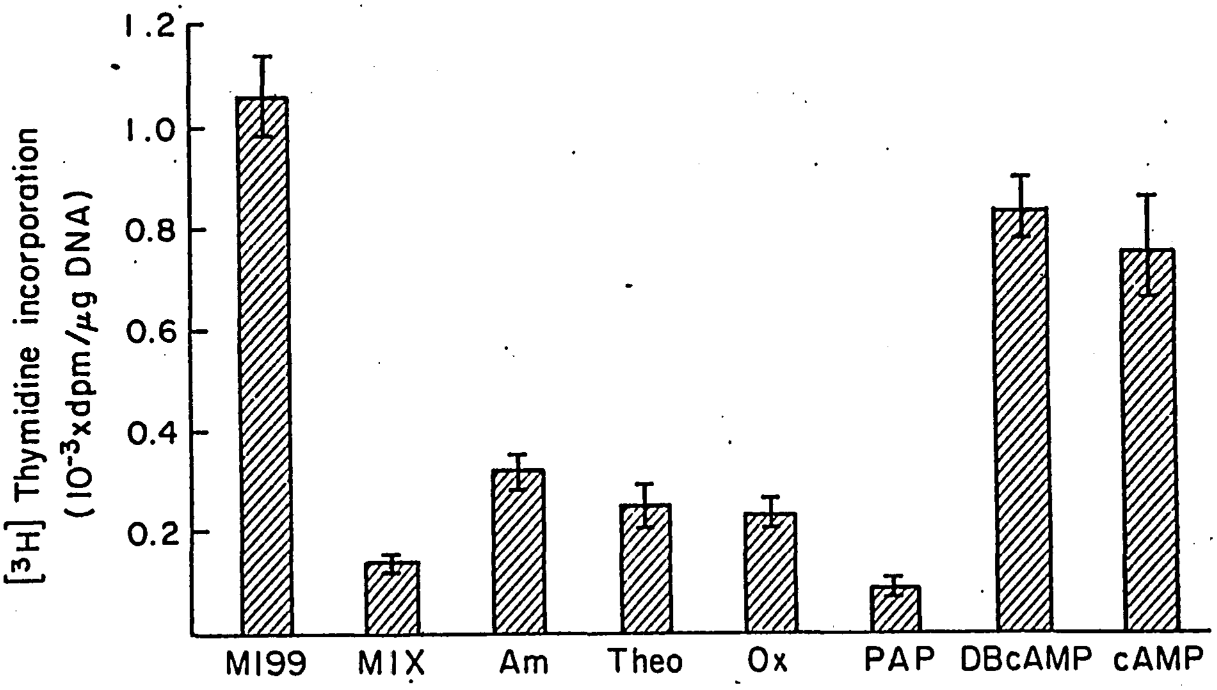

Figure 10. Effects of cAMP, DBcAMP, and cAMP phosphodiesterase inhibitors on DNA synthesis in organ-cultured MTW9. Tumor explants were incubated for 24 hours in Medium 199 alone. After this initial period the indicated components were added at a concentration of 1 mM, and DNA synthesis was measured after 48 hours as described in Materials and Methods. M199, Medium 199; MIX, 1-methyl-3-isobutylxanthine; Am, aminophylline; Theo, theophylline; PAP, papaverine. Each value is the mean ± S.E. (n=5).

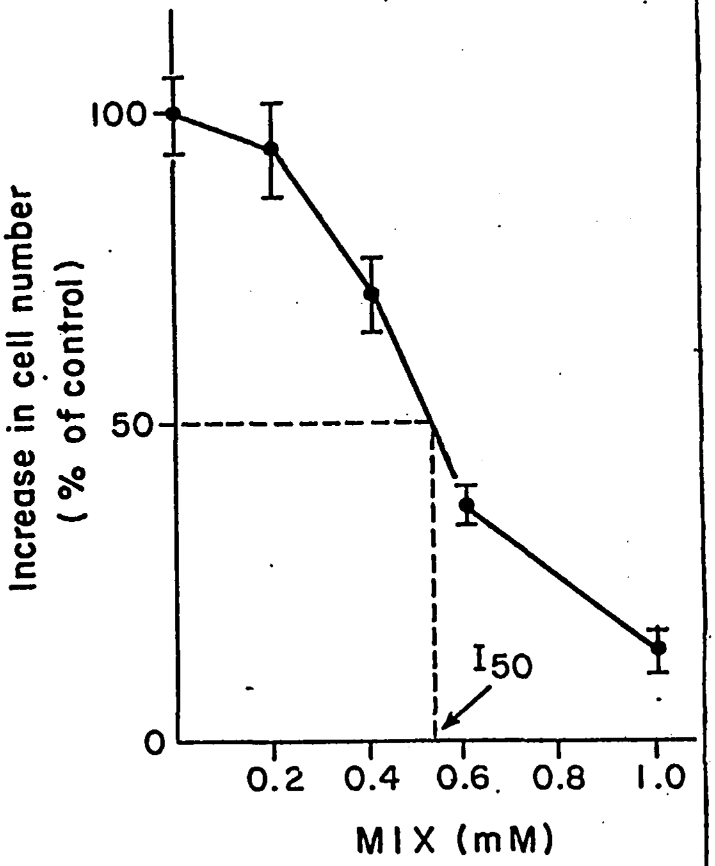

Figure 11. Inhibition of MCF-7 cell growth by 1-methyl-3-isobutylxanthine (MIX). Cells were cultured and cell proliferation was measured as described in Materials and Methods. For each experimental condition the medium consisted of SMEM containing 1% FCS and the indicated amount of MIX. Each point represents the mean ± S.E. of triplicate determinations. I_{50}, concentration of MIX resulting in 50% inhibition of cell growth.

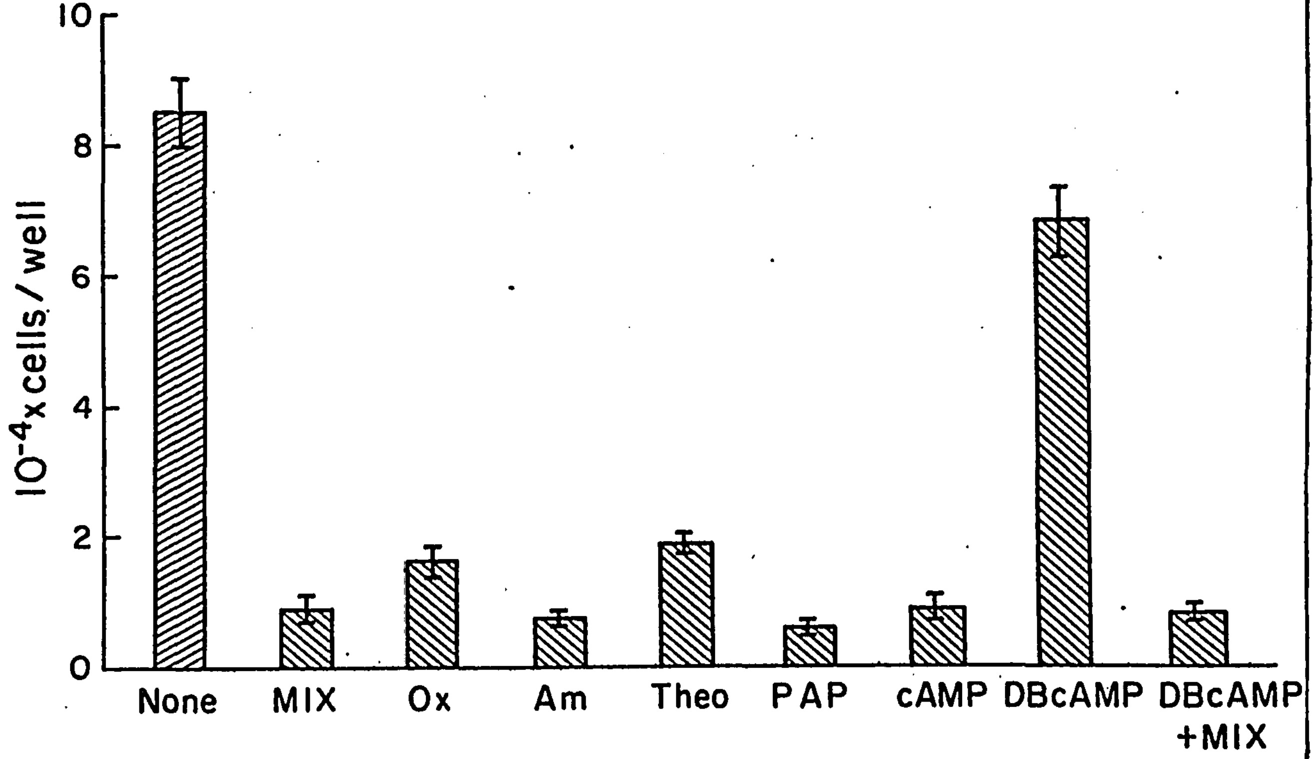

Figure 12. Inhibition of MCF-7 cell growth by cAMP and cAMP phosphodiesterase inhibitors. Cells were cultured and cell proliferation was measured as described in Materials and Methods. For each experimental condition the medium consisted of SMEM containing 1% FCS and the indicated component(s). MIX, 1 mM MIX; Ox, 1 mM oxtriphylline; Am, 1 mM aminophylline; Theo, 1 mM theophylline; Pap, 0.1 mM papaverine; cAMP, 1 mM cAMP; DBcAMP, 1 mM DBcAMP; DBcAMP + MIX, 1 mM DBcAMP + 0.1 mM MIX. Each value is the mean ± S.E. of triplicate determinations.

MIX (0.1 mM) was unexpectedly effective in suppressing cell proliferation.

Reversal of MIX-Inhibited DNA Synthesis in MTW9 by MtT Serum

Since hormone-dependent mammary tumor growth may be at least partially regulated by an endocrine-cAMP antagonism (Bodwin, 1978; Shafie, 1979), various hormones and rat sera were tested for their ability to block the inhibitory action of MIX on tumor DNA synthesis. The addition of MtT serum to the incubation medium was found to substantially antagonize the inhibitory action of MIX on tumor DNA synthesis (Figure 13). By contrast, male and female rat sera, MHS, and rat prolactin elicited only modest increases in nucleotide incorporation in the presence of MIX. The combination of MHS with female rat serum did not significantly alter the effect of either component alone. Likewise, in the presence of 1 mM MIX, insulin stimulation of DNA synthesis was only 15% of its stimulation without MIX.

Reversal of MIX-Inhibited MCF-7 Cell Growth by MtT Serum

Evidence from other laboratories suggests that cAMP and growth-stimulating hormones may oppose each other in

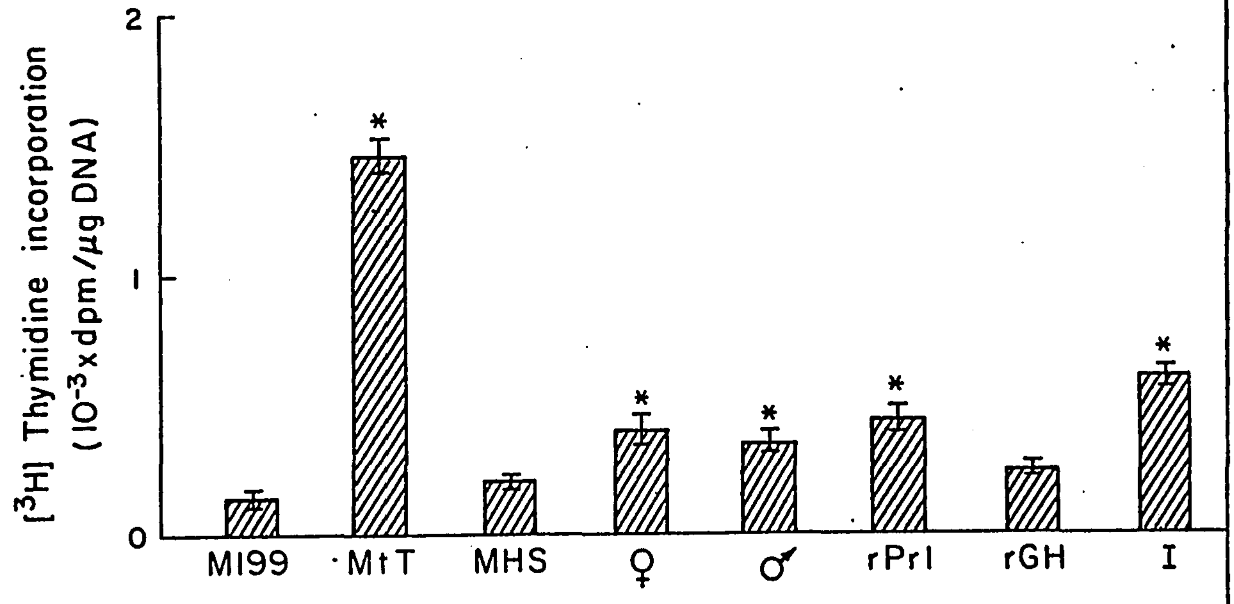

Figure 13. Effects of various hormones and sera on DNA synthesis in organ-cultured MTW9 in the presence of MIX. The indicated components were present in the medium from the beginning of the culture period. 1 mM MIX was added at 24 hours and DNA synthesis was measured after 48 hours as described in Materials and Methods. Abbreviations, symbols, and concentrations are described in Figure 6. Statistical analysis was performed using Student's t test. *, significantly different from control value ($p < 0.01$). Each value is the mean ± S.E. (n=5).

the control of mammary tumor growth (Shafie, 1979; Bodwin, 1978). The ability of various sera to antagonize the inhibitory action of MIX on MCF-7 cell growth was examined by increasing the concentration of sera in cell cultures (Figure 14). Elevated concentrations of FCS and of normal female rat serum elicited only modest increases in cell growth, whereas 3% MtT serum substantially antagonized the inhibitory action of 1 mM MIX. A combination of hormones simulating the known endocrine components of MtT serum (MHS) combined with 0.2% FCS also failed to significantly reverse growth inhibition by 1 mM MIX.

To help resolve the question of whether MtT serum was directly interfering with the action of MIX or with that of DBcAMP, MtT serum was added to cultures containing 1 mM DBcAMP together with a very low concentration of MIX (Figure 15). Elevated concentrations of MtT serum were ineffective in preventing the inhibitory effects of this preparation. Likewise, MtT serum was also unable to counteract the suppression of cell growth by cAMP.

Decreased Sensitivity to MIX in MTW9B

The inhibitory actions of methylxanthines on tumor explant DNA synthesis were compared in MTW9 and its autonomous subline, MTW9B. Dose-response curves show that

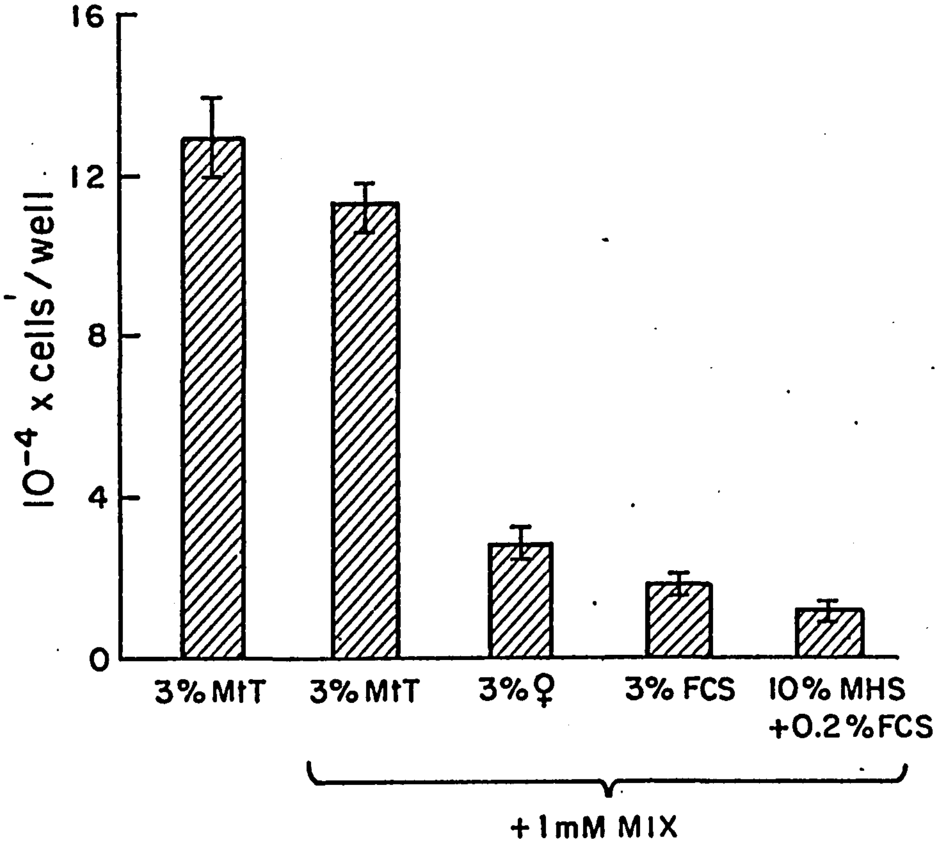

Figure 14. Reversal of MIX-inhibited MCF-7 cell growth by MtT serum. Cells were cultured and cell proliferation was measured as described in Materials and Methods. For each experimental condition the medium consisted of SMEM containing the indicated component. MtT, MtT serum; ♀, normal female rat serum; FCS, fetal calf serum; MHS, MtT hormone simulant. Each value is the mean ± S.E. of triplicate determinations.

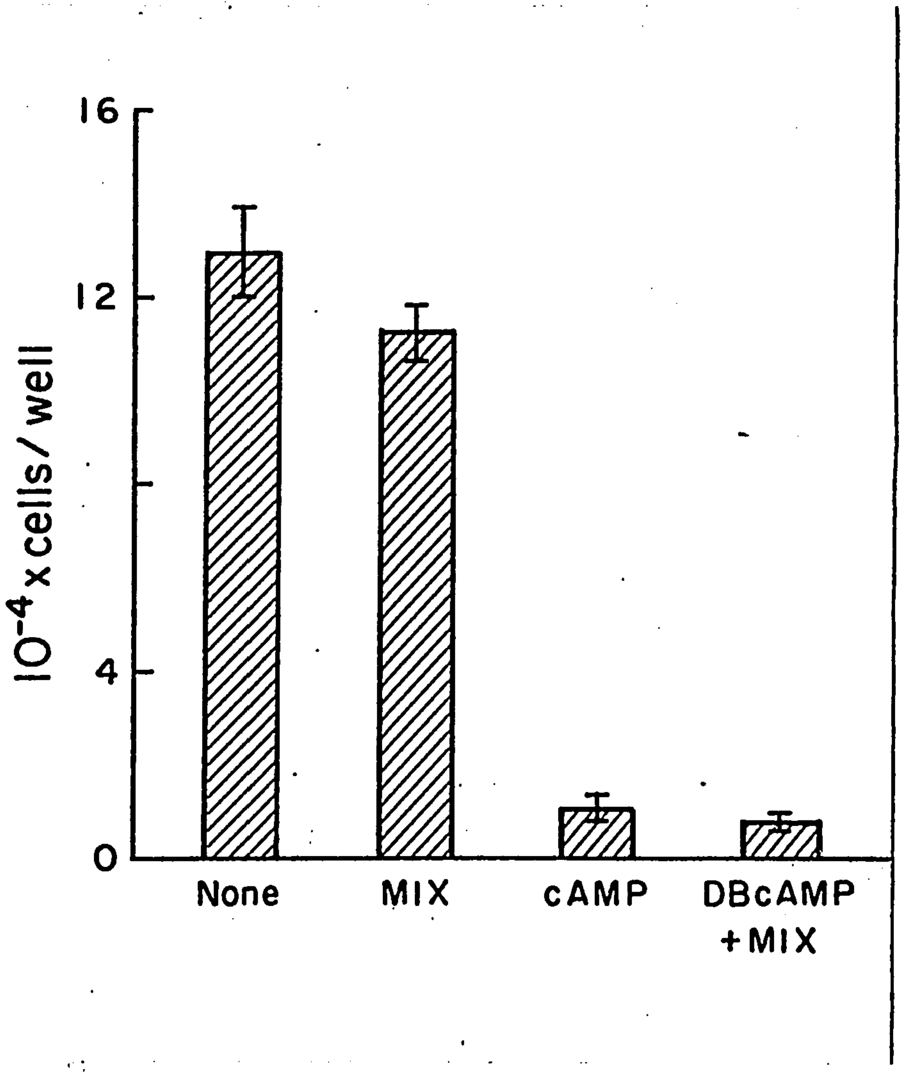

Figure 15. Inability of MtT serum to reverse the inhibition of MCF-7 cell growth by cAMP or by DBcAMP + MIX. Cells were cultured and cell proliferation was measured as described in Materials and Methods. For each experimental condition the medium consisted of SMEM containing 3% MtT serum and the indicated component(s). MIX, 1 mM MIX; cAMP, 1 mM cAMP; DBcAMP + MIX, 1 mM DBcAMP + 0.1 mM MIX. Each value is the mean ± S.E. of triplicate determinations.

MTW9 is markedly more sensitive to the action of MIX (Figure 16). Notably, DNA synthesis in MTW9 explants was suppressed by more than 70% at 0.2 mM MIX, whereas this concentration of phosphodiesterase inhibitor did not significantly affect (^{3}H)thymidine incorporation into MTW9B explants. However, very high levels of MIX (5 mM) were able to fully suppress DNA synthesis in MTW9B. The concentrations of MIX required to achieve 50% inhibition of DNA synthesis (I_{50}) in MTW9 and MTW9B were .08 and .73 mM, respectively. Similar patterns of inhibition were seen using the other methylxanthine cAMP phosphodiesterase inhibitors, aminophylline and oxtriphylline.

Diminished cAMP Phosphodiesterase Activity in MTW9B

Alterations in cAMP phosphodiesterase activity have been found to be correlated with changes in the growth status and cAMP-sensitivity of several tissues (see Introduction). Since MTW9 was much more sensitive than MTW9B to the inhibitory action of MIX (Figure 16), and it is probable that MIX exerts its effects through cellular phosphodiesterases, it was important to compare phosphodiesterase activity in MTW9 and MTW9B.

Biphasic kinetics were observed when cAMP phosphodiesterase activity in MTW9 homogenates was subjected to

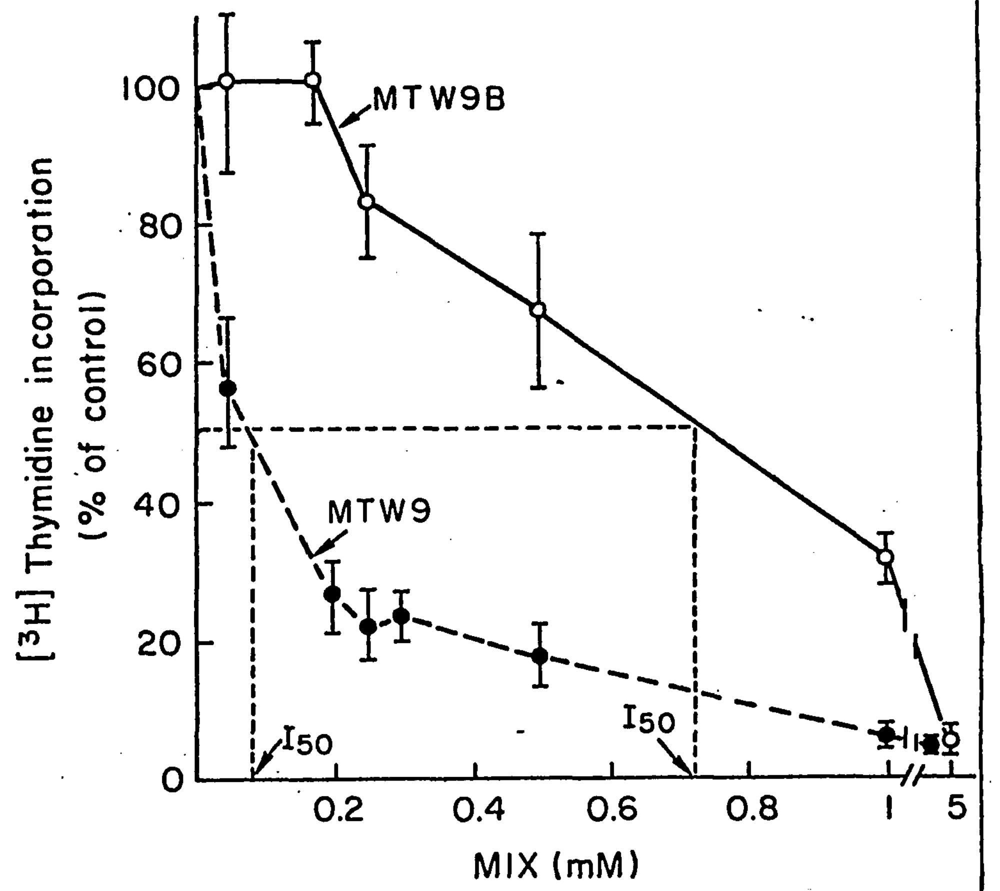

Figure 16. Comparison of the sensitivities of MTW9 and MTW9B to the inhibitory action of MIX. Experimental procedures identical to those described in Figure 9(B).

double-reciprocal analyses (Figures 17 and 18). Apparent Michaelis constants (Km's) and apparent maximum velocities (Vmax's) were determined from the linear portions of the plots (Lineweaver and Burk, 1934). Although other interpretations are possible, e.g., positive cooperativity, these kinetic patterns are most probably due to the presence of two major enzymatic forms, a low-Km and a high-Km enzyme, which have been observed in several other tissues (Thompson, 1978; Levin, 1978). In contrast, kinetic analysis of phosphodiesterase activity in MTW9B homogenates reveals the presence of only a single, low-Km enzyme (Figure 19).

Comparisons of cAMP phosphodiesterase kinetics in MTW9 and MTW9B homogenates reveal several important differences (Table 3). Whereas the high-Km enzyme is not detectable in the autonomous tumor, MTW9B, there is a substantial concentration of this form in the hormone-dependent tumor, MTW9. Also, the low-Km enzyme has significantly enhanced tissue concentration and greater affinity for cAMP in MTW9.

When MTW9 and MTW9B homogenates were mixed together for 1 hour at 22°C, no significant change from the expected additive values in phosphodiesterase activity was observed (data not shown). Thus, it is improbable that a readily

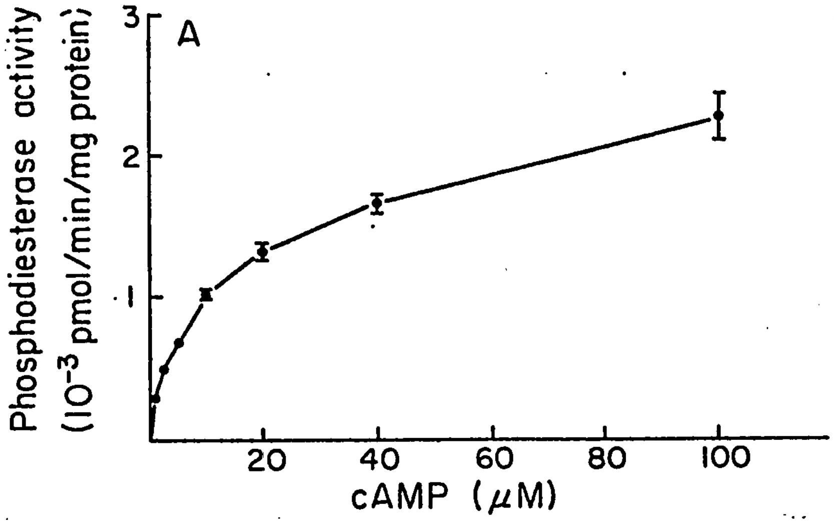

Figure 17. Hydrolysis of (^{3}H)cAMP by MTW9 as a function of substrate concentration. Enzyme activity was measured immediately after the preparation of tumor homogenates as described in Materials and Methods using substrate concentrations in the range, 1 - 100 μM. Results are expressed as the means ± S.E. for one experiment and equivalent results were obtained in three separate experiments.

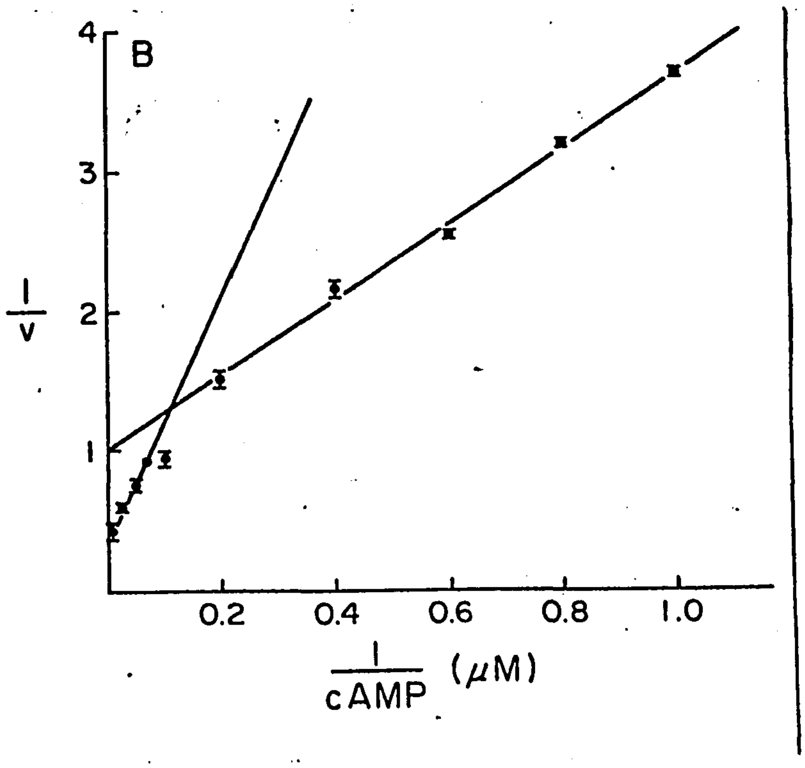

Figure 18. Kinetic analysis of (^{3}H)cAMP hydrolysis by MTW9. Enzyme activity was measured as described in Figure 17. Velocity (V) is expressed as (pmol/min/mg protein) x 10^{-3}. Apparent Michaelis constants (Km's) and apparent maximum velocities (Vmax's) were determined from the linear portions of the plots by regression analysis. Results are expressed as the means ± S.E. for one experiment and equivalent results were obtained in three separate experiments.

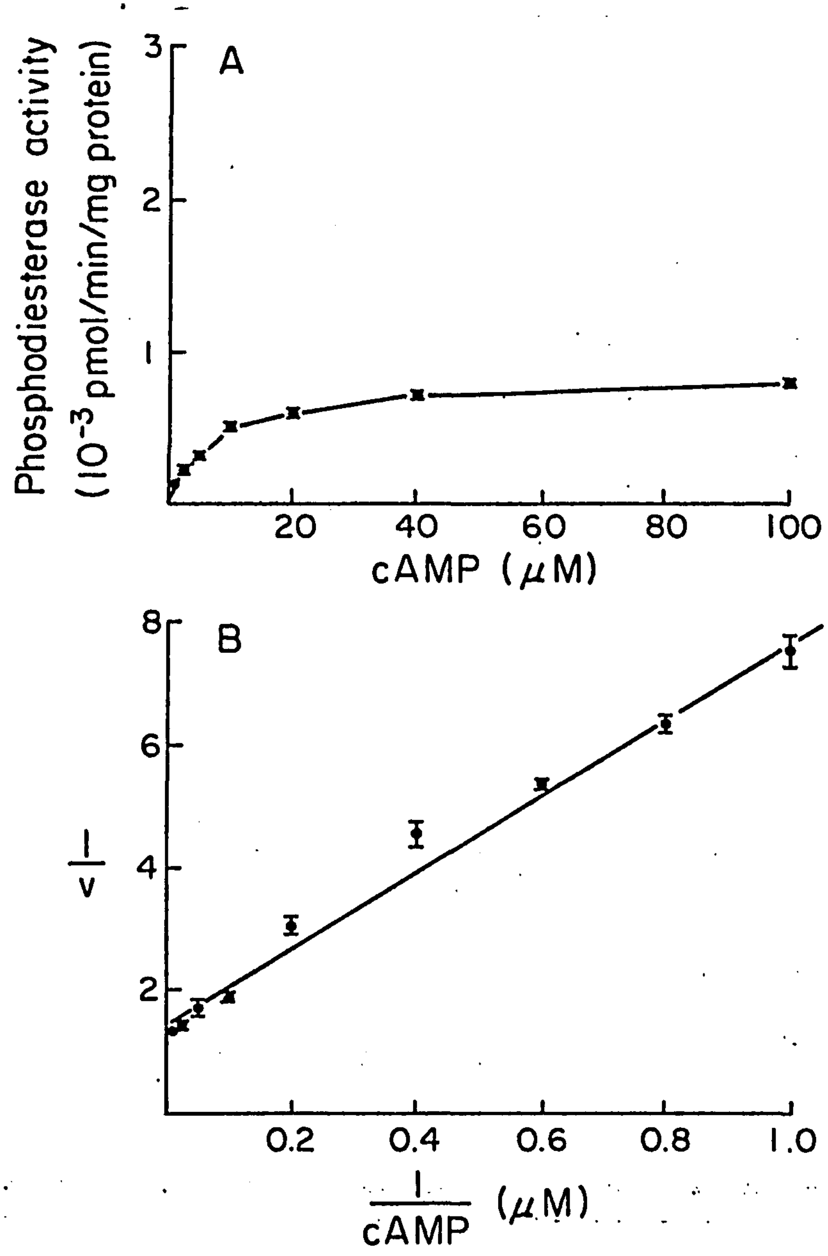

Figure 19. (A). Hydrolysis of (^{3}H)cAMP by MTW9B as a function of substrate concentration. Experimental details identical to those described in Figure 17. (B). Kinetic analysis of (^{3}H)cAMP hydrolysis by MTW9B. Experimental details identical to those described in Figure 18.

TABLE 3

Kinetic parameters for cAMP hydrolysis by tumor homogenates

cAMP phosphodiesterase activity was determined in tumor homogenates as described in Figure 17. Kinetic parameters were determined by Lineweaver-Burk analysis. The respective units are: Km, μM cAMP; Vmax, pmol/min/mg protein. ND, not detectable.

	cAMP phosphodiesterases			
	Low-Km enzyme		High-Km enzyme	
Tumor	Km	Vmax	Km	Vmax
MTW9	2.5[a]	970	22	2485
MTW9B	4.5[b]	640[b]	ND	ND

[a] Each value represents the mean of three determinations and S.E.'s were less than 15%.

[b] Significantly different from corresponding MTW9 values ($p < 0.01$).

diffusible factor, either stimulatory or inhibitory, accounts for the differences in cAMP phosphodiesterase activities between these two tumors.

Altered cAMP-Binding and cAMP-Dependent Protein Kinase Activities in MTW9B

Approximately 80% of the cAMP-dependent protein kinase activity in MTW9 was associated with the cytosol fraction (Figure 20). The activation ratio ranged from .2 to .3 in growing tumors. A substantial amount of the total activity (15%) is associated with the particulate fraction, and membrane solubilization is required to detect most of that activity. Further study could distinguish between endogenously bound kinases and any activity which may have become trapped during fractionation. A small percentage of the total protein kinase activity (5%) was found in the nuclear fraction, was not enhanced by treatment with detergent, and was largely solubilized under physiological salt conditions.

Casein has been widely used as a substrate for the study of cAMP-independent protein kinase activity, and casein phosphorylation in the presence of subcellular fractions of MTW9 was similarly independent of cAMP. Subcellular distribution of casein kinase activity was similar

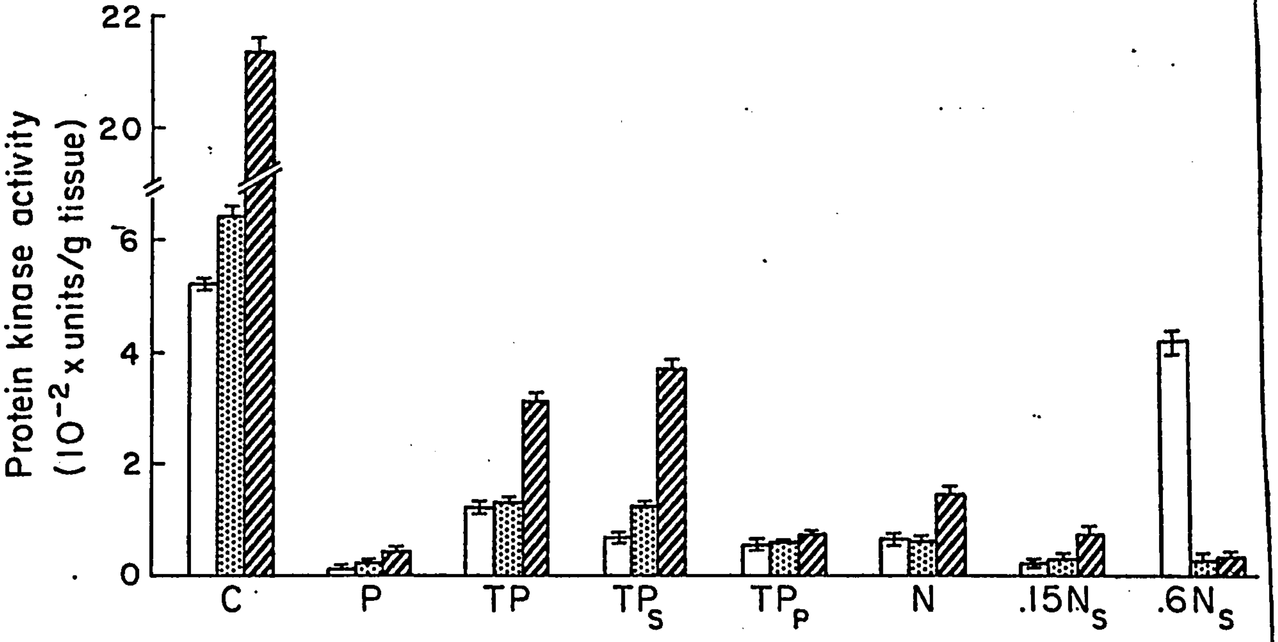

Figure 20. Distribution of protein kinases in subcellular fractions of MTW9. Subcellular fractionation and the measurement of enzyme activity were performed as described in Materials and Methods except kinase activity was determined using casein as substrate (□) or histone as substrate in the presence (▒) or absence (▨) of 2.5 μM cAMP. The fractions are: C, 100,000 x g cytosol; P, crude particulate fraction; TP, P + 0.2% Triton X-100; TP$_s$, 30,000 x g supernatant fraction from TP; TP$_p$, 30,000 x g pellet from TP resuspended in 10 volumes homogenization buffer; N, purified nuclear fraction; .15 N$_s$, saline nuclear extract; .6 N$_s$, 0.6 M NaCl chromatin extract. Results are expressed as means ± S.E. (n=3).

to that of histone kinase, with the exception that high salt extraction (.6 M) of the .15 M NaCl-extracted nuclear preparation unmasked greater nuclear casein kinase activity, and quantitatively separated this activity from that of the histone kinases.

Anion-exchange chromatography of MTW9 cytosol preparations resolved cAMP-dependent protein kinase activity into the two major apparent molecular forms, Types I and II (Figure 21), which have been identified in several other tissues. These activities elute at approximately 0.1 M and 0.2 M potassium phosphate, respectively, and each coincides with a peak of high-affinity cAMP-binding. Type II activity was enhanced 100% in MTW9B. This increase was most probably due to an elevation in holoenzyme since the activity was highly dependent upon cAMP, and there was a parallel increment in cAMP binding. A different elution pattern was observed when MTW9B cytosol preparations were chromatographed. Since other investigators have reported similar changes associated with developing and neoplastic tissues (Lee, 1975; Kuo, 1975), it is important to note the change in PKII/PKI ratio between MTW9 (0.87) and MTW9B (1.92).

Kinetic analysis of cAMP-binding by the method of Scatchard (1974) and protein kinase activity by the method

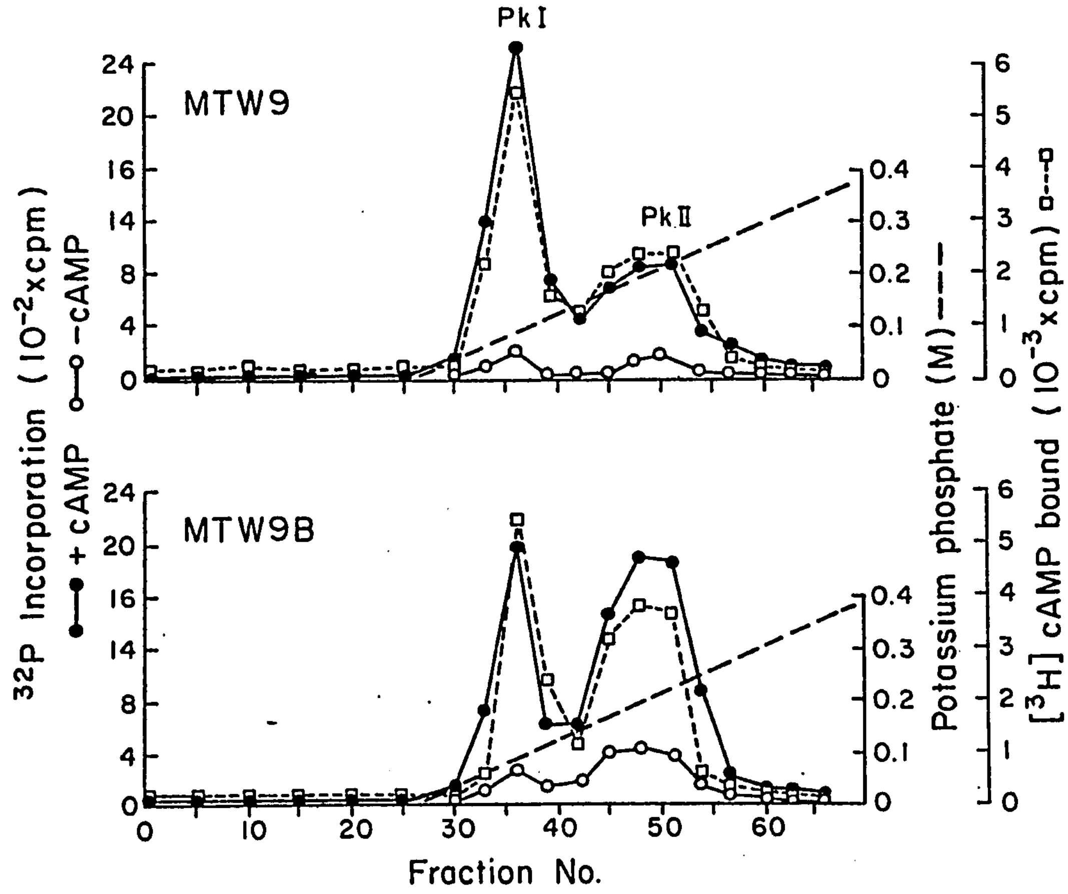

Figure 21. Comparison of anion-exchange elution patterns of protein kinase and cAMP-binding activities in MTW9 and MTW9B. Tumor cytosols were prepared and chromatographed as described in Materials and Methods. Aliquots of 10 µl were assayed for protein kinase activity in the presence (—●—) and absence (—○—) of 2.5 µM cAMP using histone as substrate. Aliquots of 50 µl were assayed for cAMP-binding activity (--□--). Representative elution patterns from individual experiments are shown and equivalent results were obtained in at least three separate experiments.

of Lineweaver and Burk (1934) revealed several additional differences between MTW9 and MTW9B (Tables 4 and 5). The total amount of cAMP-binding and cAMP-dependent protein kinase activity in MTW9B were higher by 20% and 40%, respectively. However, the apparent cAMP-binding affinity associated with PKII was 100% lower and histone kinase affinity was significantly decreased in MTW9B. As has been generally found in other tissues (Rubin, 1975), histone kinase affinities for both major enzyme forms were similar.

To examine whether the decrease in sensitivity to MIX in MTW9B might be attributable to a defect in the functioning of the cAMP system, the ability of MIX to rapidly activate cAMP-dependent protein kinases was compared in MTW9 and MTW9B. Addition of 1 mM MIX to MTW9 and MTW9B explants after 24 hours in culture promoted a dose-dependent activation of cAMP-dependent protein kinases within 1 hour (Table 6). However, the pattern of activation was similar in both tumors under the conditions of this experiment.

Since previous results had shown that MtT serum effectively opposed the inhibitory action of MIX on MTW9 explants (Figure 13) and on MCF-7 cells (Figure 14), the possible influence of this serum on the activation of protein kinases in MTW9 was explored. It was observed that inclusion of 10% MtT serum in the incubation medium from the

TABLE 4

Comparison of cAMP-binding and protein kinase activities in MTW9 and MTW9B

The preparation of tumor cytosols and measurement of cAMP-binding and protein kinase activities were performed as described in Materials and Methods.

Tumor	cAMP-binding (pmol/mg protein)	Protein kinase activity (units/mg protein) (-)cAMP	(+)cAMP
MTW9	15.9 ± 1.2[a]	140 ± 13	451 ± 27
MTW9B	20.4 ± 1.6[b]	155 ± 11	623 ± 32[b]

[a] Each value represents the mean ± S.E. of four determinations.

[b] Significantly greater than corresponding MTW9 value ($p < 0.01$).

TABLE 5

Comparison of apparent cAMP-binding and protein kinase substrate affinities in MTW9 and MTW9B

The preparation of tumor cytosols and measurement of cAMP-binding and protein kinase activities were performed as described in Materials and Methods. cAMP-binding affinities were determined by Scatchard analysis and kinase substrate affinities were calculated using Lineweaver-Burk analysis.

	cAMP-binding affinity Kd (nM cAMP)		Protein kinase substrate affinity Km (μg histone)	
Tumor	PKI	PKII	PKI	PKII
MTW9	5.9[a]	21	28	30
MTW9B	6.2	39[b]	53[b]	56[b]

[a] Each value represents the mean of three determinations.

[b] Significantly greater than corresponding MTW9 value ($p < 0.01$).

TABLE 6

Activation of protein kinases in tumor explants by MIX

Organ cultures of rat mammary tumors, MTW9 and MTW9B, were prepared and maintained as described in Materials and Methods. At 24 hours, the indicated concentrations of MIX were added to the cultures. After 1 hour, cytosols were prepared and protein kinase activity measured as described in Materials and Methods.

		Protein kinase activity (pmol/min/mg protein)		
Tumor	MIX (mM)	(-)cAMP	(+)cAMP	Activation Ratio (-cAMP/+cAMP)
MTW9	0	131 ± 7[a]	506 ± 26	.26
	.25	185 ± 14	476 ± 22	.39
	1	240 ± 21	438 ± 33	.55
MTW9B	0	151 ± 9	601 ± 27	.25
	.25	261 ± 15	544 ± 19	.48
	1	262 ± 21	466 ± 14	.56

[a] Each value represents the mean ± S.E. of three determinations.

beginning of the culture period did not appreciably affect the subsequent activation of protein kinases by MIX under the conditions described in Table 6.

Effect of MtT Resection on the cAMP System in MTW9

Surgical removal (resection) of the supportive mammosomatotropic tumor (MtTW10) in an MTW9-bearing animal results in the rapid regression of MTW9 (Hollander, 1978). Tumor sizes were decreased 20% and tumor DNA synthesis was dramatically reduced by the fourth day following resection (Table 7). Concomitantly, there were substantial changes in the tumor cAMP system. cAMP-binding and cAMP-dependent protein kinase activities in tumor cytosols were reduced 30%. These alterations were most pronounced when compared to the activities observed in the autonomous tumor, MTW9B. cAMP-binding and protein kinase activities were diminished 2-fold in the regressing tumor when compared to MTW9B. Significant changes in cAMP phosphodiesterase activities were also detected in the regressing mammary tumor (Figure 22). Both low- and high-Km enzymes were diminished 40% in the homogenates and 80% in the purified particulate fractions of regressing tumors.

In experiments similar to those described in Table 7, it was observed that (^{3}H)thymidine incorporation into MTW9

TABLE 7

Changes in DNA synthesis, cAMP-binding, and protein kinase activities during MTW9 regression

Four days after MtT resection, when mammary tumors had decreased about 20% in size, they were excised and divided into two portions. One portion was rapidly prepared for organ culture and DNA synthesis was measured during the first four hours in culture as described in Materials and Methods. Cytosol was prepared from the remaining tumor tissue and cAMP-binding and protein kinase activity were measured as described in Materials and Methods.

	DNA synthesis	cAMP-binding	Protein kinase activity (units/mg protein)	
Tumors	(dpm/µg DNA)	(pmol/mg protein)	(-)cAMP	(+)cAMP
MTW9	1058 ± 117[a]	15.9 ± 1.2	140 ± 13	451 ± 27
MTW9 - Resect	94 ± 12[b]	10.8 ± 0.9[b]	137 ± 4	315 ± 13[b]

[a] Each value represents the mean ± S.E. of four determinations.

[b] Significantly less than corresponding MTW9 value ($p < 0.01$).

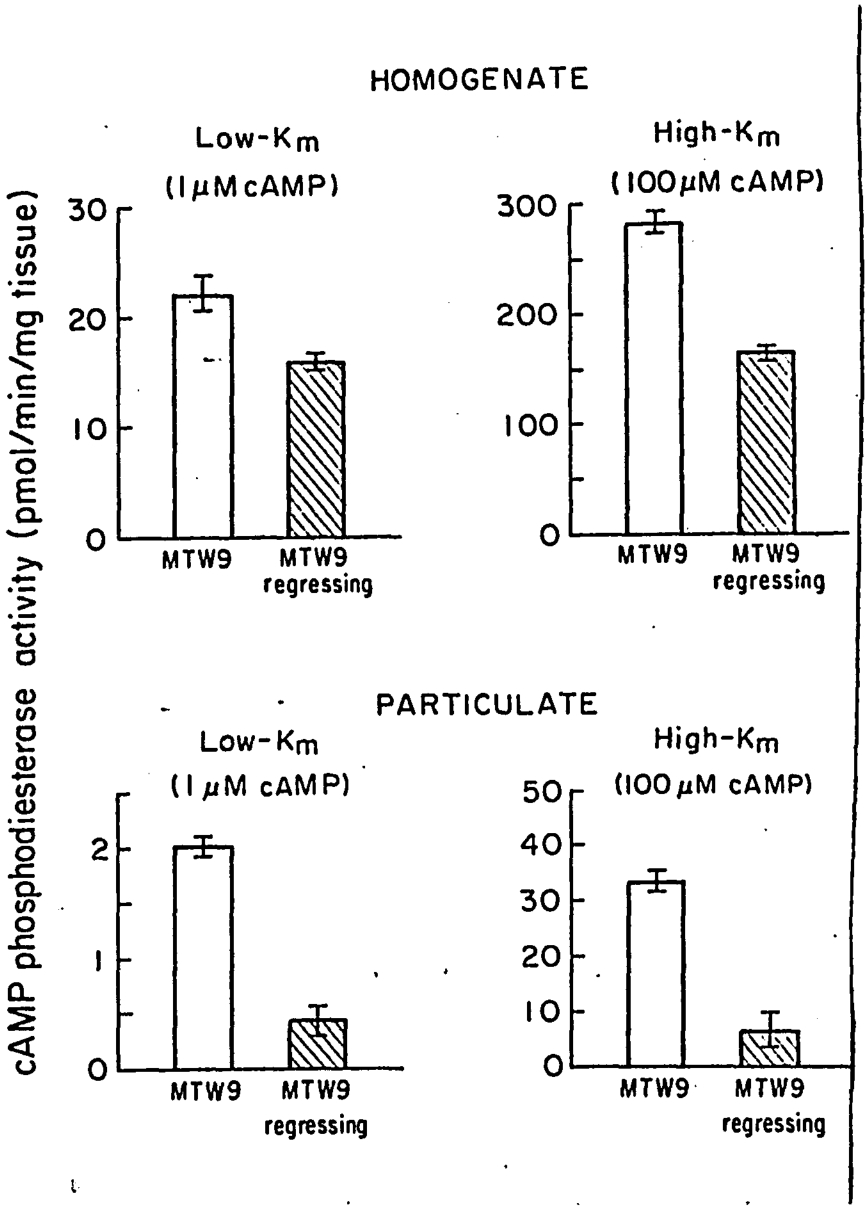

Figure 22. Changes in cAMP phosphodiesterase activities during MTW9 regression. Four days after MtT resection, when mammary tumors had decreased about 20% in size, they were excised and cAMP phosphodiesterase activity was measured in homogenates and purified particulate fractions as described in Materials and Methods. Each value represents the mean ± S.E. (n=4). In all cases values obtained with regressing MTW9 tumors were significantly less than values obtained with growing MTW9 tumors ($p < 0.01$).

explants was significantly decreased 24 hours after the <u>in vivo</u> administration of 10 mg DBcAMP (from 860 to 410 dpm/µg DNA).

DISCUSSION

Effects of Sera and Hormones on DNA Synthesis in MTW9 and on MCF-7 Cell Growth

Establishing an organ culture method which demonstrated reproducible endocrine effects on (^{3}H)thymidine incorporation into DNA proved to be a formidable task. The difficult and time-consuming procedure described in Materials and Methods was developed only after careful evaluation of a variety of techniques. The primary purpose for developing the culture methods used in this study was to examine the direct effects of substances which increase cellular concentrations of cAMP upon the growth of mammary cancers. While optimizing culture conditions, I also evaluated the stimulatory actions of appropriately selected hormones and sera on DNA synthesis in MTW9 explants and on MCF-7 cell growth.

Growth and differentiation of the normal mammary gland are thought to be regulated by complex endocrine interactions involving estrogens, progestins, adrenal cortical hormones, prolactin, insulin (Wood, 1975), and probably additional humoral mediators which remain to be defined (Gospodarowicz, 1976; Sirbasku, 1978; Kano-Sueoka, 1978). Many human and animal mammary tumors exhibit similar hormone dependencies and regress in response to various endocrine manipulations, e.g., ovariectomy (Diamond, 1976), hypophysectomy (Huggins, 1959), anti-hormone treatments (Heuson, 1971), and experimental pancreatic diabetes (Heuson, 1972). However, our knowledge of the endocrine mechanisms which are involved in the control of mammary tumor growth is very limited.

The transplantable rat mammary adenocarcinoma, MTW9, has proven to be a useful tool for the study of tumor hormone dependencies (Hollander, 1978; Diamond, 1979). Growth of this tumor can be stimulated either by coimplantation with a pituitary mammosomatotropic tumor, MtTW10, or by chronic administration of perphenazine, a dopamine antagonist which promotes host secretion of prolactin.

This is the first study to my knowledge of direct hormonal effects on DNA synthesis in MTW9. MtT serum and insulin have previously been shown to stimulate (^{3}H)thymidine incorporation in organ-cultured MTW9A, a subline of MTW9 which grows in normal female rats without an MtTW10 coimplant (Takizawa, 1970a; 1970b). Similarly, I observed that both MtT serum and insulin increased DNA synthesis in MTW9 (Figure 6). Insulin has been shown to stimulate DNA synthesis in a variety of mammary organ (Elias, 1959; Heuson, 1967) and cell (DeMeyts, 1976) cultures, and sensitivity to its action in MTW9 observed in the present studies suggests that endocrine responsiveness was at least partially preserved. However, since pharmacologic concentrations of insulin are generally required for these in vitro effects, the physiological significance of insulin's action on DNA synthesis remains unclear. Also, the mechanisms whereby insulin exerts these effects have not been defined. Of possible importance to this study, insulin has been shown to enhance the activities of cAMP phosphodiesterases in several tissues (House, 1972; Iliano, 1972; Kono, 1975) and to inhibit adenylate cyclase systems in others (Londos, 1978).

A combination of hormones resembling the known endocrine composition of MtT serum (MHS) failed to mimic the action of MtT serum on DNA synthesis in MTW9, whereas similar combinations have been shown to be stimulatory in several other neoplastic mammary tissues in vitro (Koyama, 1972; Lewis, 1974). Although there are reports indicating that prolactin can directly enhance DNA synthesis in cultured mammary tissue (Hallowes, 1977; Rudland, 1977), I found that neither ovine nor rat prolactin enhanced thymidine incorporation when used alone or in combination with MHS, normal female serum, or MtT serum.

Hormonal and serum effects on DNA synthesis observed in organ culture experiments with MTW9 were further evaluated in the human breast cancer cell line, MCF-7. The growth-stimulating potencies of complex solutions, e.g., sera and tissue extracts, can be assessed by sequential dilution. A comparative dose-response study of the effects of MtT, normal female rat, and fetal calf sera revealed significantly different potencies for promoting MCF-7 cell growth (Figure 7). The order of activity was MtT > female > FCS. FCS stimulates the growth of most cell lines, and is a standard supplement to tissue culture media. Growth-stimulating activities in FCS have not been fully characterized. From the data obtained, it is not possible to determine whether the differences observed among MtT, female rat, and fetal calf sera are due to variations in the concentrations of common sera components or to the presence of unique growth factors.

The endocrine requirements of hormone-dependent human breast cancers have not been clearly defined (Matsumoto, 1978). Prolactin and estrogen have long been thought to play dominant roles in the control of hormone-dependent mammary tumor growth (Costlow, 1978). However, to my knowledge, there is little evidence that either estrogen or prolactin is directly mitogenic to mammary cells. Methods used to measure the direct effects of estrogen and prolactin on cell growth have generally been limited to short-term (^{3}H)thymidine incorporation (Hallowes, 1977; Rudland, 1977), which may represent terminal rounds of DNA synthesis prior to differentiation rather than continued proliferation (Topper, 1974). Since the growth of serum-maintained cell cultures may require several serum components, the study of the effect of a single substance upon cell proliferation is often best evaluated in the presence of a growth-limiting concentration of serum. Although MCF-7 cell growth has been reported to be responsive to estrogen (Lippman, 1976b) and prolactin (Shafie, 1977), in agreement with other reports (Barnes, 1979), I found that neither estrogen nor prolactin directly stimulated these cells when combined with a growth-limiting concentration of FCS (Figure 8).

The presence of mammosomatotropic tumors in animals creates a unique serum endocrine composition which has been only partially analyzed (Diamond, 1979). It is possible that elevated serum prolactin stimulates the production of growth factors in MtTW10-bearing animals. It has been proposed that the *in vivo* growth-promoting activity of estrogen is mediated through induced growth factors (Sirbasku, 1978). A combination of hormones simulating the endocrine composition of MtT serum (MHS) not only failed to promote cell growth, but was actually mildly inhibitory. It was previously observed that a hormone combination similar to MHS failed to mimic the ability of the MtT tumor to prevent ovariectomy-induced regression of MTW9 (Diamond, 1978). This finding, coupled with my observations, is consistent with the concept that MtT serum contains an as yet unidentified component which is capable of stimulating tumor growth. Alternatively, it is possible that estrogen or prolactin promotes _in vivo_ tumor growth by altering other physiological processes, e.g., immunological mechanisms (Kim, 1979), tumor blood supply through angiotropic factors (Brem, 1977), or induction of plasminogen activator in tumor cells (Laug, 1975).

Effects of cAMP, DBcAMP, and Inhibitors of cAMP Phosphodiesterase on DNA Synthesis in MTW9 and on MCF-7 Cell Growth

The observation that several cAMP phosphodiesterase inhibitors suppressed (^{3}H)thymidine incorporation in organ-cultured MTW9 (Figures 9 and 10) is consistent with the reported inhibition of growth of MTW9 and several other rat mammary tumors by the _in vivo_ administration of cAMP analogs (Cho-Chung, 1974). I also found that prior _in vivo_ administration of 10 mg DBcAMP inhibited subsequent (^{3}H)thymidine incorporation after 24 hours. MIX was observed to inhibit DNA synthesis in several other tumors in organ culture: MTW9-P (perphenazine-maintained MTW9 tumors), MT449 (a metastasizing, autonomous rat mammary adenocarcinoma), and DMBA-induced rat mammary tumors (data not shown).

Whereas MTW9 growth in vivo is rapidly suppressed by DBcAMP (Cho-Chung, 1974), addition of DBcAMP to organ-cultured MTW9 failed to significantly alter DNA synthesis. Consistent with these data is the concept that this nucleotide influences in vivo tumor growth indirectly, perhaps by altering metabolic or immunologic mechanisms in host animals (Webb, 1972; Kim, 1970; Black, 1976). Alternatively, it is possible that DBcAMP may require prior activation by butyrate removal (Koontz, 1976), may be actively extruded from these cells (Brunton, 1979), or may be metabolized by rapidly-induced phosphodiesterases (Raska, 1973).

To my knowledge, only Shafie et al. (1977) have studied the effects of cAMP analogs and phosphodiesterase inhibitors on the growth of MCF-7 cells. A direct comparison with the present study is difficult since these investigators measured growth by short-term (^{3}H)thymidine incorporation. Their observations of marked inhibition of growth with 1 mM theophylline, and significantly less inhibition with 0.1 mM DBcAMP, are consistent with my results. They also found that high concentrations of insulin (10 μg/ml) reversed the inhibitory action of theophylline.

In this study, 50% inhibition of cell growth (I_{50}) was achieved using 0.5 mM MIX (Figure 11). Although the molecular mechanisms whereby MIX represses cell growth are not known, the most likely pathway involves alterations in cAMP degradation (Williams, 1976) that result in the activation of cAMP-dependent protein kinases. The ability of cAMP analogs to inhibit cell growth has been correlated with their ability to activate protein kinases in several tissues (Tisdale, 1979). Decreased sensitivity to the action of cAMP analogs is often associated with molecular defects in cAMP-binding proteins and cAMP-dependent protein kinases (Masui, 1978; Insel, 1975). MIX inhibition of adenylate cyclase activity has also been reported (Londes, 1978).

Though MCF-7 cell proliferation was repressed by 1 mM cAMP, DBcAMP had no significant effect at this concentration (Figure 12). Growth was suppressed by the combination of 1 mM DBcAMP together with a low concentration of MIX, although neither agent was effective when added individually. These data support the concept that MIX potentiates the effect of DBcAMP through inhibition of cellular phosphodiesterases. Naseem and Hollander (1973) observed that cAMP and PGE_1, but not DBcAMP, inhibited the growth of MPC-11 cells. It has been shown that DBcAMP is less active than cAMP in activating protein kinases in some tissues (Tisdale, 1979). Prior butyrate removal is necessary for some of the metabolic effects of DBcAMP (Kaukel, 1972), and a deficiency in DBcAMP metabolism might account for its lower activity in MCF-7 cells. Alternatively, MCF-7 cells may also be able to metabolize DBcAMP more efficiently than cAMP to an inactive product. Although it is likely that most cells are more permeable to the lipid-soluble DBcAMP (Boumendil-Podevin, 1977), it is possible that MCF-7 breast cancer cells extrude DBcAMP more efficiently than they do cAMP (Brunton, 1979). It is also possible that these nucleotides act at different sites in MCF-7 cells, as has been found in some tissues (Solomon, 1970; Bosmann, 1971). The production of butyryl derivatives and the greater lipid-solubility of DBcAMP may also account for some of the differences in activity between these nucleotides. The inhibitory actions of MIX and cAMP upon cell growth were not attributable to cytotoxic effects; cell growth rapidly resumed when these agents were removed from cultures of growth-inhibited cells.

Reversal of the Inhibitory Actions of MIX by MtT Serum

Growth of MTW9 can be stimulated either by coimplantation with a pituitary mammosomatotropic tumor, MtTW10, or by chronic administration of perphenazine, a dopamine antagonist which promotes host secretion of prolactin (Hollander, 1978). Tumors whose growth is supported by perphenazine administration contain estrogen receptors, and they regress in response to ovariectomy with or without continued perphenazine treatment. By contrast, tumors stimulated by the MtTW10 coimplant fail to regress after ovariectomy, although they too contain estrogen receptors. Attempts to simulate the endocrine environment of MtTW10-bearing animals by administration of hormones to perphenazine-maintained animals have failed to block ovariectomy-induced regression (Diamond, 1978). Since a substantial number (40%) of human breast cancers which contain receptors for estradiol fail to respond to endocrine therapy (Matsumoto, 1978), the mechanisms whereby the mammosomatotropic tumor stimulates growth and blocks ovariectomy-induced regression of MTW9 are of considerable interest.

Since mammary tumor growth may be at least partially regulated by antagonisms between cAMP and stimulatory hormones (Shafie, 1979), I thought it important to examine the direct effects of MtT serum upon the inhibitory actions of MIX that were demonstrated in previous experiments. The addition of MtT serum was observed to substantially block the effects of MIX on DNA synthesis in MTW9 (Figure 13) and on MCF-7 cell growth (Figure 14). However, MtT serum did not significantly reverse the inhibition of MCF-7 cell growth by the combination of 1 mM DBcAMP with a very low concentration of MIX (Figure 15). These data suggest that MtT serum does not interfere directly with the actions of either DBcAMP or MIX, but rather that a component of MtT serum opposes the synthesis or accumulation of intracellular cAMP. Consistent with this concept is the observation that MtT serum is not effective in reversing growth inhibition by cAMP alone (Figure 15).

Naseem and Hollander (1973) first observed that high concentrations of insulin (0.5 U/ml) could reverse growth inhibition by cAMP in PCT-11 cells. Shafie <u>et al</u>. (1977) showed that insulin (10 μg/ml) could also prevent the inhibition of (^{3}H)thymidine incorporation into MCF-7 cells by theophylline. Likewise, I found that high concentrations of insulin (10 μg/ml $\cong$ 0.25 U/ml) were able to partially antagonize the inhibitory action of MIX on DNA synthesis in MTW9 explants. However, the significance of these results with respect to the growth of spontaneous mammary tumors is uncertain because pharmacological levels of insulin were required. Yet, these results, coupled with the observation that rat mammary tumor regression in response to DBcAMP administration, ovariectomy, and pancreatic diabetes, may share a common mechanism (Shafie, 1979), suggest that an endocrine-cAMP antagonism may play a role in the control of tumor growth.

MtT serum may interfere with MIX action at any of several possible molecular sites, e.g., cellular uptake of MIX, phosphodiesterase inhibition by MIX, and degradation of MIX. It is possible that a component of MtT serum inhibits adenylate cyclase activity. Alternatively, MtT serum may antagonize growth inhibition by MIX at a step distal to the cAMP system. Exogenous cAMP, cAMP analogs, and phosphodiesterase inhibitors have been shown to inhibit the *in vivo* growth of several experimental tumors (Cho-Chung, 1974), and my results indicate that these agents could have potential therapeutic value in at least some human cancers. Theophylline has been used effectively in the treatment of human desmoid tumors (Waddell, 1975); and clinical trials using papaverine in patients with neuroblastomas have been successful (Helson, 1975; 1976). My data suggest that the combination of a cAMP analog with a phosphodiesterase inhibitor may be particularly effective against the growth of some tumors.

The failure of various sera and hormone combinations to mimic the ability of MtT serum to reverse MIX actions further supports the hypothesis that MtT serum contains an unidentified tumor-stimulating activity. The putative mitogenic activity of classical growth-stimulating hormones, e.g., estrogen, prolactin, and ACTH, has recently been seriously challenged (Sirbasku, 1978; Simonian, 1979). Evidence has been provided for the concept that these hormones stimulate tissue growth through intermediate growth factors (Sirbasku, 1978). From the data presented in this report, it is possible that the mammosomatotropic tumor produces a growth factor directly, or induces its production in the intact animal. In addition, somatomedins have been shown to be elevated in MtT serum (Chochinov, 1977), and they might contribute to growth-promotion by this serum.

Characterization of cAMP Phosphodiesterase, cAMP-Binding, and Protein Kinase Activities in MTW9

This is the first study to my knowledge of the cAMP system in the rat mammary adenocarcinoma, MTW9. Intracellular concentrations of cAMP were not evaluated in this study since such measurements have not been found to correlate well with the growth status of tissues and with cAMP-mediated effects. This lack of correlation can be reasonably explained in view of the dynamics of cAMP metabolism; increases in nucleotide synthesis may be balanced by cAMP binding to receptor proteins, resulting in no detectable increases in free nucleotide.

For determining cAMP phosphodiesterase activity, I chose the highly sensitive assay developed by Rangel-Aldao _et al_. (1978). Thin-layer chromatography on PEI-cellulose permitted the identification and full quantitation of the products of cAMP hydrolysis (Figure 3). The commonly used resin assay (Thompson, 1971) has been shown to significantly underestimate phosphodiesterase activity, especially if 5'-nucleotidase and adenosine deaminase are present (Ong, 1976).

Since phosphodiesterases are unstable after purification, most investigators have studied their properties by kinetic analysis of activities in whole homogenates or subcellular fractions. In most tissues examined, two major forms of cAMP phosphodiesterase activity, high- and low-affinity enzymes, have been resolved by physical or kinetic methods (Thompson, 1978; Levin, 1978). Similarly, I found that phosphodiesterase activity in MTW9 homogenates shows complex kinetics which is resolvable into high- and low-affinity activities (Figures 17 and 18). Whereas the Michaelis constants for these activities agree well with values found in mammary and other tissue types, the maximum activity levels in MTW9 are elevated when compared with other normal and neoplastic mammary tissues (Singer, 1976; Cohen, 1976; Chatterjee, 1975; Cho-Chung, 1977a; Clark, 1973). Chatterjee and Kim (1975) studied phosphodiesterase activity in MTW9B and MTW9A. Two enzyme activities were distinguished by kinetic analysis in each tumor homogenate. In MTW9B, the low and high Michaelis constants for high and low affinity enzymes were 3 μM and 50 μM, respectively, and maximum velocities were 175 and 1250 pmol/min/mg protein, respectively. In MTW9A, the activity levels for low and high Km enzymes were 69.9 and 1455 pmol/min/mg protein, respectively. In MTW9, I found low- and high-Km enzymes to have Michaelis constants of 2.5 μM and 22 μM, respectively, and maximum velocities of 970 and 2485 pmol/min/mg protein, respectively. Although MTW9 and MTW9A have been shown to differ from each other in many biochemical characteristics, the high phosphodiesterase activity found in MTW9 in this study may be partially related to the differences in assay methods employed.

As has been found in other tissues (Klinesmith, 1975; Rubin, 1975), subcellular fractionation shows that most of the protein kinase activity in MTW9 is associated with the cytosol fraction (Figure 20). A similar level of cytosol protein kinase activity was also found in MTW9-P (perphenazine-maintained MTW9 tumors) and MTW9-C (ovariectomy-responsive variant of MTW9 which grows in normal female rats). Most of the nuclear activity was active only on casein, was cAMP-independent, and was substantially activated by 0.6 M NaCl extraction. These results agree with other studies of mammary tissue (Majumder, 1977b; Desjardins, 1975). Protein kinase activities in MTW9 cytosols were separated into two major forms by DE-52 chromatography (Figure 21). The degree of activation of each by cAMP and also the elution with coincidental cAMP-binding peaks, suggest that these activities represent cAMP-dependent protein kinases. Whereas identical elution patterns were observed when protamine was used as the substrate, the kinases were less dependent upon cAMP. This could be related to the observation that protamine activates cAMP-dependent protein kinases (Rubin, 1975). Though two major peaks of protein kinase activity were reported in mouse mammary gland and in C3HBA mouse mammary adenocarcinomas, Type I was identified as a cAMP-independent catalytic subunit (Majumder, 1977).

To measure total cAMP-binding in tumor cytosol preparations, binding activity was examined under conditions optimizing cAMP exchange (Wilchek, 1971; DoKhac, 1973).

Cytosol binding activity was found to be 15.9 pmol/mg protein in MTW9 (Table 4), which is in the range commonly observed for other tissues (1-20 pmol/mg protein) (Talmadge, 1975; Cho-Chung, 1977b; Granner, 1972). The specificity of the binding sites for cAMP was confirmed by competitive binding studies (Table 2). (^{3}H)cAMP-binding was significantly diminished only by non-radioactive cAMP and 8-Br-cAMP. As reported by other authors (Menon, 1978), I observed DBcAMP to be a weak competitor for cAMP-binding sites. When the binding data from each DE-52 peak was analyzed by the Scatchard method (Scatchard, 1974), the results agreed with the presence of a single set of specific binding sites in each peak (Table 5). The equilibrium dissociation constants for Types I and II were 5.9×10^{-9} M and 2.1×10^{-8} M, respectively. When fully activated by cAMP, the protein kinases in Types I and II had similar affinities for histone as has been reported for most other tissues studied (Rosen, 1975; Hofmann, 1975; Walsh, 1979).

Alterations in the cAMP System Concomitant with Progression to Hormone Autonomy in MTW9

Changes in the growth status (proliferating, static, regressing) of tissues during developmental and neoplastic processes are often associated with changes in the concentrations and activity levels of specific cellular constituents, such as enzymes, hormone-receptors, membrane constituents, ions, and small molecules. There are many reports of alterations in the proteins controlling cAMP metabolism and protein phosphorylation concomitant with developmental changes (Jungmann, 1975; Kuo, 1975; Knight, 1977) and neoplastic transformation and progression (see Introduction). However, to my knowledge no other work has specifically focused upon possible alterations in these components after the development of hormone-autonomy in a single tumor line. MTW9 is markedly dependent upon hormones for growth, and progression of this tumor to a hormone-autonomous variant, MTW9B, is thought by some investigators to be due to sequential clonal selection (Kim, 1975). In this study, I compared several aspects of cAMP metabolism and action in MTW9 and MTW9B.

MTW9 explants were found to be much more sensitive to the inhibitory action of MIX upon DNA synthesis when compared to MTW9B explants (Figure 16). Since phosphodiesterases are the most likely sites for action of MIX, it is possible that the different sensitivities of MTW9 and MTW9B to MIX are due to molecular defects in phosphodiesterases in MTW9B. This idea was tested by comparing the soluble phosphodiesterase activity in these tumors by kinetic analysis (Table 3). A high-Km enzyme was not detectable in MTW9B. Also, the low-Km enzyme in MTW9B had a significantly decreased apparent Michaelis constant and maximum velocity. These differences could not be attributed to a diffusible factor. There is evidence that altered substrate affinity plays a role in regulating phosphodiesterase activity (Pledger, 1976). If there is some defect in cAMP control of growth in this tumor, one might expect a related defect in cAMP induction of phosphodiesterases. However, it is unclear how diminished phosphodiesterase activity in MTW9B could be causally related to either the hormone-independent behavior or decreased sensitivity to MIX in this tumor.

Sapag-Hagar <u>et al</u>. (1974) have suggested that increases in high-Km phosphodiesterase in rat mammary gland in preparation for lactation play a role in differentiation of the gland. Proliferation and differentiation are usually considered to be antagonistic processes in cells, and the loss of high-Km phosphodiesterase in MTW9B may be causally related to the loss of endocrine responsiveness. Alternatively, since cellular concentrations of cAMP are more likely to be regulated by the low-Km phosphodiesterase (Cohen, 1976), it is possible that the high-Km activity performs an additional cellular function, unrelated to cAMP hydrolysis. Analogous to the hormone-receptor deficiencies in this tumor, diminished phosphodiesterase activity in MTW9B may reflect a more generalized deficiency in the cellular cAMP system, which renders these cells unresponsive to growth regulation by hormones.

cAMP-binding and protein kinase activities were also altered in MTW9B as compared with MTW9. Both cAMP-binding and cAMP-dependent protein kinase activities were enhanced in MTW9B. Although apparent cAMP-binding affinities associated with Type I enzymes were similar in these tumors, the binding affinity of Type II was 2-fold lower in MTW9B. Substrate affinity was also diminished 2-fold in both Types I and II in MTW9B (Figure 21; Tables 4 and 5). Type II protein kinase activity was enhanced 2-fold in MTW9B. Byus _et al_. (1977) have suggested that differential activation of protein kinase isozymes may play a role in growth control. The difference in the Type II/Type I activity ratio between MTW9 (0.87) and MTW9B (1.92) could possibly play a role in the differences in growth control between these tumors. However, from these data it is not possible to assign a specific role to the altered cAMP-binding and protein kinase activities in MTW9B.

Understanding the cellular role of different cAMP-dependent protein kinases is complicated by the concept that the holoenzyme is the inactive form of the enzyme. Also, since catalytic subunits have generally been found to be identical, isozyme patterns in tissues should be attributable more to the distribution of regulatory subunits than to catalytic subunits. Consistent with this reasoning is the possibility that the greater activity of Type II in MTW9B represents increased cell content of regulatory subunit of Type II, and that this subunit plays a role in the unique growth characteristics of MTW9B. Important approaches to understanding the role of different enzyme forms include the study of changes in cellular distribution of these molecules during cell cycle. In CHO cells Type I activity was observed to be elevated during mitosis, while Type II activity was increased at the end of G_1 (Costa, 1976b).

Experiments were designed to detect possible defects in the functioning of the cAMP system in MTW9B by examining the competence of tumor explants for rapid protein kinase activation. In vitro activation of protein kinases by MIX was similar in MTW9 and MTW9B (Table 6). These results provide support for the concept that both of these tumors have functional cAMP systems, and that cAMP mediates the inhibitory actions of methylxanthines on tumor DNA synthesis. However, a similar dose-dependent activation was observed in each of these tumors, and no differences between the tumors were detectable under these experimental conditions.

Inclusion of 10% MtT serum in the incubation medium failed to influence the pattern of protein kinase activation in MTW9. Thus, the ability of this serum to antagonize the inhibitory effects of MIX upon MTW9 explants (Figure 13) cannot be accounted for by a mechanism which precludes the rapid activation of protein kinases. These results suggest that MtT serum does not alter adenylate cyclase or protein kinase activities. Speculations on possible explanations of these data include:

(1) MtT serum may oppose the action of MIX at a site distal to the activation of protein kinases;

(2) MtT serum may cause a rapid de-activation of protein kinases; or

(3) the inhibitory action of MIX might not be mediated by cAMP or protein kinase activation.

However, no data are presently available to support or refute these possibilities.

It is likely that endogenous concentrations of cAMP are controlled primarily by opposing synthetic and degradative processes in these tumors. MIX inhibition of phosphodiesterase activity in these tumors probably permits the accumulation of cAMP and subsequent activation of protein kinases due to ongoing adenylate cyclase activity. While optimizing conditions for the protein kinase assay, I observed that methylxanthines did not directly affect protein kinase activity. However, these data do not rule out the possibility that MIX may also stimulate adenylate cyclase or exert other effects, i.e., on membrane transport, in these tissues.

If MIX inhibits DNA synthesis through protein kinase activation, it is possible that differential isozyme activation may account for the different sensitivities to MIX in MTW9 and MTW9B. Also, MTW9B may be able to lower cAMP levels by extruding the nucleotide from the cell (Brunton, 1979), by inactivating it by rapidly induced phosphodiesterase activity (Raska, 1973), or by inhibiting adenylate cyclase activity.

Although there are significant changes in phosphodiesterase, cAMP-binding, and cAMP-dependent protein kinase activities associated with progression in MTW9, it is not possible to unequivocally attribute the decreased sensitivity to MIX and altered hormone responsiveness in MTW9B to any specific defect in the cAMP system.

Alterations in the cAMP System Concomitant with Regression in MTW9

A striking feature of human breast cancer is the remarkable remission sometimes obtained with endocrine therapy. Measurement of the concentration of estrogen receptors in breast cancers has become a major prognostic tool in predicting successful responses to endocrine therapy. However, many human breast cancers still fail to respond to endocrine therapy, although they contain receptors for estrogen (Matsumoto, 1978). Also, in most responsive patients the disease eventually recurs, and is usually then unresponsive to endocrine therapy. In spite of the clinical importance and considerable effort in this field, the specific hormones and endocrine mechanisms involved in breast cancer growth and regression remain obscure.

There is evidence that the cAMP system may play a role in ovariectomy-induced regression of rat mammary tumors (Cho-Chung, 1978; Shafie, 1979). I investigated possible changes in key components in the cAMP system in MTW9 concomitant with tumor regression. MTW9 regresses rapidly in response to the surgical removal of the pituitary tumor coimplant, MtTW10 (Hollander, 1978). Phosphodiesterase, cAMP-binding, and protein kinase activities were found to be significantly reduced in regressing tumor cytosols. In contrast, cAMP-binding and protein kinase activities have been reported to be increased in DMBA-induced rat mammary carcinomas during regression induced by ovariectomy, DBcAMP administration, or streptozotocin-induced diabetes (Shafie, 1979). Shafie et al. (1979) have found consistent changes in the cAMP system in regressing tumors, and they are investigating the possible role of the cAMP system in mammary tumor regression. However, a direct comparison of data is difficult for the following reasons: the changes reported by these authors vary with regard to the duration of regression; the interpretation of their data involves a controversy concerning nuclear translocation of substantial amounts of cAMP-binding and protein kinase activities; and there are important differences between MTW9 and the models used by these authors.

It is possible that the decreases in cAMP-binding and protein kinase activity seen in MTW9 cytosols during regression are due to a partial translocation of these activities to the nucleus. Similar changes in the compartmentation of these activities have been reported by several authors (Costa, 1976a; Jungmann, 1974; Lastagna, 1975). Exposure of MTW9 explants to MIX resulted in the activation of protein kinases, with subsequent decreases in regulatory and catalytic subunits in the cytosol (Table 6). While other interpretations are possible, e.g., extrusion and association with membranous material, these data are consistent with the concept of nuclear translocation. However, other investigators (Keely, 1975; Zick, 1979) have questioned the physiological significance of reported nuclear translocation, and to my knowledge, no definitive evidence for translocation under physiological conditions has been presented. Also, in cell fractionation studies using MTW9, most of the cAMP-dependent protein kinase activity associated with nuclei could be solubilized by physiological salt concentrations (Figure 20).

Pledger _et_ _al_. (1976) have observed that serum rapidly stimulates phosphodiesterase activity (both maximum velocity and affinity) in quiescent BHK cells. These authors postulate that a serum factor may regulate phosphodiesterase activity, and that subsequent growth stimulation in these cells may result from decreased cAMP concentrations. cAMP concentrations have been found to be increased during rat mammary tumor regression following DBcAMP administration (Bodwin, 1978), insulin deficiency (Matusik, 1976), inhibition of prolactin secretion (Matusik, 1976), and ovariectomy (Bodwin, 1978). Bodwin _et_ _al_. (1980) have suggested that estrogen and prolactin may stimulate cell growth by lowering cAMP concentrations in cells. I found that phosphodiesterase activities are decreased in homogenates and purified particulate fractions of regressing MTW9 tumors. Particulate fractions were prepared by a method employed by other investigators in the isolation of specific phosphodiesterase activities which are optimally responsive to modulation by hormones (Thompson, 1978; Levin, 1978).

Turkington et al. (1973) reported that prolactin induces cAMP-binding and cAMP-dependent protein kinase activities in mouse mammary gland. Prolactin concentrations in MtT sera are dramatically elevated (Table 1), and diminished prolactin concentrations due to the removal of MtTW10 coimplants could be related to decreased cAMP-binding and protein kinase activities found in regressing tumors.

These data suggest possible mechanisms whereby hormones in MtT serum may influence mammary tumor growth by regulating the activity of key components in the cAMP system.

SUMMARY AND CONCLUSIONS

Sera from animals bearing mammosomatotropic tumors (MtT serum) was observed to stimulate both DNA synthesis in MTW9 explants and proliferation of MCF-7 cells. These activities could not be mimicked by normal female rat serum or by combinations of hormones known to be present in MtT serum. Methylxanthine inhibitors of cAMP phosphodiesterase, but not DBcAMP, suppressed DNA synthesis in MTW9 explants and the proliferation of MCF-7 cells. However, DBcAMP potentiated the inhibitory actions of very low doses of methylxanthines. Addition of MtT serum to cultures blocked the action of phosphodiesterase inhibitors, but not that of DBcAMP plus MIX. Appropriately selected sera and hormones failed to duplicate these actions of MtT serum.

A comparison of cAMP action and metabolism in MTW9 and MTW9B revealed several differences between these tumors. The hormone-dependent tumor, MTW9, was markedly more sensitive to the inhibitory effects of methylxanthines on DNA synthesis. cAMP phosphodiesterase activity was reduced and both cAMP-binding and cAMP-dependent protein kinase activities were elevated in the autonomous tumor, MTW9B. Decreases in cAMP phosphodiesterase, cAMP-binding, and protein kinase activities were found in MTW9 tumors during regression induced by the removal of supportive mammosomatotropic coimplants. cAMP phosphodiesterase activity was especially low in purified particulate fractions from regressing tumors.

In conclusion, activation of the cAMP system under the conditions employed in this study inhibits DNA synthesis in several rat mammary tumors and proliferation of MCF-7 human breast cancer cells. The combination of a cAMP analog with an inhibitor of cAMP phosphodiesterase is particularly effective. It is possible that sera from MtTW10-bearing rats contain an activity, unidentified at present, which is capable of antagonizing the growth-inhibitory action of the cAMP system, and which may participate in the regulation of mammary tumor growth. Substantive differences in sensitivity to cAMP and in cAMP metabolism found between MTW9 and MTW9B are consistent with the concept that the cAMP system plays a role in the hormone dependence of MTW9. Observed alterations in the cAMP system in regressing tumors provides further support for this idea. Endocrine mechanisms involved in the regulation of tumor growth are not well understood. Although these experimental results do not provide a definitive conclusion concerning the role of cAMP in endocrine-responsive cancers, they do support the concept that hormone-dependent tumor growth may be at least partially regulated by an antagonistic interplay between supportive hormones and the cAMP system.

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