MICRO-ANALYSIS OF ADENYLATE CYCLASE AND CYCLIC AMP-DEPENDENT PROTEIN KINASE ACTIVITIES IN HUMAN MAMMARY TUMORS

by

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This dissertation is dedicated

to my mother, whose loss inspired my work,

and to my father, each for their strength,

encouragement, and continued

faith in me.

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LIST OF ABBREVIATIONS

AC	-Adenyl cyclase (E.C. No. 4.6.1.1.)
AMP	-Adenosine-5'-monophosphate
ATP	-Adenosine-5'-triphosphate
BSA	-Bovine serum albumin
B_2cAMP	-N^6,$O^{2'}$-dibutyryl adenosine 3':5'-monophosphate
cAMP	-Cyclic adenosine 3':5'-monophosphate
cAMPdPK	-Cyclic AMP-dependent protein kinase (E.C. No. 2.7.1.37)
CAT	-Protein kinase catalytic subunit
cCMP	-Cyclic cytosine 3':5'-monophosphate
cGMP	-Cyclic guanosine 3':5'-monophosphate
cIMP	-Cyclic inosine 3':5'-monophosphate
DTT	-Dithiothreitol
FA	-Fibroadenoma
FSH	-Follicle-stimulating hormone
G/F	-Guanine nucleotide, fluoride stimulated regulatory subunit of AC
Gpp(NH)p	-Guanyl 5'-yl-imidodiphasphate
GTP	-Guanosine-5'-triphosphate
hCG	-Human chorionic gonadotropin
H & E	-Hematoxylin and eosin
HEPES	-N-2-hydroxyethyl piperazine-N^1-2 ethane sulfonic acid
HK	-Hexokinase (E.C. No. 2.7.1.1.)
IDC	-Infiltrating ductal carcinoma

LH	-Luteinizing Hormone
MES	-2-(N-morpholino)ethane sulfonic acid
MOPS	-3-(N-morpholino)propane sulfonic acid
PK	-Protein kinase
NAD+	-Nicotinamide adenine dinucleotide (oxidized form)
NADH	-Nicotinamide adenide dinucleotide (reduced form)
NADP+	-Nicotinamide adenine dinucleotide phosphate (oxidized form)
NADPH	-Nicotinamide adenine dinucleotide phosphate (reduced form)
$NaDodSO_4$	-Sodium dodecyl sulfate
PDE	-Cyclic 3':5'-nucleotide phosphodiesterase (E.C. No. 3.1.4.17)
PGE_1	-Prostaglandin E_1
PL	-Prolactin
REG	-Protein kinase regulatory subunit
RIA	-Radioimmunoassay
ScAMP-TME	-Succinyl cyclic AMP tyrosine methylester
TSH	-Thyrotropin-stimulating hormone
Tris	-Tris(hydroxymethyl)aminoethane

1. INTRODUCTION

The pathogenesis of human mammary carcinoma remains largely unknown despite the fact that breast cancer is the most frequent and fatal neoplasm in women of the Western world (Moore and Charney, 1975; McDivitt, Stewart, and Berg, 1968). Notable change in the mortality rate for carcinoma of the breast has not been obtained over the past eighty years although dramatic advancements have been made in the development of early diagnostic procedures, such as xerography, and mammography, as well as in the refinement of surgical techniques, radiation treatment, and adjuvant chemotherapy.

Just as each example of neoplastic disease seems to be the outcome of a unique set of circumstances, the etiology of human breast cancer also appears to be multifactorial and sufficiently obscure so that rational approaches to prevention of the disease have not as yet been possible. Genetic susceptibility almost certainly underlies the onset of this neoplastic disease. Viral association, carcinogens, exogenous and endogenous hormones, ionizing radiation, and reduced immunological competence associated with aging are among the factors which have been implicated to varying degrees in the induction or potentiation of human mammary carcinoma.

Although a major emphasis of pathological studies related to neoplastic tissue continues to be histological, it has become increasingly clear that these methods do not possess the degree of accuracy necessary for optimal clinical diagnosis, particularly with respect to the biological grading of tumors. Oncological investigations

directed towards correlating biochemical descriptions of cancerous tissue with the degree of neoplasticity based upon histopathology have provided new insights for understanding the etiology of the carcinogenetic process. Integration of information gained from such an approach may eventually define the nature of the premalignent condition, allow predictions to be made concerning the biological behavior of cancerous tissue, and serve as a rational basis for treatment of both the primary and metastatic disease.

The failure of neoplastic cells to respond to homeostatic control of the host, their ability to invade surrounding tissues, and their ability for metastasis suggests that a unique molecular basis must underlie such aberrant biological behavior. In recent years, the observation that many tumors can form and release various substances which are ectopic, particularly hormones and fetal-associated macromolecules, has received increasing attention (Tormey et al., 1975). In addition, deviant metabolic patterns and the occurrence of distinct isoenzyme profiles in cancerous tissue illustrates the important changes in gene expression which occur during cell transformation (Weber, 1976). The use of a regulatory enzyme system as a biological marker for cell transformation events may lend understanding to the molecular nature of neoplastic disease.

Three major obstacles occur in biochemical studies of carcinogenesis, applicable to work with human mammary carcinoma, and other forms of neoplastic growth. First of all, while a variety of animal model systems of graded neoplasms and hormone-dependent tumors are

available for biochemical examination, as well as a limited number of human breast cancer cell lines, the specific relevance these hold for human mammary cancer is unclear. A question which remains to be answered is whether malignant transformation induced by exogenous carcinogens, viruses, or tumor transplants is analogous to the carcinogenic process in humans.

A second point to be made is that a strict discrimination between those metabolic events which are associated with tumor initiation and those which accompany tumor progression and tissue de-differentiation is necessary in order that the biochemical relevance of such change and its application to the principles of tumor biology be elucidated. Such a cause-and-effect analysis is indeed extremely difficult.

Thirdly, in light of the cellular heterogeneity of tumors, a meaningful interpretation of biochemical differences related to cell transformation requires stringent separation between tumor and normal cell populations in order to delineate metabolic alterations associated with tumorigenesis. The gross heterogeneity of breast tissue demands special attention in order to assess the tumor-specific events occurring.

Cyclic adenosine 3':5' monophosphate (cAMP) has been implicated as a primary regulator of cellular proliferation. While cyclic nucleotide metabolism may be only a single parameter of the neoplastic process, its total significance has yet to be appreciated. Since cAMP system defects have been suggested to have potential importance in neoplasia, it is logical to study this system as a unit in mammary

tumors, especially since there is little integrated data available on enzymes involved in cAMP biosynthetic or effector functions from human mammary tumors.

It is hoped that the information gained from this study will be useful in clarifying the concepts of human mammary tissue neoplasia.

1.1. Description of the Normal and Neoplastic Mammary Gland System

1.1.1. Normal Glandular Tissue: The human mammary gland is heterogeneous and consists of an epithelial parenchyma of acina and ducts, adipose tissue; blood vessels, nerves, and lymphatics; supported by fascial elements and muscle. Epithelial glandular tissue is divided by connective tissue into fifteen to twenty-four wedge-shaped lobes, each supplied by an individual lactiferous duct lined by pseudostratified columnar and double-layered cuboidal epithelium. Ducts converge towards the areola where dilation occurs to form lactiferous sinuses prior to opening onto the skin.

Each lobe is subdivided into numerous lobules surrounded by a collagen sheath. Lobules consist of ten to a hundred or more acini radiating from an intralobular branch of the lactiferous duct. Acini are lined by a single layer of cylindrical or cuboidal epithelial cells which, in conjunction with a second layer of myoepithelial cells, contract in response to oxytocin, thereby forcing milk from acini and collecting ducts. During prelactation, intralobular ducts and acini branch and increase in diameter in response to estrogen and progesterone from the placenta. The secretion of milk proteins results from the production of prolactin by the anterior pituitary

triggered by reduced blood levels of estrogen and progesterone.

1.1.2. Neoplastic Glandular Tissue: The histogenesis of mammary cancer, based upon ultrastructural and cytochemical studies, suggest that these neoplasms can arise from any of three cell types which constitute the breast secretory unit; stromal or ductal epithelial, and myoepithelial cells. Several attempts to ascertain that a relationship exists between the cell of origin of a neoplasm and its degree of hormone dependency have been made, with little success. Although characters separating the benign from malignant breast tumor are not clearly defined, the general progression of the disease is still considered to follow the pattern suggested by Broders (1932):

normal epithelium ⟶ hyperplastic epithelium ⟶ intraductal carcinoma ⟶ invasive ductal carcinoma

1.1.2.1. Fibroadenoma (FA): Fibroadenoma is the third most frequent breast tumor in the American female population, exceeded by carcinoma and fibrocystic disease. It most frequently occurs in women less than thirty years of age and is seldom associated with malignant epithelial change; therefore, fibroadenoma is considered benign. Epidemiologic studies reveal little correlation between fibroadenoma and most risk factors associated with breast cancer (Normura, Comstock, and Tonascia, 1977).

Mammary fibroadenoma is considered to be of lobular, rather than true ductal origin. It has been suggested that tumors originate in the smallest epithelial-lined tubules analogous to acini (Wellings, Jensen, and Marcum, 1975). The tumor is not encapsulated, but is

sharply delineated and composed of a proliferating connective tissue stroma and an abundance of acini and ducts.

The histological pattern is one of delicate, cellular, fibroblastic stroma enclosing glandular and cystic spaces lined by epithelium. The connective tissue tends to have a loose, reticulated appearance. In fact, round to oval gland spaces may be present, lined by single or multiple layers of cells. The connective tissue surrounding these spaces tends to be compressed and denser than the intervening stroma. In some areas, the connective tissue stroma may appear to have undergone proliferation. As a consequence, glandular lamina are collapsed or compressed in slit-like, irregular clefts. Structural variations exist in this tumor type and the extreme conditions may be classified as "adenoma" (epithelium predominates) or "fibroma" (connective tissue predominates).

1.1.2.2. Infiltrating Ductal Carcinoma (IDC): The tumor groups within this classification represent the most common form of mammary cancer, comprising approximately 80% of all malignant breast tumors. Again, extremes in tissue composition occur, with those lesions which are extremely cellular designated as "carcinoma simplex" and those which are more fibrotic termed "fibrocarcinoma."

The tumor is composed principally of dense, collagenous, hyaline, fibrous stroma, in which are found scattered, isolated nests or filaments of irregular epithelial cells. Small sheets or masses may create gland patterns. Individual cells are round to polygonal or compressed, and contain small, deeply chromatic nuclei that are

uniform in size and shape. At the margins of the tumor mass, neoplastic cells may infiltrate into the surrounding fibro-fatty tissue and frequently invade perivascular and perineural spaces as well as blood vessels. In tumors characterized by dense fibrosis, neoplastic cells may be found in great numbers around the periphery of the fibrotic mass.

The origin of the tumor is considered to be the mammary duct which becomes dilated and occluded by carcinoma cells. The comedocarcinoma originates from anaplastic proliferations of the ductal epithelium in which large, hyperchromatic cells fill and eventually dilate the lactiferous ducts. Cells rimming the duct remain viable and often appear in regularly spaced glandular or papillary growth patterns. The centermost cells die and dislocate from the mass to form a lumen consisting of cellular debris and nuclear fragments. As the lesion advances, neoplastic growth extends through the basement membrane and is then considered to be an infiltrating ductal carcinoma. Epithelial cells of these carcinomas may be found scattered, isolated in small nests, or projected in columns through a densely fibrous stroma.

1.2. Cyclic 3':5' Adenosine Monophosphate: Function and Implications in Carcinogenesis

Cyclic AMP, synthesized by adenylate cyclase (AC), is recognized as a regulatory agent which controls numerous cellular processes. Current physiological models project an involvement of cAMP not only as the second messenger of hormone action, but also in carbohydrate and lipid

metabolism, activation of regulatory enzymes, mediation of membrane permeability, phosphorylation of histones, and determination of DNA synthetic rates (Folco and Paoletti, 1978). An additional role of cAMP appears to be the control of normal cellular proliferation, and alterations in cAMP metabolism have been associated with the cancerous state (Pastan, 1975).

In 1973, Goldberg formalized the Yin Yang concept according to which in bidirectional systems a reciprocal relationship exists between cyclic nucleotides during the cell cycle. It is proposed that cyclic guanosine 3':5' monophosphate (cGMP) participates in the induction of cell division, whereas cAMP is associated with inhibition of cell proliferation, and differentiation. Current evidence implicates cAMP and cGMP in the regulation of gene expression through cyclic nucleotide-dependent protein kinase modulation of transcription.

Cell cycle specific fluctuations in cAMP have been shown to occur in mammalian cells, with a three to five-fold increase during the G_1 to S phase transition in Chinese hamster cells (Russell and Stambrook, 1975). The escape from growth control is a primary step in the deviation from normal cellular behavior and is associated with alterations in the cell surface properties including surface adhesive forces, polarity, surface shape and movement, saturation density, biochemical composition and cell junctions and intercellular communication (Hynes, 1975). Since cAMP synthesis is catalyzed by a cell membrane-associated enzyme, alterations in membrane properties associated with cell transformation are suggested to modulate

adenylate cyclase activity, which in turn, may alter local concentrations of calcium, which may ultimately control changes in the activity and structure of microfilaments and microtubules, allowing for the growth characteristics and behavior associated with malignantly-transformed cells. Microtubule-associated protein (MAP_2) purified from brain cytosol has been identified as a substrate protein for cAMP-dependent protein kinase-catalyzed phosphorylation (Lohmann, Walter and Greengard, 1980). Increased phosphorylation of intermediate filaments was observed during mitotic arrest in CHO cells (Robinson, et al., 1981).

A fundamental property of normal cells is that upon reaching confluency, a steady-state level of cAMP is maintained which is not subject to feedback response at either the synthetic or degradative level (Otten, et al., 1971). It is suggested that the reduction of contact inhibition of tumor cells may be associated with reduced levels of AC activity (Burke, 1968). High levels of cAMP are associated in some studies with a reduced growth rate for cells in monolayer culture. The level of cAMP increases when non-malignant cells reach confluency, suggesting that cell to cell contact plays a role in the activation of AC and the regulation of growth by cAMP. Teel and Hall (1973), using KB cells, indicated that plating density was reduced three-fold on addition of millimolar levels of N^6, O^2-dibutyryl cAMP, (B_2 cAMP) due to a suggested inhibition during the G_2 phase of the cell cycle. Burger et al. (1972) demonstrated that B_2 cAMP functioned to block growth increase induced by concanavalin A or pronase treatment of untransformed cells.

The possible involvement of cyclic nucleotides in neoplastic growth is suggested by experiments in which restoration of normal growth characteristics to transformed cells has been observed by treatment with cAMP or analogs of cAMP (Ryan and Heidrick, 1968; Abell and Monahan, 1973). For instance, growth of cells at a lower saturation density was obtained by treatment of polyoma-transformed 3T3 cells with theophylline, an inhibitor of cAMP-phosphodiesterase, or B_2 cAMP (Sheppard, 1972). Growth arrest of a hormone-dependent, metastatic human breast cancer cell line, MCF-7, was preceded by increases in the cellular concentration of cAMP, AC, and type II cAMPdPK activities (Cho-Chung, et al., 1972). It was suggested that growth of human breast cancer cells is subject to cAMP-mediated regulation, and demonstrated that arginine may play a role in this process by stimulation of NAD-dependent ribosylation functional in AC activation.

Cyclic AMP also inhibits growth in solid tumors and has been shown to induce tumor regression (Gericke and Chandra, 1969; Cho-Chung, 1979). That cAMP acts as a signal to suppress proliferation in mammary neoplasia is supported by the observation that regression in rat mammary tumors can be induced by treatment with B_2 cAMP (Cho-Chung and Gullino, 1974).

It should be emphasized that dramatic changes in cyclic nucleotide levels have been reported to occur during cell proliferation both in tissue culture and <u>in vivo</u> (Costa, Gerrer and Russell, 1976; Otten, Johnson and Pastan, 1971; Ryan and Heidrick, 1974; Sheppard and

Prescott, 1972). If malignant transformation is viewed as an abnormality in cellular differentiation or the replicative potential of the cell, an overall increase in the concentration of cAMP in cells undergoing oncogenic transformation would be predicted. In 1974, Minton, et al., reported that cAMP levels were fifteen-fold higher in human breast carcinoma tissue than in adjacent normal breast tissue, when expressed on a wet tissue weight basis. Kung et al., (1977) observed cAMP levels seven-fold higher in normal than neoplastic tissue when calculated on the basis of wet weight, but levels of cAMP were significantly lower in neoplastic tissue when expressed in terms of cell number. In an analysis of normal, dysplastic and neoplastic human breast tissue, results of Bechtel et al. (1978) indicate that the specific levels of cAMP and cAMPdPK activity are significantly elevated in neoplastic tissue. However, when the data were expressed on a per cell basis, then carcinoma tissue is characterized by markedly lower values. It is difficult to assess these results in view of a number of factors which differ in normal and neoplastic tissues such as differences in cell density, intracellular volumes of the cells, and differences in the relative abundance of various types of cells.

In addition to difficulties in interpreting results in a meaningful manner, measurements of intracellular cAMP levels in transplantable as well as carcinogen-induced tumors have given conflicting results, observable in both animal and human mammary tumors. This discrepancy, plus the variation in cAMP levels reported, lends doubt as to whether low cAMP levels are associated with neoplastic growth in mammary

tissue. It may be speculated that intracellular cyclic nucleotide levels may be more closely related to specialized metabolic functions of cancer cells, just as in normal cells, cAMP is known to affect a wide range of cellular processes. Perhaps, then, enzymes that control the levels of cAMP, and those which mediate its function are of greater interest and relevance.

Cyclic AMP is synthesized by a membrane-bound, multicomponent enzyme, adenylate cyclase [ATP pyrophosphate lyase (cyclizing), E.C.4.6.1.1.]. Given the alterations of the plasma membrane found in neoplastic cells, the association of AC with the plasma membrane, and the frequent hormonal-dependency of tumor development in breast tissue, it is logical that a modulation of AC activity levels or alteration in its response to circulating or ectopically-produced hormones, may be important signals for early stages of cell transformation. Therefore, determination of AC activity in malignant and benign tissue indicative of the potential rate of cAMP formation may be very meaningful in human mammary tumors.

In vitro evidence suggests that malignant transformation is associated with low AC activity (Sheppard, et al., 1971). However, again, conflicting reports of AC activity in tumor tissue emphasizes the insensitivity and restrictive nature of the assay techniques employed (Brown, et al., 1969; Rillema et al., 1978). More recent work by Eppenberger et al. (1977) has demonstrated that decreased cellular AC and cAMP-binding activities occur in neoplastic breast tissue. Again, analysis of normal and neoplastic mammary tissue is

complicated by the same factors observed with cAMP levels. Elevated AC levels occur in neoplastic tissue when expressed on a weight or protein basis, while significantly lower activities are obtained in terms of cell number.

Although there is only indirect evidence for alterations in the catalytic subunit of AC in neoplastic tissue, the loss or defect of the hormone receptor function or the guanine nucleotide (G/F) regulatory subunit have been demonstrated in a number of malignant cultured cell lines of animal and human origin, in human renal and adrenal cortical carcinoma, and murine hepatoma (Schimmer, 1972; Kelly and Butcher, 1975). Recently, both basal adenylate cyclase activity and response to parathyroid hormone PGE, glucagon and fluoride were shown to be modified in a particulate preparation from renal cortical carcinoma indicating that a high rate of cAMP production may actually occur in malignant cells (Hunt et al., 1978). Stimulation of adenylate cyclase by MSH, ACTH, and PGE_1 was examined in intact mouse melanoma cells of varying metastatic potential (Niles and Makarski, 1978). Cells of low metastatic potential demonstrated greater cAMP potential. In broken cell preparations, the degree of hormonal stimulation was virtually identical in the three metastatic ranges. A complete loss of [1251]-iodoglucagon binding and glucagon stimulated adenylate cyclase activity occurred in poorly differentiated rat and mouse hepatomas in comparison to normal tissue and well-differentiated tumor tissue; although the enzyme was still activated by fluoride and epinephrine (Mirel et al., 1978). Tell et al. (1978) showed that

particulate fractions of three human adrenocortical tumors demonstrating ACTH and PGE_1 receptors can be independently affected, with the catalytic subunit and GTP-sensitive subunits unaltered by the selective tumoral process. Finally a lack of PGE_1 responsiveness of AC by a cAMP-unresponsive rat mammary carcinoma, in contrast to activation of AC from a cAMP responsive tumor, has been documented (Cho-Chung, 1979). The molecular mechanisms resulting in a loss of hormone sensitivity may involve altered hormone affinity and number of receptors, coupling of the receptor to the enzyme, or in the catalytic subunit itself, or a combination of these possibilities.

Notable regression of metastatic breast cancer lesions has been demonstrated after ovariectomy, adrenalectomy, or hypophosectomy, or following endocrine therapy (Papaioannou, 1974; McGuire, 1978; Rose, 1979). This, along with the presence of hormone receptors in mammary carcinomas, suggests the potential for hormone-dependency of tumor development. The current classification system of breast tumors as hormone dependent or independent, based upon the presence of estrogen receptors, draws attention to the effect of steroid hormones on cAMP levels. This area of study needs further research. In contrast to the second messenger system involving nonsteroid hormones, steroid hormones, particularly estrogen and progesterone, have been shown to interact with specific cell receptors and are functional in the stimulation of DNA, RNA, and protein synthesis. Since protein synthesis, specifically related to enzyme induction, is involved in normal cell differentiation, modulation of transcription might signal

an early stage of cellular malignancy. Recently, a possible link between steroid and nonsteroid hormones has been found in a specific protein called SCARP (steroid and cAMP-regulated phosphoprotein) which is dephosphorylated by the effect of 17-B-estradiol (Liv and Greengard, 1976). The endogenous phosphorylation and dephosphorylation of this particular protein is also regulated by cAMP.

In addition to androgenic, adrenal, and ovarian steroids, protein hormones including prolactin, growth hormone, thyroid hormone, and insulin also play a significant role in normal breast development, suggesting the presence of multiple hormone receptors. Recently, an immunochemical demonstration of human chorionic gonadotropin has been made in tissue of breast carcinoma (Castro, et al., 1980). A high frequency of elevated serum thyroid stimulating hormone (TSH) and prolactin-releasing hormone (PRL) has also been observed in breast cancer patients (Pineda, 1980). Studies using membrane-associated AC from murine mammary carcinoma have shown stimulation by epinephrine and PGE_1 (Schorr and Russel, 1974).

The catabolism of cAMP occurs by its conversion to 5' AMP catalyzed by cAMP-phosphodiesterase. Two isozymes of cAMP-phosphodiesterase, high and low affinity forms, have been examined in human malignant breast tumors, rat mammary tumors, and rat mammary tumor culture. Work of Kung et al. (1977), Singer et al. (1976); Chatterjee and Kim (1975) and recent work by Larner and Rutherford (1981) indicate an enrichment of the low-Km cAMP enzyme in neoplastic mammary tissues.

Cyclic AMP-dependent protein kinases (ATP: protein phosphorans-ferase, E.C.2.7.1.37; cAMPdPK) are proposed to translate most, if not

all, the intracellular effects of cAMP. A well-defined function of cAMP is to facilitate the phosphorylation of proteins by liberating the catalytic subunit of cAMPdPK. In addition to phosphorylation, and regulation of numerous metabolic enzymes such as pyruvate kinase, hormone-sensitive lipase, acetyl CoA carboxylase, glycogen synthetase, glycogen phosphorylase, lactate dehydrogenase, hydroxymethylglutaryl CoA reductase, ATP citrate lyase and cholesterol ester hydrolase, protein kinases also catalyze the phosphorylation of contractile, cytoskeletal, and membrane proteins, as well as histones, ribosomal proteins and the eukaryotic initiation factor, eIF-2 (Lincoln and Corbin, 1978; Glass and Krebs, 1977; Datta et al., 1977; Issinger et al., 1980). The phosphorylation of histone and nonhistone chromosomal proteins may regulate chromosomal condensation, gene expression, and mitosis, consequently cellular proliferation in mammalian tissues. Phosphorylation of nonhistone and histone chromosomal proteins increases in cells following viral and chemically-induced transformation (Blat, Bowroukhoff, and Harel, 1977; Link and Marks, 1981). That protein kinases play a significant role in cell transformation events is supported by the characterization of the Avian src gene product p60 src, and simian vacuolating (SV40) virus T antigen which are independently required for oncogenic transformation in RNA and DNA tumor viral-infected cells, respectively, and possess phosphotransferase activity (Witte, Dasgupta and Baltimore, 1980). In addition, the serum component, epidermal growth factor (EGF) which is essential for normal, but not transformed, cell growth

in tissue culture systems, has been shown to activate a membrane-associated protein kinase (Cohen et al., 1980). A membrane-bound ecto-protein kinase capable of phosphorylating surface proteins has recently been characterized in Hela cells (Kubler, Pyerun, and Kinzel, 1982).

Two isoenzymes of cAMPdPK have been identified in various tissues whose regulatory subunits differ with respect to molecular weight, charge, and autophosphorylation (Rosen et al., 1977). Costa (1978) has found that a differential expression of type I and type II cAMPdPK occurs during the cell cycle. The activation of cAMPdPK is transiently increased two-fold as a function of G1 progression in mitotically synchronized Chinese hamster ovary (CHO) cells, with the type II kinase increasing during G_1 transit and the type I kinase increasing during cAMP-induced growth arrest. The fact that malignantly-transformed cells lack normal growth characteristics and are not subject to phase specific regulation suggests that alteration of the protein kinase system is probable.

A close coupling of cAMP production with protein kinase activation and increasedproduction of enzymes such as tyrosine amino transferase and PEP carboxylase in Reuber hepatoma (H35) cells suggests that activation of protein kinase is functionally linked to the induction of specific enzymes (Culpepper and Liu, 1981). Preferential phosphorylation of endogenous histone HI has also been shown to increase in relation to this enzyme induction after treatment with cholera toxin, or a combination of cholera toxin and dexamethasone.

One function of cellular cAMP-binding proteins (BP) is to serve as regulatory subunits for cAMP-dependent protein kinases. In many tumor systems, cAMPdPK and cAMP-BPs demonstrate a parallel decrease in activity as compared to normal tissues. Bechtel et al. (1978) has shown that PK activity was three-fold higher in normal than in primary mammary carcinoma on a cellular basis. In a cAMP-resistant S49 lymphosarcoma, either no detectable cAMPdPK activity or cAMP-BP was obtained or the cAMPdPK had a much reduced affinity for cAMP or lower Vmax than normal (Steinberg et al., 1977). Lasser and Daniel (1976) demonstrated that the transition from a cAMP-sensitive to cAMP-resistant lymphoma cell phenotype is related to a structural alteration in the regulatory subunit of cAMPdPK which also affected its affinity for cAMP and interaction with the catalytic subunit. Regulatory and catalytic subunits of the cAMP-sensitive lymphoma kinase were demonstrated to be more thermal labile.

According to Cho-Chung et al. (1979) a difference in the ability of two types of W256 rat mammary carcinomas to respond to B_2cAMP treatment in vivo is due to a qualitative difference in cAMP-BP, the regulatory subunit of the cAMPdPK. A correlation existed between cAMP unresponsiveness and altered cAMP binding by tumor cytosol. Cytoplasmic cAMP-BP and cAMPdPK accumulated in nuclei of regressing tumor cells, but not in nuclei of nonregressing tumor cells. The lack of nuclear binding of cytoplasmic BP was suggested to be due to a molecular lesion in the cytoplasmic cAMP-BP. This is also supported by temperature and pH instabilities. In addition, the major cAMP-BPs from responsive tumor cytosol showed electrophoretic mobilities

distinct from those from nonresponsive tumor cytosol. Nuclear uptake of a 56,000 dalton cAMP-receptor complex and phosphorylation of a 76,000 dalton nuclear protein was shown. Nuclear cAMP binding was increased three-fold after B2cAMP treatment of responsive mammary carcinoma, accompanied by a 50% loss in total cytoplasmic cAMP binding. These results suggest that nuclear accumulation of cAMP-BP may play an important role in the nuclear events and cAMP-mediated control of growth in mammary tumors. Anderson and Mendelson (1975) observed different binding patterns of cAMP to nuclear extracts from mammary glands of normal rats and a transplantable R-35 mammary tumor and suggested that an altered cyclic nucleotide binding pattern may be a fundamental defect associated with mammary carcinogenesis.

Another study demonstrated that nuclear uptake of the cAMP receptor protein is related to the phosphorylation of a 76,000-dalton nuclear protein and provides additional evidence that cAMP is involved in the nuclear events of hormone-dependent mammary tumor cells. Injection of 17-β-estradiol induced tumor regrowth, increased the number of estrogen receptors and decreased cAMP binding which suggests an interaction between the steroid hormone and cAMP during regression of mammary carcinomas (Cho-Chung et al., 1979).

Little data is available on cAMPdPK in human mammary tumors. Bechtel et al. (1978) observed that progressive dedifferentiation and neoplasia were associated with increased specific levels of cAMP and cAMP-dependent protein kinase in human mammary tumors. Eppenberger et al. (1977) reported higher cAMP binding activity and cAMPdPK activity in human primary mammary carcinomas when expressed on the

basis of wet tissue weight or protein content, but lower than adjacent normal tissue on the basis of cell number. Based on the DNA content of tissues, Majumber (1977) observed significantly lower cAMP-binding and cAMPdPK activities in C3H mouse mammary carcinoma.

1.3. Characterization of Adenylate Cyclase

Adenylate cyclase is a highly regulated, complex enzyme, and an integral component of the plasma membrane (Sutherland and Rodbell, 1958). Rodbell (1969) has proposed a three component model of hormone-responsive membrane-bound AC. The receptor, located on the exterior membrane surface of the target cell, serves as the binding site for the effector. The catalytic site on the inner membrane surface possesses binding sites for ATP and Mg^{2+}, and generates cAMP at the cytoplasmic face. An intermediate coupler, a GTP-sensitive site (G/F subunit) serves to transmit the message initiated by hormone binding to the catalytic site for inactivation of AC. The exact mode of information transduction is unknown. The "floating receptor" model of Cuatrecasas (1974) and DeHaen (1976) proposes that the receptor and catalytic unit of adenylate cyclase exist as independent molecules capable of noncovalent interaction with each other by lateral diffusion within the plasma membrane. Upon binding a regulatory ligand, a conformation change is induced in the receptor which enhances its potential for complex formation with the catalytic subunit, thereby increasing activity. In support of this model, Houslay et al. (1977) used irradiation inactivation by an electron beam to determine the apparent target size of the glucagon receptor and catalytic subunit in hepatic membranes. The simplest

model compatible with their data is that the receptor and catalytic subunits are physically and functionally separate and only couple in the presence of hormone. This model suggests hormone stimulation may be dependent upon membrane viscosity. Other evidence for this is as follows: (1) cell variants sensitive to β-adrenergic effectors have been selected that lack either the β-adrenergic receptor or the catalytic subunit (Ross and Gillman, 1977); (2) using the technique of cell fusion, a variety of hormone receptors have been transferred from one cell type to couple with adenylate cyclase of a different cell type (Schulster et al., 1978); and (3) by column chromatography of differentially detergent solubilized plasma membrane preparations. The catalytic subunit and binding sites for the regulatory ligands have been physically separated (Limburg and Lefkowitz, 1977). Although it is not clear how the subunits interact, the binding process is separate and distinct from the activation step. The activation requires the simultaneous binding of hormone to receptor and intracellular GTP to the guanyl nucleotide regulatory subunit. Most studies on the role of GTP employ the more stable GTP analog guanyl-yl-imidodiphosphate [Gpp(NH)p]. In the case of β-adrenergic receptor, the hormone signal is terminated concomitantly with the hydrolysis of GTP and GDP and inorganic phosphate at the regulatory site. The continued presence of both ligands is essential to maintain steady-state levels of active adenylate cyclase. This mechanism also accounts for the observation that non-hydrolysable Gpp(NH)p induces a permanent active state of cyclase in the presence of hormone. The rate of

Gpp(NH)p activation is accelerated by hormone and, in turn, the concentration of hormone required for half-maximal activation is reduced in the presence of guanine nucleotide.

Stoichiometric analysis of the cyclase reaction indicates that ATP is converted to equimolar amounts of cAMP and pyrophosphate, with an equilibrium constant of 0.065M at pH 7.3 and 25°C (Greengard et al., 1969). The enzyme requires bound Mg^{2+} at two sites, one of which is the catalytic subunit. Binding of the second Mg^{2+} is influenced by the action of positive effectors and allosterically enhances the reaction of the catalytic subunit with Mg-ATP. Maximum cAMP production is obtained with a Mg:ATP ratio close to two, while ATP in excess of Mg^{2+} is inhibitory (Wolff and Jones, 1971). The Km for ATP, as determined from a variety of tissues, is approximately 1-5 x 10^{-4}M (Birnbaumer et al., 1969).

Although Mg-ATP appears to be the substrate for AC, the divalent cation requirement can be satisfied by Mn^{2+} and partially by Co^{2+}. Increased activity occurring in the presence of Mn^{2+} would be due to a greater affinity of Mn^{2+} for ATP or that the Vmax of the reaction is greater with Mn-ATP than Mg-ATP. Monovalent cations such as Na^{+}, K^{+}, and Li^{+} may be either inhibitory or stimulatory (Dousa, 1972; Birnbaumer et al., 1969).

Calcium has variable effects on hormonal stimulation of AC. It is essential for ACTH activation of AC in adrenal and lipocyte membranes (Birnbaumer et al., 1970), as well as for solubilized brain AC (Johnson and Sutherland, 1973), while inhibiting LH stimulation of corpus luteum AC; and PGE_1, isoproterenol, and histamine stimulation of

polymorphonuclear leukocyte AC (Stolc, 1977). At one time Ca^{2+} effects were thought to reflect membrane alteration rather than induction of specific regulatory events. However, a low molecular weight Ca^{2+}-binding protein which mediates some of the regulatory effects of Ca^{2+} has been isolated from various tissues (Bromstom et al., 1976; Cheung et al., 1975). This polypeptide (m.w. 16,500) binds four moles of Ca^{2+} per mole protein and is referred to as calmodulin or calcium-dependent regulator (CDR). Formation of the enzyme-activator complex is dependent upon Ca^{2+} binding which confers conformation change to the activator enabling it to interact with the apoenzyme to form an active holoenzyme. Both particulate and solubilized AC, as well as soluble phosphodiesterase, have been shown to require calmodulin for full activity in some cases (Cheung et al., 1975; Lynch et al., 1977). More recently, calmodulin has been shown to exhibit other regulatory functions including the activation of certain protein kinases (Cohen et al., 1978; Schulman and Greengard, 1978). With respect to AC activity, calmodulin has been shown to increase the velocity of the reaction without affecting its apparent Km for ATP, and in its presence, the enzyme is more stable against thermal inactivation (Lynch et al., 1975). The effects of calmodulin, plus fluoride or guanine nucleotides, were additive.

Wallace et al. (1978) report an endogenous protein inhibitor of bovine brain adenylate cyclase which is Ca^{2+}-dependent. Adenylate cyclase activity of a particle fraction from rat cerebral cortex was shown to be composed of at least two components, one of which required

calmodulin (Brostrom et al., 1977). Each component was activated by GTP and synthetic analogs of GTP, although concentrations of GTP required for maximum activation of the calmodulin-dependent AC decreased with increasing calmodulin concentrations. The calmodulin-dependent component was stabilized by calmodulin, responded to increasing free Ca^{2+} concentrations biphasically (activation, then inhibition), was inhibited by a high Mg^{2+}/Ca^{2+} ratio, and composed approximately 80% of the cyclase activity as determined by NaCl-EGTA extraction. Adenylate cyclase activity not dependent upon calmodulin was inhibited by increasing free Ca^{2+} concentrations, and had elevated activity at high Mg^{2+}/Ca^{2+} ratios.

Regulation of AC by Ca^{2+} and calmodulin has also been examined in C-6 rat glial tumor cells (Brostrom et al., 1976). A biphasic response to changes in Ca^{2+} concentrations was observed for basal and epinephrine-stimulated [from 1 μm to 100 μm] AC in cell homogenates. Particulate enzyme was stimulated approximately 40% by addition of a soluble, heat stable, dialyzable component from the particulate preparation, which co-migrated with calmodulin on SDS polyacrylamide gels. Elevated levels of Ca^{2+}-calmodulin (100 to 1000 mg) were inhibitory to the cyclase reaction.

A wide variety of regulatory ligands, including peptide and glycoprotein hormones, neurotransmitters, prostaglandins, as well as fluoride and guanyl nucleotides, are thought to interact with AC for the transmission of information intracellularly. Almost all

eukaryotic AC systems are stimulated by fluoride whose effect is direct and not related to its capacity to inhibit ATPase. The mode of action of F^- is unknown, although stimulation requires the presence of Mg^{2+} (Mn^{2+}) and is poorly reversible. Membrane fragmentation is proposed to alter enzyme structure, making it susceptible to F^- stimulation, since F^- has not been shown to induce an accumulation of cAMP in intact cells.

Differences between F^- and hormonal stimulation of cAMP production include (1) the magnitude of the F^- response is more strongly temperature-dependent, (2) F^- concentration: effect curves are hyperbolic, and (3) F^- activation is less dependent on the membrane-lipid environment (Birnbaumer et al., 1969, 1970).

Stimulatory effects of hormones and F^- are noncompetitive (Birnbaumer et al., 1971). Previously, it was thought that F^- stimulated AC systems maximally, since the enzyme from most tissues was not responsive to hormones in the presence of F^-. Recently, it has been possible to achieve hormonal, as well as 5' guanyl-yl-imidodiphosphate [Gpp(NH)p]-stimulated activities to levels higher than obtained in the presence of F^-, demonstrating that F^- activation should not necessarily be considered as an expression of the catalytic capacity of the enzyme. Work by Drummond et al. (1971) indicated that F^- activation was due to an increase in Vmax of the reaction with no significant effect on the apparent Km for Mg-ATP. Alternatively, it has been postulated that F^- may stimulate AC by reducing the apparent Kd for Mg^{2+} at the proposed second binding site on the enzyme (Birnbaumer et al., 1969).

Hebdon et al. (1978) demonstrated the reconstitution of Gpp(NH)p and fluoride-stimulated adenylate cyclase activity by addition of one or more proteins solubilized from membrane preparations and separated by gel filtration in Gpp(NH)p and fluoride insensitive brain particulate adenylate prepared by differential detergent extraction. Thermal and trypsin inactivation of the reconstituted activity suggest that the two activities are functionally separable. Gpp(NH)p and fluoride interaction was a reversible and divalent cation-independent step which when followed by a divalent cation-dependent step, leads to a persistent state of enzyme activation.

A crucial step in activation of AC is the binding of guanyl nucleotides at the regulatory site. Occupation of this site by GTP or synthetic analogs sensitizes both the receptor and catalytic subunit for coupling which is promoted by hormone binding at the receptor site. Like F^- activation, the rate of GTP [or Gpp(NH)p] activation is concentration and temperature dependent. In a membrane preparation from rabbit ventricle, activation by Gpp(NH)p or fluoride reduced the Ka for Mg^{2+} (Drummond and Dunham, 1978). In membrane preparations from rat liver, guanyl nucleotides inhibited the response of adenylate cyclase to fluoride ion over the same concentration range which stimulated the response of the enzyme to glucagon (Rodbell et al., 1971). Gpp(NH)p at concentrations as low as 10 nM caused a 50% inhibition in fluoride stimulated activity. It was proposed that guanyl nucleotides regulate the action of fluoride in the system.

GTP binding has been shown to convert glucagon receptors associated with turkey erythrocyte AC into two forms: one form binds glucagon with high affinity, displays positive co-operativity of binding, and during 10-15% occupancy, demonstrates an 80% activation level; the other form possesses a lower affinity for glucagon binding (Kd shift from 2nM to 10nM) and comprises the bulk of the receptors. According to Rodbell (1975), the first class of receptors represents the coupled receptor-enzyme complex, and the latter class receptors were uncoupled.

Guanine nucleotide effects on AC have been demonstrated with GTP, but more often with GTP analogs which are resistant to hydrolysis [guanyl-5'yl-imidodiphosphate, Gpp(NH)p] and guanyl-5'yl'methylenediphosphate [Gpp(CH_2)p]. The GTP effect is transient, owing to hydrolysis by a membrane-associated GTPase activity which apparently resides at the guanyl nucleotide regulatory site serving to terminate the activation of the enzyme (Cassel and Selinger, 1976). Hydrolysis of GTP to GDP results in dissociation of the coupled subunits and decay of AC activity to its ground state level. The release of GDP is thought to be the rate-limiting step of the reaction sequence (Rodbell et al., 1975). Cholera toxin (CT), specifically the A_1 subunit, has been shown to catalyze the ADP-ribosylation of the regulatory subunit in the presence of NAD, thereby inhibiting GTPase and stimulating cAMP production. Preincubation with GTP and CTA_1 results in the same "persistent" state of activation observed after preincubation of the enzyme with Gpp(NH)p (Cassel and Selinger, 1977).

The action of GTP analogs appear to be irreversible, in contrast to GTP, so that stabilization of the activated state is of significance in isolation experiments. Following solubilization, the GTP binding component has been separated from the catalytic subunit using GTP affinity chromotography (Spiegel et al., 1979; Pfeuffer and Helmreich, 1975). Work by Iyengar et al. (1979) on hepatic AC indicated that two distinct nucleotide sites function to regulate coupling of the hormone-receptor complex to the AC moiety.

Rodbell (1975) has suggested a three-state model for the steady state kinetics of catalysis and regulation of AC by guanyl nucleotides. The enzyme can exist in (a) the basal or E state, (b) affected by nucleotides, but having low activity (transient or E' state) or (c) in equilibrium with the second and in a highly active state (final or E" state). Activation by Gpp(NH)p proceeds with a lag presumably due to slow isomerization of E' to E". Hormones, such as glucagon in the case of liver AC, stimulate enzymic activity by accelerating this E' to E" isomerization, and by shifting the equilibrium between these two states towards the highly active E" form. Evidence that activation by Gpp(NH)p may be associated with the dissociation of a guanyl regulatory subunit has been obtained by studies of the effects of guanyl nucleotides in catecholamine-sensitive AC of erythrocyte membranes (Pfeuffer and Helmreich, 1975). In studying activation of adipose tissue AC, Cuatrecasas et al. (1975) proposed that activation by guanyl nucleotides is the result of the formation of a covalent enzyme-PP or enzyme-P(NH)P complex. Based on kinetic data, it is suggested that normal stimulation of AC by

hormones, in the presence of GTP, is the result of an increased rate of formation of a highly active, unstable enzyme-PP complex. Activation of the enzyme by Gpp(NH)p is both slow and irreversible because of low rates for formation of the active and highly stable enzyme-P(NH)p complex, coupled with little complex decay.

Nonnucleotide cellular regulators in addition to calmodulin have been discovered which influence β-adrenergic catecholamine-responsive AC in rat reticulocytes and erythrocytes (Shane et al., 1981). Rat liver cytosolic fractions have also been reported to stimulate membrane-associated AC (Pecker and Hanoune, 1977).

The isolation of cAMP by Sutherland and Rall (1958) marked the first step in the development of a comprehensive theory of hormone action. Intracellular cAMP levels are usually less than 0.1 μM in the unstimulated state, increasing a hundred fold or more in the presence of hormones (Robinson, 1968). Most of the actions of peptide and protein hormones, as well as many biogenic amines, result from primary interaction with a membrane-bound receptor and the catalytic and regulatory subunits of AC, leading to the formation of the second messenger, cAMP (Robison et al., 1968). An excellent account of the biochemical properties of hormone-sensitive AC is given by Ross and Gilman (1980). The mechanism by which the occupied hormone receptor stimulated cyclase activity is unknown, particularly the process which assures hormonal specificity and the nature of the signal: effect coupling which leads to AC activation. Singer (1976) has proposed that ligands may induce "clustering" of receptors in

membranes. Microtubules, as well as microfilaments, are closely associated with the plasma membrane, and are probably involved in the control of receptor mobility, redistribution, and hormonal responsiveness of AC by amplification of the associated enzyme reaction involved (Zor, Strulovici and Lindner, 1978).

Adenylate cyclase in broken cell preparations of membrane fractions is hormonally sensitive in vivo. For example, the enzyme from particulate preparations of myocardium is stimulated by catecholamines and glucagon (Murad et al., 1962); from kidney by vasopressin and parathyroid hormone (Melson et al., 1970); from liver by catecholamines and glucagon (Ray et al., 1970); from erythrocytes by epinephrine (Rosen and Rosen, 1969) and lipocytes by a variety of lipolytic hormones (Birnbaumer and Rodbell, 1969). The interaction of hormones with their receptors resulting in the activation of AC is totally and rapidly reversible as originally demonstrated in glucagon-stimulated AC from rat liver plasma membranes (Birnbaumer et al., 1972). The concentration effect curves for hormonal activation of AC appears to follow Michaelis-Menten kinetics with Hill coefficients close to one.

Phospholipids play a key role in hormone-induced activation of AC and solubilization of AC from brain, heart, skeletal muscle, and liver tissue abolishes or diminishes hormone responsiveness (Sutherland et al., 1962). The effects of varying membrane lipid composition and viscosity on AC activity in mouse fibroblasts has been examined (Engelhard et al., 1976). The relationship of membrane phospholipids to hormone-responsive AC in liver, thyroid, and kidney have also been

intensively investigated. Loss of [^{125}I] glucagon binding and glucagon activation of AC in liver membranes due to phospholipase treatment could be partially restored upon addition of membrane lipid extracts or pure phospholipids (Birnbaumer et al., 1971; Rodbell et al., 1971b; Pohl et al., 1971). Restoration of noradrenalin/catecholamine and glucagon/histamine responsiveness to solubilized myocardial AC could be obtained by addition of monophosphatidyl inositol and phosphatidyl serine, respectively (Levey, 1973). TSH activation of thyroid AC is partially restored with phosphatidyl choline (Yamashita and Field, 1973). In contrast, work by Neer (1973) with Lubrol PX-solubilized renal medulla AC demonstrated that removal of detergent by DEAE cellulose chromatography restored vasopressin-responsiveness without the addition of exogenous phospholipids.

Membrane phospholipids are considered to act primarily at the coupler site. The catalytic site appears to be independent of phospholipds since solubilized or phospholipase-treated AC retain F^- responsiveness under mild treatment, and hormone binding remains intact (Levey, 1973).

The concept that hormonal activation of AC is regulated by nucleotides is based on observations that using low ATP or AMP-P(NH)P concentrations, hepatic plasma membrane AC exhibits an almost absolute requirement for GTP to show activation by glucagon (Rodbell et al., 1971). Similar findings have been reported for other AC systems (Rodbell et al., 1971b). Cuatrecasas et al. (1975) proposed that

hormonal activation is the result of a two-step process with the formation of the hormone-receptor complex, immediately followed by coupling to the catalytic unit of AC. Constantopoulos and Najjar (1973) proposed that activation of this enzyme is the result of a dephosphorylation reaction. They also suggested that inactive or basal adenylate cyclase activity is given by a phospho-form of the enzyme. Direct support for these two hypotheses is lacking.

Another model of adenylate cyclase activation suggests that cyclase systems are 'restrained'. The restraining elements would be subunits of the system, such as free hormonal receptors (Levey et al., 1974), a nucleotide-binding protein (Pfeuffer and Helmreich, 1975), a fluoride-binding component (Schramm and Naim, 1970) or the phospholipid matrix of the membrane itself. According to this model, the appearance of hormone-responsiveness is associated with a decrease in basal activity, as documented by LH activation of AC in developing rat ovaries (Hunzicker-Dunn and Birnbaumer, 1976). Coupling of the hormone receptor to AC may result in restriction of catalytic activity, so that hormonal stimulation occurs due to release of inhibition via uncoupling of the receptor-catalytic subunit complex, similar to the mode of activation of protein kinase by cAMP (Channing and Kammerman, 1973; Brostrom et al., 1970). Levey et al. (1974) have provided evidence in favor of dissociation being involved in glucagon activation of cardiac AC. Following Lubrol PX solubilization, AC activity and glucagon binding co-migrated on Sephadex G-100 chromatography (Mr 100,000-200,000). Preincubation with glucagon prior to

chromatography resulted in loss of co-migration and a complex of 28,000 daltons containing bound glucagon, suggesting that interaction of the hormone with the solubilized complex results in dissociation of a glucagon-binding component.

Finally, the classical model of hormone regulation of AC interjects a system composed of regulatory and catalytic subunits with allosteric receptor sites for hormones, regulatory nucleotides, and possibly divalent cations and F^-. Robison (1967) originally proposed activation by hormones to be a result of hormone:receptor coupling. The key difference from the models of Rodbell et al. (1975) and Cuatrecasas et al. (1975) is that activity seen in the presence of nucleotide and hormone is the result of a tertiary complex and that nucleotides do not necessarily play an intrinsic obligatory role in hormonal stimulation.

The mechanism of action of prostagladins is unclear, although Kuehl and Humes (1972) speculate that these fatty acids interact with either the coupler or catalytic site of AC. Prostaglandins may act as allosteric effectors of AC by inducing phase changes in membrane components, specifically phosphatidyl serine and phosphatidyl inositol, resulting in enzyme activation (Johnson and Ramwell, 1973).

Mammalian AC have been solubilized from membranes following the initial studies of Sutherland et al. (1972) and progress in purification is being made. Nonionic detergents such as Triton, digitonin, and particularly Lubrol PX, an ethylene oxide condensate of dodecanol, have been used to extract AC from heart, brain, liver, testis, kidney,

adrenal cortex and erythrocytes. Detergent-free molecular weight estimates in the 150,000 to 200,000 dalton range have been made, but these results from crude preparations are as yet tentative (Robison, et al., 1968).

Soluble activity is generally expressed as the percent of activity determined in the particulate fraction. A characteristic feature of the soluble enzyme is loss of hormonal responsiveness, presumably due to the uncoupling of the hormone receptor and catalytic unit. Other properties may also be affected. For example, the solubilized, unstabilized enzyme from rat hepatic membranes demonstrated properties different from the solubilized enzyme from membranes pretreated with Gpp(NH)p such as weak stimulation by Gpp(NH)p, decreased inhibition by adenosine, a strong inhibition by phosphate and pyrophosphate, and the apparent loss of regulation by divalent cations (Londos et al., 1979).

Success in solubilization of active adenylate cyclase from membranes seems to depend on the state of activation of the enzyme. Preincubation of the particulate fraction with Gpp(NH)p or fluoride results in an increase in the amount of activity solubilized, as well as a more stable preparation, although the percent yield may still be quite low.

Treatment of mouse LM (fibroblast) membranes with nonionic detergents, organic solvents or phospholipases changes either the basal or hormonal-stimulated adenylate cyclase activity or both (Engelhard et al., 1976). Treatment with ethanolamine, elaidate,

choline, or lineolate supplements restored these activities to a degree dependent upon the nature of the phospholipid polar head. Actual increases in fluoride and PGE_1-stimulated activity could be demonstrated. Houslay (1977) has fused phospholipids with membranes and observed changes in activity and temperature dependence of adenylate cyclase. Using a solubilized preparation from rat heart, Levey (1971) has demonstrated a critical requirement for acidic phospholipids in the activation of adenylate cyclase following ligand binding, i.e., phosphatidyl serine was required for glucagon and histamine activation of detergent-free myocardial enzyme, whereas inositol was required for catecholamine activation.

Neer (1974) observed that soluble renal AC binds small amounts of detergent (approximately 0.2 ng/mg protein) and that no greater than 5% of the enzyme surface is involved in hydrophobic interaction with renal membrane components. This may indicate that AC is attached to the inner membrane surface, not embedded deeply in the lipid matrix. In this scheme, the hormone receptor on the exterior surface of the cell would have a large intrinsic hydrophobic portion which interconnects with AC.

Examples where the reconstitution of functional AC systems has been successful include: (1) activation of ram sperm AC through restoration of guanine nucleotide regulatory component prostaglandin receptors in human erythrocyte membranes (Stengel and Hanoune, 1981), (2) restoration of hormone, guanine-nucleotide and F^- stimulated AC to membranes of S49 lymphoma (cyc-) cells by addition of

detergent extracts of membranes containing the guanine-nucleotide regulatory component (Howlett et al., 1979), and (3) coupling of the catecholamine receptor in turkey erythrocytes to the catalytic subunit in mouse erythroleukemia friend cells (Orly and Schramm, 1976).

1.4. Characterization of Cyclic AMP-Dependent Protein Kinase

Phosphorylation of proteins is recognized as a process of reversible covalent modification that functions in the rapid regulation of a variety of cellular processes. Protein kinases catalyze the transfer of phosphate to serine, threonine (tyrosine) residues in substrate proteins. Features which make a substrate susceptible to phosphorylation by a given kinase are just on the verge of being understood, and are most complex in nature, as exemplified by muscle glycogen synthetase which is phosphorylated by at least three different kinases at six specific sites (Embi et al., 1980). Local primary protein structure seems to play a major role in determining substrate specificity of cAMP-dependent protein kinase (Kemp et al., 1975). Amino acid sequence analysis of substrate proteins suggests the presence of two adjacent basic amino acids on the N-terminal side of the susceptible serine or threonine residue may be critical for recognition (Yeaman et al., 1977). The molecular basis of substrate specificity has been studied by use of synthetic peptide analogs corresponding to phosphorylatable regions of native proteins (Chan et al., 1982). It appears that secondary structure may also play an important role in that NMR studies indicate that cAMP-dependent protein kinase prefers peptide substrates with either β-turn or β-coil

conformation (Graves et al., 1978). Two well-defined structural domains appear to retain the tertiary structure of cAMP-protein kinase type I (Potter and Taylor, 1979). Following limited proteolysis, the larger domain retained cAMP-binding activity, corresponding to the COOH-terminal end of the peptide chain, whereas the smaller domain was associated with maintaining the dimeric structure of the regulatory subunit. Characterization of small cAMP-binding fragments of cAMP-dependent protein kinase has aided in defining the minimal structure necessary for cAMP-binding (Rannels and Corbin, 1979).

Cyclic AMP-dependent protein kinase (cAMPdPK) exists as two isoenzyme forms in most mammalian tissues, designated type I and type II on the basis of their elution profiles from DEAE cellulose (Krebs, 1972; Rubin and Rosen, 1975). Prototype enzymes characterized for types I and II PK were isolated from rabbit skeletal muscle and bovine heart, respectively. The ratio of type I/type II kinase activity varies dramatically in tissues, although cAMPdPK concentrations remain similar (Walter et al., 1978). In contrast to the universal occurrence and relatively fixed concentrations of cytosolic protein kinases, the abundance of membrane-associated protein kinases is highly variable, being virtually absent in some tissues, but accounting for a significant proportion of total kinase activity in other instances [(e.g. brain, human erythrocytes, rabbit heart, corpus luteum (Rubin, 1979)]. Physicochemical properties of membrane-bound protein kinase closely parallel characteristics of cytosolic protein

kinase I, but are dissimilar from those of soluble type II enzyme. Both types of holoenzymes are tetrameric, consisting of a regulatory dimer (R_2) and two catalytic (C) subunits. Dimers of purified regulatory subunits have been shown to be covalently cross-linked by interchain disulfide bonding (Zick and Taylor, 1982). The tissue concentration of subunits range from 0.3 μM to 1.0 μM. In the presence of saturating concentrations of cAMP, enzyme activation occurs by the binding of four moles of cAMP to each mole of PK to form an R_2 $(cAMP)_4$ complex. The stoichiometry of the reaction is thus: $R_2C_2 + 4$ cAMP $\rightleftharpoons R_2(cAMP)_4 + 2C$. This dissociation of the holoenzyme occurs concomittant to the activation of the C subunits for phosphorylation of substrate proteins (Corbin et al., 1978; Beavo and Mumbly, 1980).

Despite similar substrate specificity and activation properties, the in vivo function of types I and II PK is thought to be determined by their intracellular location (Corbin et al., 1972). In addition to different elution patterns from anion exchange resins, type I and II kinase differ with respect to association-dissociation properties (Hofman et al., 1975), effect of Mg.ATP (Corbin, Keely, and Park, 1975), and autophosphorylation (Rosen and Erlichman, 1975). Type I PK elutes from DEAE chromatography at relatively low salt concentrations (approximately 60 mM NaCl), and is easily dissociated by 0.5 M Nacl or histone. Type II PK elutes with 130-160 mM NaCl and dissociates slowly upon addition of 0.5 M NaCl or histone (Hofmann, 1975). Kupfer et al. (1980) showed that salt-induced conformation change of the catalytic subunit of cAMPdPK occurs under conditions of neutral

pH and physiological ionic strength. An additional difference is the binding of cAMP at low pH (Haddox, Nicol, and Goldberg, 1973).

Major structural differences supposedly occur only in the R subunits, as both types of kinase have nearly identical C subunits (Zoller et al., 1975). R^1 and R^2 have apparent molecular weights of 49,000 [R^1(49)] and 54,000 [R^2(54)], respectively. R^2 is autophosphorylated by the C subunit (Rosen and Erlichman, 1975), whereas R^1 is phosphorylated by a cGMP-dependent protein kinase. The amino acid sequence surrounding this site of phosphorylation by cGMPdPK has been determined (Hashimoto et al., 1981). That R^1 and R^2 differ has been documented by amino acid composition and tryptic-peptide mapping. The two subunit types differ antigenically, and occur in both soluble and particulate forms (Corbin et al., 1977).

Catalytic and regulatory subunits from unrelated species can form heterologous holoenzyme hybrids (Miyamoto et al., 1973). Evidence indicates conservation of the C subunit, as well as the C-binding domains of R subunits (Schwoch et al., 1980; Weber et al., 1980). The heterogeneity of R subunits demonstrated in some tissues may be due to limited proteolysis (Talmadge et al., 1977; Weber and Holz, 1978). Tissue specific patterns of R^1 and R^2 subunit variants have been demonstrated in normal and leukemic human lymphocytes, human renal and mammary tumors, and Hela cells using R-type specific antibodies and a photoaffinity labeling technique involving [^{32}P] azido-cAMP (Weber et al., 1980). The principal R subunit identified in human mammary tumors was R^1 (49), although an additional R^1 variant,

a 37,000-m.w. component, was also found. R^2 subunit variants include 34,000, 49,000 and 50-000-m.w. isoproteins. Of interest is the observation that R^2 (50) existed in all human tumors tested, and in several cases, represents the only R^2 type regulatory subunit.

The question arises whether differentiation and cell proliferation are determinants of R-isoprotein patterns. Changes in the ratio of PKI/PKII during the cell cycle (Costa et al., 1976); in differentiation processes (Richard and Rolfes, 1980; Eppenberger et al., 1979); in hormone-induced hypertrophy (Russel, 1978); in hormone-responsive mammary tumors (Cho-Chung et al., 1979); and viral transformed 3T3 cells (Gharret et al., 1976) have supported the idea that PKII is primarily involved in the differentiation process, where as PKI relates to cell proliferation. Other data suggest association of PKII with cell proliferation (Boynton and Whitfield, 1980), however, it appears that attribution of altered ratios of kinase exclusively to one or the other of these processes is impossible. It is interesting that R^1 and R^2 may be under separate hormone control, and are differentially expressed in response to steroid hormones in various tissues (Fuller et al., 1978). Genetic analysis of somatic cell mutants unable to respond to cAMP have been studied in an effort to understand the mechanism of cAMP action in cultured mammalian cells. Mutant Chinese hamster ovary (CHO) cells which were resistant to growth inhibitory effects of cAMP and are characterized by having a defective catalytic subunit, and consequently, a cAMP-insensitive protein kinase, provides strong evidence that growth inhibitory and

morphological effects of cAMP are mediated by cAMP-dependent protein kinase (Evain et al., 1979).

Although biochemical and structural changes associated with cellular differentiation and proliferation do not appear to be associated with alterations of only one type of cAMPdPK, type specificity may be related to translocation of soluble PK activity. Work from several laboratories suggest that the type II kinase or R^2 subunit exhibits an affinity for nuclear or nuclei-containing particle fractions (Schwartz and Costa, 1980; Boynton and Whitfield, 1980).

Cyclic AMP receptor proteins have been identified and characterized by the use of a photoaffinity label 8-azido [^{32}P] cAMP (Walter et al., 1978) and purified by cAMP affinity chromatography (Walter et al., 1977; Dills et al., 1979). The function of R^1 and R^2 as inhibitory subunits of cAMPdPK is well established (Corbin et al., 1981; Walter et al., 1981). Recent findings indicate that R^1 and R^2 may exist as free cAMP binding proteins, allowing protection of cAMP from destruction by phosphodiesterase (Prashad, 1981). Two heat stable inhibitors, thought to be altered regulatory subunits of cAMPdPK, a type I inhibitor isolated from rabbit skeletal muscle, and a type II inhibitor from bovine heart tissue, have been purified and partially characterized (Walsh et al., 1971; Krebs et al., 1979). The inhibitor from skeletal muscle promotes a five-fold increase in the binding constant of cAMP to PK. Heat stable TCA precipitable inhibitors have also been resolved in other tissues (Szmigielski et al., 1977; Talmadge et al., 1975). The existence of free cAMP-binding

proteins other than the regulatory subunits of PK has been reported (Tsuzuki and Kiger, 1975). In mouse neuroblastoma cells, the amount of cAMP-binding protein, designated R^1 was increased by papaverine (PDE inhibitor), PGE, and B_2cAMP without affecting either the levels of PKR subunits or PK activity (Prashad et al., 1980). R^1 was shown to differ on the basis of (1) peptide mapping, (2) isoelectric point, (3) mobility on urea gels, (4) susceptibility to guanidine HCl, (5) reconstitution with the C subunit, and (6) turnover rates. The R^1-cAMP complex protects cAMP against phosphodiesterase, and may be functional in maintaining intracellular concentrations of cAMP (Cheung and Patrick, 1974).

The binding of cAMP to regulatory dimers of type I cAMPdPK (R_2^1) occurs readily under physiological conditions (apparent Kd for cAMP—6nM) and exhibits slight positive cooperativity. This is lost when ATP, which binds with high affinity to the type I holoenzyme (apparent Kd—35nM), is present (Hoppe et al., 1978). According to Hofman et al. (1975), ATP decreases the affinity of the enzyme for cAMP by a factor of 40 (to approximately 0.23 μM). No comparable high affinity site has been detected with bovine heart type II kinase. Isolated subunits of type I kinase recombine at stoichiometric concentrations slowly, or not at all in the absence of MgATP, but in the presence of MgATP, recombination occurs readily, suggesting that MgATP does not exert its effect on cAMP binding by competing with cAMP for a binding site, but does so by facilitating recombination of dissociated subunits.

The binding of cAMP to the type II cAMPdPK regulatory dimer does not exhibit cooperativity, whereas cAMP binding to the type II holoenzyme exhibits negative cooperativity (Buss et al., 1979; Hofman et al., 1975). Binding of cAMP to type II kinase requires rather high concentrations of cAMP (apparent Kd—2.8 μM) when physiological concentrations of enzyme are used. Addition of MgATP, resulting in phosphorylation of the R^2 subunit lowers the apparent Kd to 0.5 μM and abolishes negative cooperative binding. Isolated subunits recombine readily in the absence of MgATP, however, phosphorylation of the R^2 subunit retards this recombination. It seems the presence of MgATP or autophosphorylation of R^2 changes the cAMP binding charcteristics by modulating the interaction of the catalytic and regulatory subunits.

Dissociation of PK holoenzymes occur both <u>in vitro</u> and <u>in vivo</u> in response to increased cellular cAMP following hormonal stimulation of tissue. Soderling et al. (1973) demonstrated that epinephrine and insulin modify the activity state of cAMPdPK through changes in cAMP concentration in adipose tissue. Glucagon, epinephrine, and MIBX, a PDE inhibitor, were shown to increase levels of cardiac cAMP and activation of both isozymes in a dose-dependent manner (Kelly et al., 1975; Corbin and Keely, 1977). Alterations in membrane protein phosphorylation were shown to mediate the effects of isoproterenol on AC activity in rat cortical membranes (Whittemore et al., 1981). Likewise, hCG and B_2 cAMP were found to induce phosphorylation of multiple proteins in Leydig cells (Dufau, Sorrell and Catt, 1981).

According to Hayes et al. (1980), a distinct pool of cAMPdPK may be activated by isoproterenol and PGE in cardiac tissue. Hormonal specificity most likely depends upon a precise spatial and temporal relationship of the receptor-AC complex, cAMP levels, PK and substrate. The arrangement of these components into functional units (or compartments) within the cell may play an important role in the regulation of metabolic events by cyclic nucleotide-dependent mechanisms.

1.5. Statement of Objectives for the Biochemical Approach

It is reasonable to assume that a regulatory enzyme system could be used as a biological marker for the early detection of cancer and to establish a grading system for different tumor types. If modulation of cAMP levels in tumor tissue does in fact occur, this may be of clinical importance as a signal for early stages of cell transformation and tumor differentiation. Therefore, the aim of this project is directed towards characterizing adenylate cyclase and cAMP-dependent protein kinase activities associated with malignant and benign human breast tumors with respect to alterations in catalytic and regulatory properties. The investigation presented in this treatise was conducted in order to establish fundamental information on the activities of two enzymes essential to cAMP metabolism in human mammary tumors, adenylate cyclase and cAMP-dependent protein kinase. In choosing this biochemical system to be studied, the following characters were considered favorable: (1) evidence for cAMP function in the regulation of cell growth, (2) membrane

association of adenylate cyclase, which synthesizes cAMP, so as to explore the potential for system effects due to cell membrane alteration during neoplasia, (3) hormonal responsiveness for adenylate cyclase, underlined by observations of endocrine and steroid hormonal modulation of tumor regression, (4) and the fact that cAMP effects mediated by cAMP-dependent protein kinases may alter nuclear function, so that possible cellular defects in the cAMP system at the membrane, cytoplasmic, and nuclear level may be examined.

The histological complexity of human breast tumors requires the adaptation of special techniques for the delineation of biochemical events characteristic of neoplastic areas and not associated with nonproliferating adipose, connective tissue and other supportive elements. Previous work has used entire specimens for use in quantitative and qualitative analyses. However, such results can be expected to supply information about the sum of tissue types represented, and not tumor-specific events.

The approach taken in this study was to investigate the enzymatic profiles of pure fractions of tumor cells isolated from stromal and ductal regions of tumor tissue samples by microdissection. With the development and application of microanalytical techniques which permit repeated analyses on visually confirmed samples of neoplastic tissue, such a characterization of AC and cAMPdPK has been possible in a series of malignant and benign human mammary tumors. The microanalytical techniques employed were supplemented by partial purification and characterization of the chosen enzymes on a larger scale,

utilizing both cytosolic and membrane particle fractions.

The proposed work will demonstrate the potential usefulness of the microtechnique described as a diagnostic tool based upon the characterization of tumor-specific enzymes and a comparison of these characteristics established with those obtained using whole tumor homogenates. The advantages to such an approach have been previously described; the limitations of the microanalysis will be discussed. It is possible that the cancer cell's ability for continuous proliferation and failure to differentiate may be related to the lack of a functional cAMP signal to suppress cell division. The hypothesis that the growth characteristics of the cancer cell are associated with an alteration in cyclic nucleotide metabolism will be examined.

The enzyme profiles will include the following parameters:

(A) <u>adenylate cyclase</u>: (1) total specific activity, (2) cytological distribution, (3) Km for ATP, (4) temperature and pH stability, (5) regulation by Ca^{2+} and calmodulin, (6) hormone responsiveness, (7) activation by GTP or GTP analogues, and (8) the effect of cholera toxin.

(B) <u>cAMP-dependent protein kinase and cAMP binding protein</u>: (1) cytological distribution, (2) rate of phosphorylation of a number of different proteins, (3) pH and temperature stability, (4) affinity constants for ATP and Mg^{2+} in the presence and absence of cyclic nucleotides, (5) affinity constants (Kb) of binding protein for cAMP, (6) extent of stimulation (Ka) by cAMP, (7) calcium regulation,

(8) presence of protein kinase "inhibitor protein", and (9) physiochemical differences in cAMP binding proteins, or regulatory subunits of PK.

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Experimental Materials: Histological stains and tissue-Tek mounting medium were obtained from Arthur H. Thomas Co. (Philadelphia, PA). Theophylline, 3' isobutyl-1' methylxanthine, adenosine, 5'AMP, histones, casein, protamine, cyclic nucleotides, ATP, GTP, GPP(NH)p, cholera toxin, succinyl cyclic AMP tyrosine methyl ester, benzamidine, cyclic AMP-dependent protein kinase, protein kinase inhibitors, regulatory and catalytic subunits, cAMP agarose, and DEAE-Sephacel were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals used were reagent grade, purchased from Sigma Chemical Co. $Na^{125}I$(10 Ci/mmole) was supplied by Collaborative Research (Waltham, MA) and anti-cAMP serum provided by Squibb (Princeton, NJ). ^{3}H-cAMP (27 Ci/mmole) and $-^{32}P$ ATP (25 Ci/mmole) were obtained from ICN Pharmaceuticals, Inc. (Irvine, CA).

Acetylating reagents, acetic anhydride and triethylamine were purchased from Fisher Scientific (Fairlawn, NJ). Hormones from human sources were generously donated by the National Pituitary Agency, NIAMDD (Baltimore, MD). Whatman 3IET paper was provided by Whatman Inc. (Clifton, NY) and Metricel GN-6 filters obtained from Gelman Filtration Products (Ann Arbor, MI). All supplies for polyacrylamide gel electrophoresis were from BioRad Laboratories (Richmond, CA), with the exception of Coomassie brilliant blue G-250 from Bakers Chemical Co. (Phillipsburg, NJ), Ampholites from LKB Corporation (Bromma, Sweden), and silver nitrate from Sigma Chemical Co. (St.

Louis, MO).

2.1.2. Tissue Specimens: Frozen specimens of human breast tumors were provided through arrangement with resident pathologists of three hospitals in the Roanoke, Virginia area. Specimens were frozen within 30 minutes after surgical removal, transported from the hospital on dry ice, and held in a Revco freezer at -76°C until sectioned. Samples of each tumor retained by the hospital were fixed, stained and evaluated by the attending physician on the basis of histological criteria for aggressiveness, i.e. degree of tissue differentiation, invasion, pleomorphism, and number of mitoses. Pertinent sections from the pathology reports were forwarded to the university laboratory for subsequent comparison with frozen tissue sections.

Other tissues were used to develope the enzyme assay techniques including rat brain, rat liver, and bovine heart. In all cases, these tissues were treated as described for the human breast tumor.

2.2. Methods

2.1.1. Tissue Sections: Histological slides were prepared for each tumor in order to identify specific intracellular areas within the lesions. Frozen hospital specimens were mounted in embedding medium and sectioned in a microtome cryostat at -28 to -30°C. Sections which were to be stained were cut to a thickness of 4 to 12 μm, depending upon the tissue consistency. Differential staining with hematoxylin and eosin followed. Five to eight thicker (28-32 μm) sections were made for each stained section in a sequential manner throughout each

specimen. The thick sections were subsequently lyophilized in groups and stored under reduced pressure at -76°C in screw cap vials (No. 10-159-10, Virtis Co., Gardiner, NY). A variety of enzymes of diverse metabolic function have been found to be quite stable in these sections for a period of years (Gutmann, 1978).

2.2.2. Sample Preparation

2.2.2.1. Tissue Homogenates: Washed particle fractions from malignant and benign breast tumors are prepared according to the procedure of Johnson and Sutherland (1973). Similar preparations from rat brain and liver are used to monitor the sensitivity of the radioimmunoassay for adenylate cyclase. Skin and fat were removed from the tumor specimens when necessary. Whole tumors or tumor pieces were homogenized with a Polytron homogenizer (PT20), followed by a Dounce ground glass hand homogenizer, at 4°C in 8 volumes of 0.1 M glycylglycine buffer, pH 7.5, containing 0.25 M sucrose and 3 mM DTT. The homogenate is centrifuged at 3000 x g for 10 minutes, and the pellet resuspended and washed twice in fresh buffer. Aliquots of washed particle suspensions were frozen and stored at -76°C.

For the preparation of tumor cytosolic fractions for analysis of PK activity, tumors were homogenized with a Polytron homogenizer at 4° C using 4 parts (w/v) of 10 mM Tris-HCl, pH 6.8, containing 1.5 mM EDTA, and 5 mM theophylline, and centrifuged at 30,000 x g or 105,000 x g for 30 or 90 minutes, respectively. In some cases, streptomycin sulfate is added to a final concentration of 2% and the

supernatant fractions clarified by centrifugation to eliminate nucleic acids. The cytosolic fractions are assayed for PK activity directly or ammonium sulfate added to a final saturation of 50%, the suspension stirred for 1 hour, centrifuged at 10,000 x g for 20 minutes, and the pellet resuspended in 10 mM Tris-HCl, pH 6.8, containing 6 mM 2-mercaptoethanol, then dialyzed against the same buffer at 4°C overnight. The streptomycin sulfate-treated or ammonium sulfate-precipitable cytosolic fraction was subjected to chromatography on DEAE-Sephacel for further resolution.

2.2.2.2. Specimen Micro-dessection: On the day an assay was to be performed, a vial containing the desired lypophilized sections was removed from the freezer and allowed to come to room temperature. Sections were removed and examined under a dissecting scope. "Landmark" features were located which correspond to those of adjacent histologically stained sections. Tandem observation of a stained section and lyophilized sections facilitated the delineation of tumor-specific regions within the non-stained material. The lyophilized section was placed atop a 4" x 4" square of Plexiglass resting freely on the stage of a dissecting microscope. To control static electricity a small piece of radium foil was suspended via a copper wire above the dissecting surface. The structure was brought into focus in the field of view at a magnification (10X-40X) and at a level of illumination providing maximum clarity of detail. Cutting was done freehand with a microscalpel, or one constructed from a small section of razor blade attached via a short length of thin spring steel wire to a

thicker copper wire anchored in a wooden dowel rod. Hair points of varying stiffness and degree of taper were similarly constructed, except that the hair was cemented directly to the copper wire.

After the desired number of samples had been obtained, they were aligned on a small strip of black cardboard and brought to a quartz fiber "fishpole" balance for weighing (Lowry and Passonneau, 1972). Samples were handled and transported on the tips of hair points or quartz fiber tips cemented to a hair tip.

2.2.3. Measurement of Dry Weight: Specific enzyme activity was expressed per unit dry weight as measured by the quartz fiber balance (Figure 1). The balance was constructed from quartz fiber mounted horizontally in a chamber made from a 3 ml test tube. To the free end of the fiber a very light glass pan was attached. A sliding glass door prevented interference by air currents. The sample was placed on the pan with a hair point, the door closed, and the displacement of the fiber tip measured with an ocular micrometer of variable magnification. Static electricity was controlled by a strip of radium foil mounted above the chamber opening. All mechanical manipulations were observed through a dissecting stereoscope using a fiber optic illuminator. The entire apparatus was solidly mounted on a heavy metal stand.

The balance was calibrated colorimetrically using p-nitrophenol as described by Lowry and Passonneau, (1972). Briefly, after selecting the appropriate objective and eye-piece lenses to give the desired magnification of the tip of the quartz fiber, small crystals of p-nitrophenol were placed on the pan. Various crystal sizes were

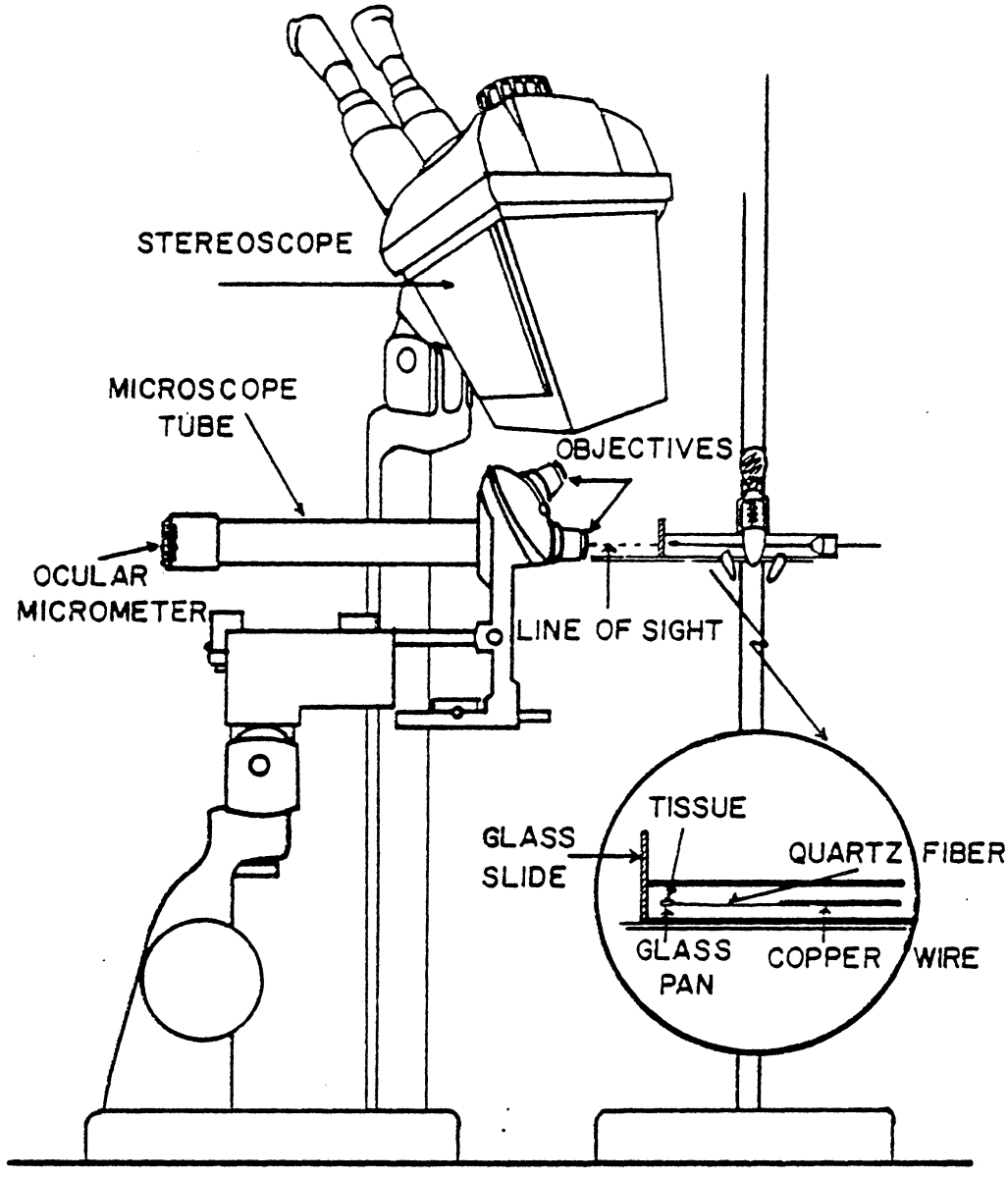


FIGURE 1

Quartz fiber balance. The apparatus was used for measuring dry weight of microgram and nanogram quantities of tissue sections as described in Materials and Methods.

used and the degrees of tip displacement recorded. The crystals were dissolved in 500 µl of 50 mM carbonate buffer (pH 10.0) and the optical density of the solution read at 500 nm. The exact weights of the crystals were calculated from readings made with a 3.6×10^{-5} p-nitrophenol standard solution. The number of micrograms dry weight per tissue sample was calculated on the basis of deflections of the quartz fiber tip. Micro-pipettes were also calibrated spectrophotometrically with p-nitrophenol standard solutions.

2.2.4. Protein Determination: Protein measurements were made according to the procedure of Lowry et al. (1951) using bovine serum albumin as the standard.

2.2.5. Preparation of ^{125}I-TME-ScAMP: Synthesis of the radioligand was accomplished by the method of Steiner et al. (1972) as described by Brooker et al. (1979). Succinyl cyclic AMP tyrosine methyl ester (ScAMP-TME) was made to 60 µM in 5 mM sodium acetate, pH 4.75. Twenty microliters of 0.5 M potassium phosphate, pH 7.0, were added to 1 mCi Na ^{125}I, followed by 20 µl of the ScAMP-TME preparation. After mixing, the iodination reaction was initiated by addition of 5 µl chloramine T (1 mg/ml in 0.5 M potassium phosphate, pH 7.0). The reaction was terminated after 60 seconds by the addition of 50 µl sodium metabisulfite to eliminate volatile $^{125}I_2$. The reaction mixture was spotted onto Whatman 31ET paper (2 x 40 cm) and developed by descending paper chromatography in 1 butanol:glacial acetic acid: water (12:3:5). The paper strip was dried, cut into 1 cm segments,

and the pieces eluted in 2.0 ml 50 mM sodium acetate, pH 4.75, and 10 µl aliquots counted for radioactivity. The second peak of activity from the origin (Rf = 0.6 - 0.7) contained immunoreactive ^{125}I-TME-ScAMP.

2.2.6. Preparation of Calmodulin: Rat cerebral tissue, or whole human mammary tumors were homogenized at 4°C in 4 volumes of 0.1 M glyclglycine buffer, pH 7.5, containing 0.25 M sucrose and 3 mM DTT. The homogenate was centrifuged at 13,000 x g for 30 minutes, and the supernatant removed and heated at 95°C for 5 minutes. After cooling on ice, denatured protein was removed by centrifugation at 1000 x g for 20 minutes. The supernatant was dialyzed against 50 mM glycyl-glycine, pH 7.5, overnight at 4°C. Solid ammonium sulfate was than added (291 g/liter) to bring the solution to 50% saturation. The pH was adjusted to neutrality with 1N NH_4OH, and the preparation stirred for 1 hour at 4°C. After centrifuging at 10,000 x g for 30 minutes, the pellet was discarded. The supernatant fraction was adjusted to pH 4.0 with 1 N H_2SO_4 in 50% ammonium sulfate and stirred on ice for 1 hour. Following centrifugation at 10,000 x g for 30 minutes, the supernatant was discarded and the pellet resuspended in 1.0 ml 50 mM glycylglycine, pH 7.5. Attempts were made to extract calmodulin from four tumor specimens, FA 1038, FA-1250, IDC-733 and IDC 457.

2.2.7. Solubilization of Adenylate Cyclase: Stabilization of tumor-particle AC was attempted by preincubation with 10 mM NaF or 100 µm

Gpp(NH)p on ice for 10 minutes, after which the preparations were diluted 1:10 with 50 mM glycylglycine, pH 7.5, containing 0.25 M sucrose and 1 mM DTT, and centrifuged at 38,000 x g for 20 minutes at 4°C. The pellet fraction was then resuspended in the same buffer containing 0.1% Lubrol PX and incubated on ice for another 20 minutes. Following centrifugation at 105,000 x g for 90 minutes at 4°C, the supernatant and pellet fractions were assayed for AC activity using radioimmunoassay.

2.2.8. Preparation of Heat Stable Inhibitor of Protein Kinase from Tumor Extracts: Supernatant fractions separated from tumor washed particle preparations were heated to 96°C over 20 minutes, cooled on ice and filtered through Whatman No. 1 paper. To the filtrate was added 100% TCA (w/v) to give a final concentration of 15%. After stirring for 60 minutes, the preparation was centrifuged at 10,000 x g for 20 minutes, and the precipitate redissolved in 50 mM Tris-HCl, pH 7.5, containing 2 mM EDTA. The pH was adjusted to neutrality with 1 N NaOH. The inhibitor preparation was dialyzed against three changes of 5 mM Tris-HCl, pH 7.0, with 1 mM EDTA, centrifuged at 34,000 x g for 20 minutes, and the supernatant stored at -15°C.

2.2.9. Analytical Techniques:

2.2.9.1. Radioimmunoassay for Adenylate Cyclase: The assay for adenylate cyclase activity was carried out in 6.0 x 50 mm disposible Kimble culture tubes. Sections of tissue (weighing 5 to 50 µg dry wt.) or 6.19 µl aliquots of the washed particle preparation (tumor

preparations were diluted from 1:2 to 1:10 to give the equivalent of 64 to 68 µg dry wt. per reaction tube) were suspended in a reaction mixture consisting of 50 mM glycylglycine buffer, pH 7.5 with 5 mM ATP, 8 mM $MgCl_2$ (or 1.5 mM ATP/4.0 mM $MgCl_2$ for tissue microsections), 10 mM theophylline, 1 mg/ml BSA and 1 mM DTT in a total volume of 26 µl. After incubation at 37°C for designated times, the reaction was terminated by acetylation of the cAMP product by addition of 4.48 µl of a freshly prepared solution of acetic anhydride and triethylamine (1:2). Each tube then received 12.2 µl of ^{125}I-labeled cAMP (1 Ci/ml) diluted 1:500 in 50 mM NaAc buffer with 0.12% Triton X-100, pH 6.2, to give approximately 20,000 cpm/tube followed by 12.2 µl of antiserum (diluted 1:250 in 50 mM NaAc buffer, pH 6.2, containing 30 mg/ml BSA). Dilution of the antibody was adjusted as needed to allow a 50% binding level in the absence of unlabeled cAMP. The samples were allowed to equilibrate overnight at 4°C and then mixed with 300 µl of 1% (w/v) activated charcoal in 50 mM NaAc buffer, pH 6.2, containing 10 mg/ml BSA. The tubes were centrifuged at 2000 x g for 15 minutes and the entire supernatant removed from each tube and the radioactivity determined using a Beckman biogamma counter. As increasing amounts of tissue-produced cAMP competed with a constant amount of labeled cAMP, adenylate cyclase activity was reflected as a decrease in antibody bound radioactivity and quantified using a standard curve ranging from 0 to 1000 fmol plotted on semi-logarithmic paper (Figure 2). Four controls were used for each treatment: a substrate blank, a tissue blank, an antibody blank and a zero time

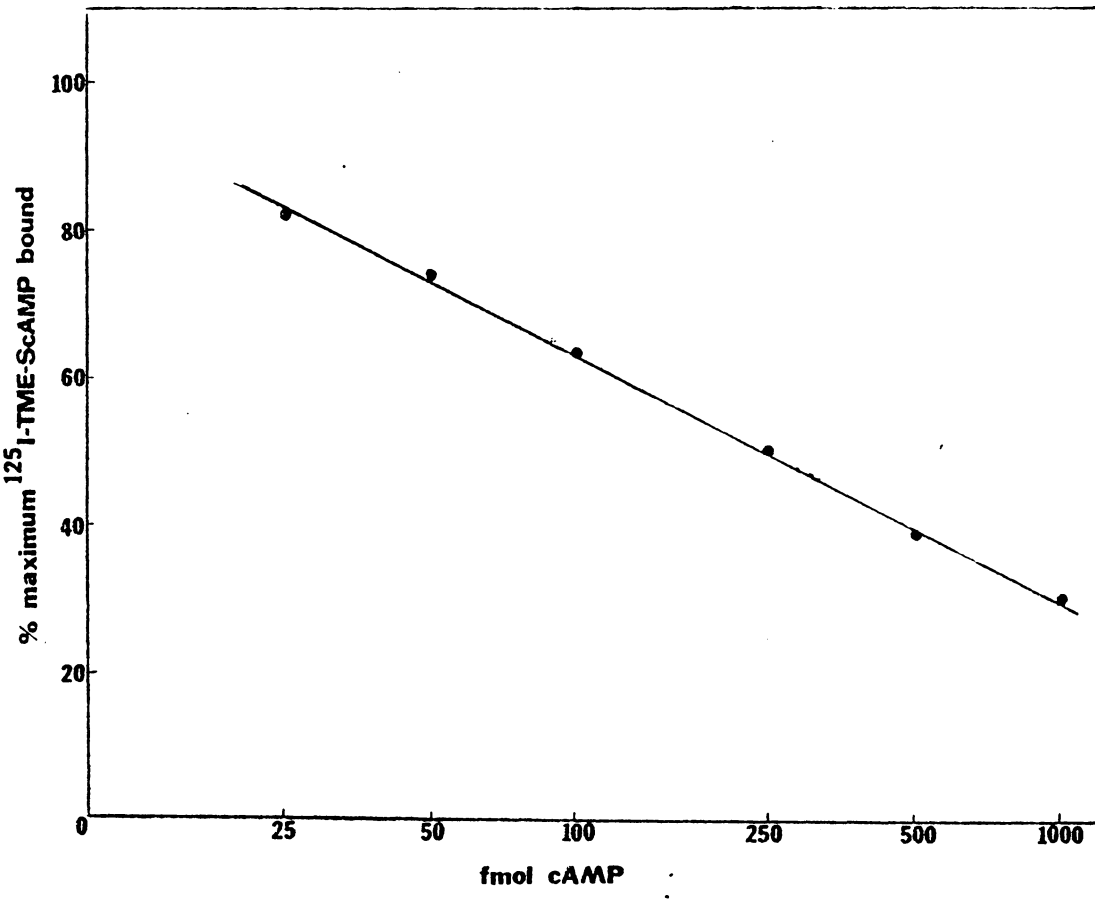


FIGURE 2

Cyclic AMP standard curve for the RIA. Values given represent the mean of duplicates for each concentration noted. Protocol given as in Materials and Methods.

reaction blank. No cross reaction of the antibody with ATP, ADP, or adenyl-yl-imidodiphosphate (AMP-PP) was observed. Separate standard curves were run for each assay condition used. Activity was corrected for endogenous cAMP levels by subtracting values obtained with substrate blanks. One unit of activity was defined as the amount of enzyme which produced one fmol cAMP per minute for tumor particle fractions and one pmol cAMP per minute for analyses of tumor microsections.

2.2.9.2. Filter Binding Assay for Cyclic 3':5' AMP-dependent Protein Kinase: The assay for cyclic AMP-dependent PK was measured using a modification of the method described by Gill and Walton, (1979). Fifteen microliter samples from 20,000 x g or in some cases, 105,000 x g supernatant fractions were added to a concentrated reaction mixture to give a final concentration of 30 µM ATP (0.4 Ci/mmole; 500,000-900,000 cpm/tube), 12.0 mM $MgCl_2$, 2.5 mM DTT, 50 mM theophylline/IBMX, and 2 mg/ml histone in 25 mM Tris-HCl, pH 8.0. Reactions were carried out in the presence and absence of 5 to 10 µm cAMP. The 6 x 50 mm tubes, containing a total volume of 30 µl, were then transferred to a 30°C water bath and incubated for 5 to 15 minutes. The reactions were stopped by transferring 27.8 µl of the reaction mixture to Whatman No. 31ET chromatography paper squares (2.0 x 2.0 cm) and the squares placed in ice-cold 10% TCA for 15 minutes. This was followed by a 2 minute wash in hot (90°C) 5% TCA, and a 15 minute wash in ice-cold 5% TCA. Finally, filters are rinsed with 95% ETOH, ETOH-acetone (1:1, v/v) and acetone. After air-drying, squares were transferred to vials containing 3 ml quantifluor and TCA-precipitable

radioactivity determined by liquid scintillation counting. Activities were corrected for enzyme and substrate blanks and expressed as either cpm ^{32}P incorporated into substrate or in units of enzyme activity; with one unit being defined as the amount of enzyme to catalyze the incorporation of one pmol ^{32}P into substrate per minute.

2.2.9.3. Spectrophotometric Assay for Adenosine Triphosphatase: The degradation of ATP with time indicative of endogenous ATPase activity in tumor cytosolic fractions and microsections was evaluated using a coupled enzyme system developed by Lowry and Passonneau (1961):

(a) ATP + glucose $\xrightarrow{\text{hexokinase}}$ ADP + glucose-6-P

(b) glucose-6-P + NADP $\xrightarrow{\text{glucose-6-P dehydrogenase}}$ 6-P-gluconate + NADPH + H+

The assay mixture consisted of 100 mM Tris-HCl, pH 7.5, containing 1 mM $MgCl_2$, 0.5 mM DTT, 0.5 mM NADP+, 1 mM glucose, 0.28 µ/ml hexokinase and 0.07 µ/ml glucose-6-phosphate dehydrogenase in a final of 280 µl. The reaction was initiated by addition of ATP (final concentration of 3.0 mM). Change in absorbance at 340 nM was observed over a 5 minute reaction period at 23°C or until the reaction reached completion. The molar extinction coefficient of NADPH(E_{340} = 6.22×10^{-3} liter · $mole^{-1}$ · cm^{-1}) was used to calculate the amount of NADPH synthesis (mM). NADPH production was corrected using a reaction blank minus ATP. A decrease in the amount of NADPH synthesis upon addition of 23 µl tumor extract (50 to 100 µg dry wt tissue) to the assay was taken to indicate endogenous ATPase activity.

2.2.9.4. Measurement of Endogenous Cyclic 3':5' AMP levels: Tissue pieces were cut from specific areas within tumor sections, weighed and placed in 6 x 50 mm tubes so that each contained 10-30 µg dry weight tissue. After the addition of 14.8 µl 5% TCA, tubes were incubated for 15 minutes at 4°C. Each tube then received 2.0 µl of 1 N HCl and the contents dried in a Virtis Bio-dryer for 30 minutes. A series of standards containing 2 to 180 fmol cAMP were treated similarly. After drying, residues were dissolved in 30 µl of 50 mM sodium acetate, pH 6.2, acetylated with 1.56 µl acetylating reagent and the radioimmunoassay performed as described above with the exception that each tube received a total of 5,000 cpm of ^{125}I-cAMP and a 1:1800 antibody dilution was used.

2.2.9.5. Quantification of Cyclic 3':5' AMP Binding Protein: Cyclic AMP binding proteins were analyzed in tumor tissue homogenates, supernatant fractions, and column fractions. Twenty-six µl of a concentrated reaction mixture were mixed with 98.7 µl of the tissue extract to give a final concentration of 5 mM DTT, 5 mM NaCl, 1 mM $MgCl_2$, 0.4 mM EGTA, and 30 nM ^{3}H-cAMP (30,000 cpm/tube) in 20 mM MES, pH 7.0. The tubes were incubated for 30 minutes at 4°C, and the binding reaction stopped by filtration on 25 mm Metricel GN-6 filters (0.45 µm pore size). Filters were washed with 20 mM MES, pH 7.0, dried, and the radioactivity was determined in 3.0 ml Scintilene Scintillation fluid (Fischer Scientific).

Column fractions were at times analyzed for cAMP binding acitivity by a modification of the above procedures. Following incubation at

4°C, 300 µl of 50 mM Tris-HCl pH 7.5 containing 6% activated charcoal and 1% BSA were added, and the assay tubes were centrifuged at 3,000 x g for 15 minutes. Supernatants were removed, transferred to vials containing 3 ml Quantifluor (Mallinckrodt) and radioactivity determined by liquid scintillation spectrometry.

2.2.9.6. Electrophoretic Analysis of Tumor Extracts: Tumor extracts, as well as column fractions, were run on 9% $NaDOdSO_4$ polyacrylamide slab gels (3% cross-linking, 0.4% SDS) at 30 mA under constant current using the discontinuous $NaDOdSO_4$/polyacrylamide gel system described by Laemmli (1970). Protein bands were visualized by staining the gels with Coomassie Brilliant Blue G-250 or by the silver stain procedure described by Merril et al. (1981).

Apparent molecular weights of polypeptides were estimated from a standard log molecular wt versus mobility curve (Weber and Osborne, 1969) which was obtained using standards of known molecular weight including bovine serum albumin, (66,000 daltons), pepsin (34,700 daltons), trypsinogen (24,000 daltons), β-lactoglobulin (14,300 daltons) and lysozyme (14,300 daltons).

2.2.9.7. Analysis of Endogenous Protein Phosphorylation: Endogenous protein phosphorylation in a cell-free system was examined using the method described by Rudolph and Krueger (1979). The phosphorylation reaction was carried out in 6 x 50 mm disposable tubes in a total volume of 200 µl. The reaction mixture consisted of 10 mM $MgCL_2$ and 5 mM theophylline in 50 mM HEPES, pH 7.5. Reactions were run in

the presence and absence of 10 µM cAMP. The phosphorylation was initiated by the addition of 20 µl of 10 µM (^{32}P)-ATP (approximately 600,000 cpm/tube) and the tubes incubated at 30°C for 20 minutes. The reaction was terminated by the addition of 100 µl of a solution containing 9% SDS, 15% glycerol, 30 mM Tris-HCl, pH 7.8, and 0.05% bromophenol blue and the tubes were mixed and immediately transferred to a boiling water bath for 5 minutes. After cooling to room temperature, 100 µl of 75 mg.ml DTT was added to each tube. Samples were covered and incubated overnight at room temperature.

Ten microliter samples were run on 9% SDS polyacrylamide slab gels (3% cross linking, 0.4% SDS) as described in the previous section. Following electrophoretic separation, gels were washed in 7% glacial acetic acid, then dried. Localization of phosphorylated polypeptides was done by autoradiography using Kodak XAR-S film. Radioactive bands localized by autoradiography were cut out of dried gels and counted by liquid scintillation spectrometry to determine the absolute amounts of ^{32}P incorporated. Alternatively, gels were scanned using densitometry.

2.2.9.8. DEAE-Sephacel Chromatography of the Protein Kinase Holoenzyme: Ammonium sulfate (291 g/liter) was slowly added to the 30,000 x g supernatant over a period of 40 minutes to a final saturation of 50%. The suspension was further stirred for one hour, then centrifuged at 10,000 x g for 15 minutes. The pellet was redissolved in an equal volume of 20 mM Tris-HCl, pH 7.8, containing 2 mM β-mercaptoethanol and dialyzed overnight against the same buffer at 4°C. After

dialysis, the preparation was centrifuged at 10,000 x g for 20 minutes and the pellet discarded. In some cases, 50% streptomycin sulfate was added over a 30 minute period to the 30,000 x g supernatant to give a final concentration of 2%, then centrifuged at 30,000 x g for 20 minutes, and the pellet discarded.

The enzyme preparation was then applied to a DEAE-Sephacel column (1.6 x 6 cm) equilibrated with 20 mM Tris-HCl, pH 7.8, containing 2 mM β-mercaptoethanol and 1 mM EDTA (Buffer A). The column was washed with 20 to 30 ml of Buffer A, and fractions (2.5 ml) collected at a flow rate of 40 ml/hr. The holoenzyme was eluted from the resin with a linear salt gradient from 0.0 to 0.3 M NaCl in Buffer A. Fractions were collected at a flow rate of 80 ml/hr. for 2 hrs. Fractions containing cAMP-dependent protein kinase and cAMP binding activity were pooled, concentrated in an Amicon ultrafiltration cell using a Diaflo PM-10 membrane to 1 to 2 ml final volume, then dialyzed against buffer A overnight at 4°C.

2.2.9.9. Separation of Regulatory and Catalytic Subunits by cAMP-Agarose Affinity Chromatography: The separation of protein kinase subunits was done using the procedure described by Dills et al. (1979) with modifications. The protein kinase holoenzyme preparation resolved by DEAE-Sephacel chromatography was applied to a 10 x 0.7 cm column (QUIK-SEP RIA column, ISOLAB, Inc. Akron, OH,) of cAMP-agarose [N^6-$H_2N(CH_2)_2$-cAMP] (8-carbon spacer) equilibrated with 5 mM MES, pH 7.5, containing 0.1 mM EDTA, 15 mM β-mercaptoethanol and 100 mM NaCl (Buffer B). After adsorption of the regulatory subunit, the

column was washed with 5 volumes of buffer B, followed by 5 volumes of buffer B containing 75 mM NaCl, and lastly, 2 additional column volumes of buffer B. Bound protein was eluted from the immobilized cyclic nucleotide by first washing the column with buffer B at 23°C, followed by a 60 minute wash with buffer B containing 0.2 mM cAMP (for PK regulatory subunit, type II) or by incubating the gel for 60 minutes at 30°C in 6 ml buffer A containing 30 mM cAMP, pH 6.5, and the gel removed by section filtration (PK regulatory subunit, type I). The cAMP washes were pooled and dialyzed exhaustively against buffer B to remove unbound cAMP prior to determination of cAMP-binding activity. The buffer and salt eluted fractions were assayed for catalytic activity in the absence of cAMP.

3. RESULTS

3.1. Adenylate Cyclase Associated with Human Mammary Tumor Particulate Fractions

3.1.1. Evaluation of Adenylate Cyclase Assay Conditions: Buffer-washed particle fractions to be used for evaluation of membrane-associated adenylate cyclase were prepared from a series of malignant and benign tumors. In order to determine the bioassay conditions for optimum cAMP production, several biochemical parameters were investigated using representative tumor preparations.

Incubation of the particulate fraction from malignant tumor S-79-3784 in 50 mM glycylglycine buffer, containing 5.0 mM ATP, 8.0 mM $MnCl_2$ and 10 µg/ml BSA, adjusted to pH values ranging from 6.0 to 9.0 indicated that maximum cAMP synthesis occurred at pH 7.5 under the assay conditions employed (Figure 3). The pH optimum was identical for benign tumor preparations. Production of cAMP was linear over a 30 minute incubation period at 37°C, after which a leveling effect was observed (Figure 4). This was presumably due to loss of enzyme activity with time since inclusion of 1 mg/ml creatine kinase and 20 mM creatine phosphate as an ATP regenerating system had no effect on cAMP formation. The observed rate of AC activity and cAMP formation was a linear function of tissue addition up to 100 µg dry wt tumor extract per reaction tube (Figure 5). Heat-inactivation of tumor preparations by preincubation at 80°C for 15 minutes followed by incubation in the AC reaction mixture resulted in no AMP production over the endogeneous tissue level present.

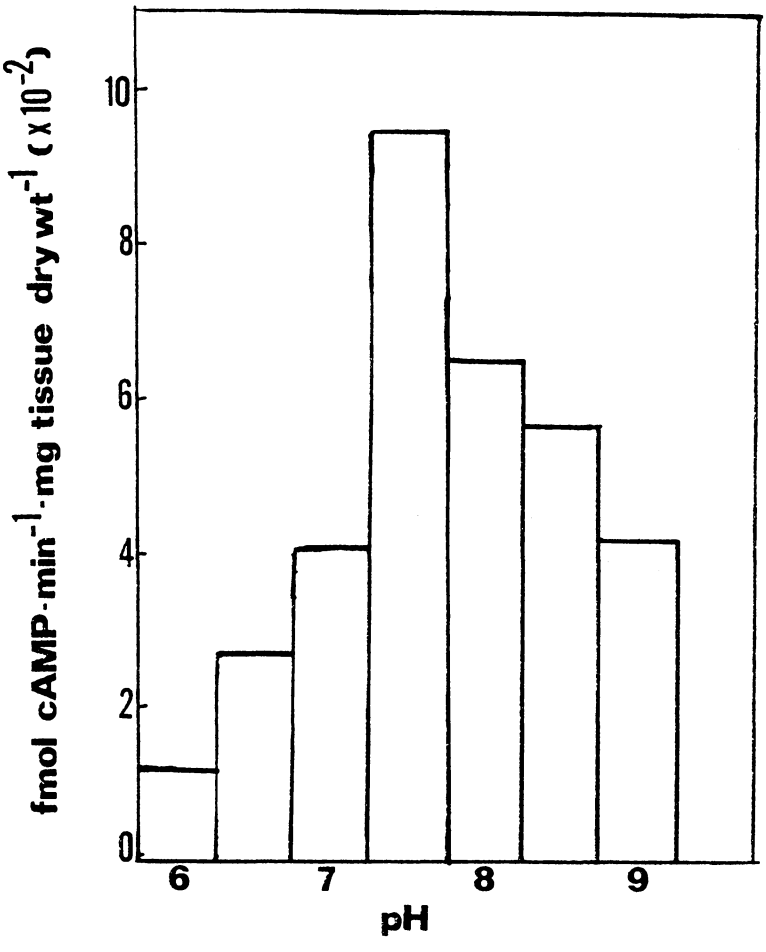


FIGURE 3

pH profile for pellet bound adenylate cyclase activity from malignant tumor IDC-3784. Incubation occurred for 30 minutes at 37°C in 50 mM glycylglycine containing 5 mM ATP, 8 mM $MnCl_2$, 1 mg/ml BSA, and 5 mM theophylline, adjusted to various pH values. Reactions were initiated by the addition of 98 µg tumor extract.

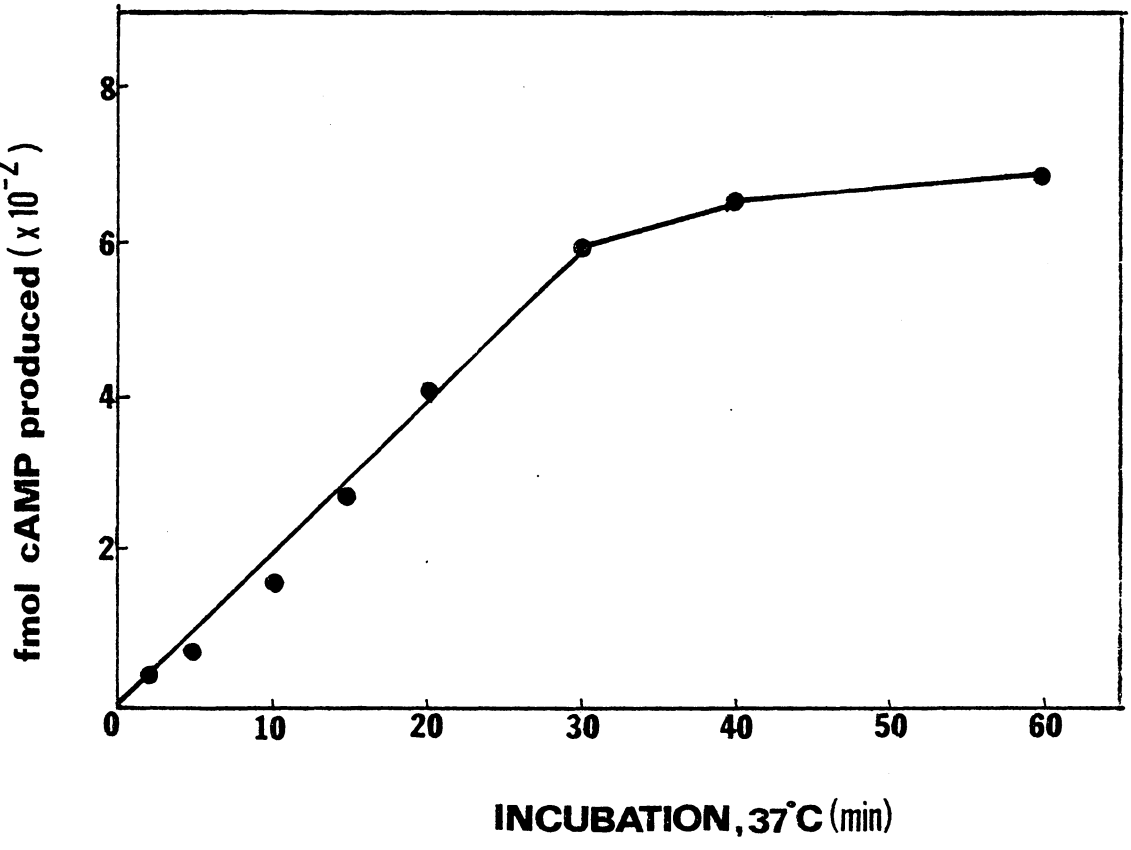


FIGURE 4

Time course for cAMP production by the particulate preparation from malignant tumor IDC-3784. The reactions were initiated by addition of ATP (final concentration, 5 mM) to 26.0 µl of the assay mixture, 50 mM glycylglycine, pH 7.5, containing 8 mM $MnCl_2$, 1 mg/ml BSA, and 68 µg tumor extract, and terminated by addition of 4.48 µl of the acetylating mixture.

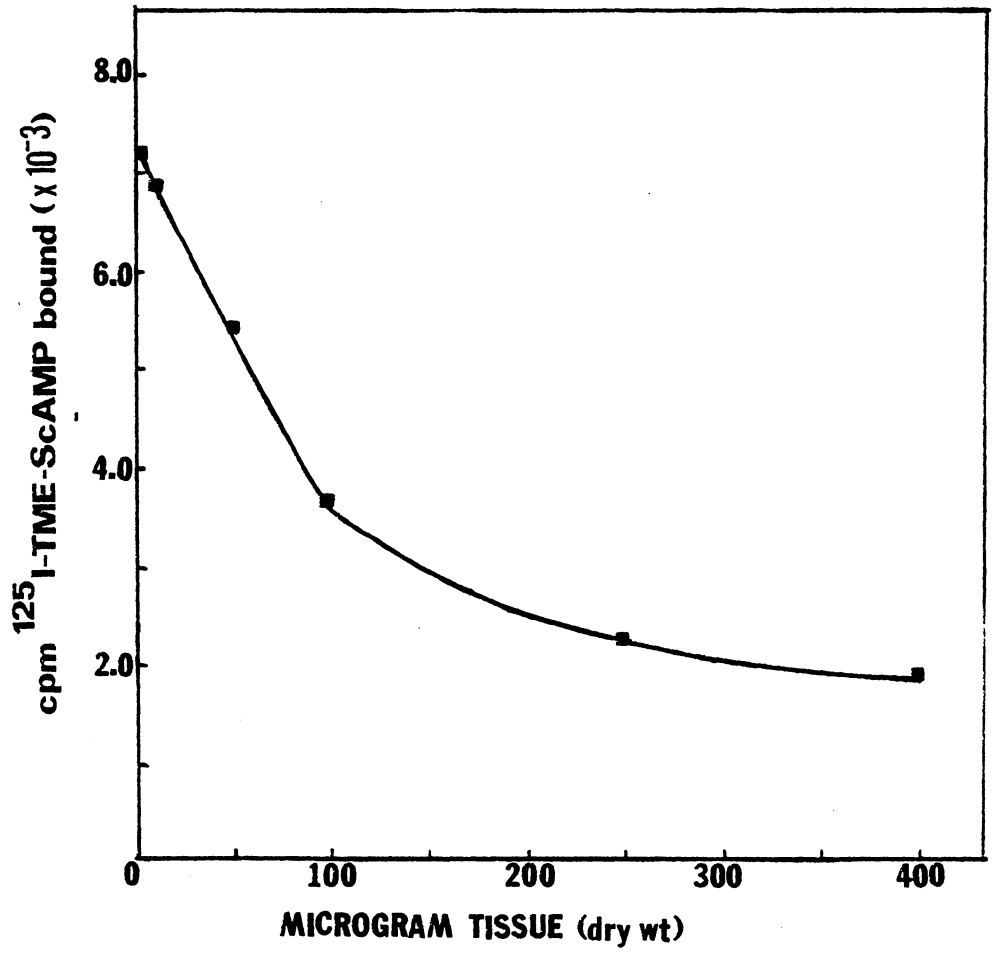


FIGURE 5

Activity curve for adenylate cyclase in a particulate fraction from malignant tumor IDC-3784 indicating the spectra of weight range which could be used in the radioimmunoassay.

That the product of the cyclase reaction was indeed cAMP was demonstrated by addition of increasing concentrations of venom cAMP-phosphodiesterase (cAMP-PDE, Sigma Chemical Co.) to the reaction mixture. Inclusion of cAMP-PDE at a final concentration of 0.12 µg/ml resulted in near total loss of cAMP production during a 30 minute incubation in the presence of 5.0 mm ATP, whereas addition of heat-inactivated cAMP-PDE at the same concentration showed no effect (Figure 6). Addition of inhibitors of PDE to the reaction mixture, 5.0 mM theophylline or 3-methyl-1-isobutylxanthine, prevented the degradation of authentic cAMP.

Initial measurements of membrane-associated AC activity from benign and malignant tumors were made in the presence of 8.0 mM Mn^{2+} and 5.0 mM ATP. Concentrations of Mn^{2+} in excess of 8.0 mM were inhibitory to cAMP production with 40% and 70% inhibition occurring in the presence of 10.0 and 15.0 mM Mn^{2+}, respectively. Since it was confirmed in these studies that free ATP was inhibitory to the cyclase reaction, the divalent cation concentration was maintained in a 3mM excess over the final ATP concentration. Increased synthesis of cAMP was observed with increasing Mn-ATP concentration (Table 1). Lineweaver - Burk plots of data from several experiments showed the apparent Km value for ATP was 650 µM.

It was of interest to evaluate the effectiveness of additional divalent cations in replacing the Mn^{2+} requirements demonstrated by AC. Activity levels were determined for Co^{2+}, Ca^{2+}, Fe^{2+}, and Zn^{2+} at four concentration levels, 0.01 mM, 0.10 mM, 1.0 mM and 8.0 mM.

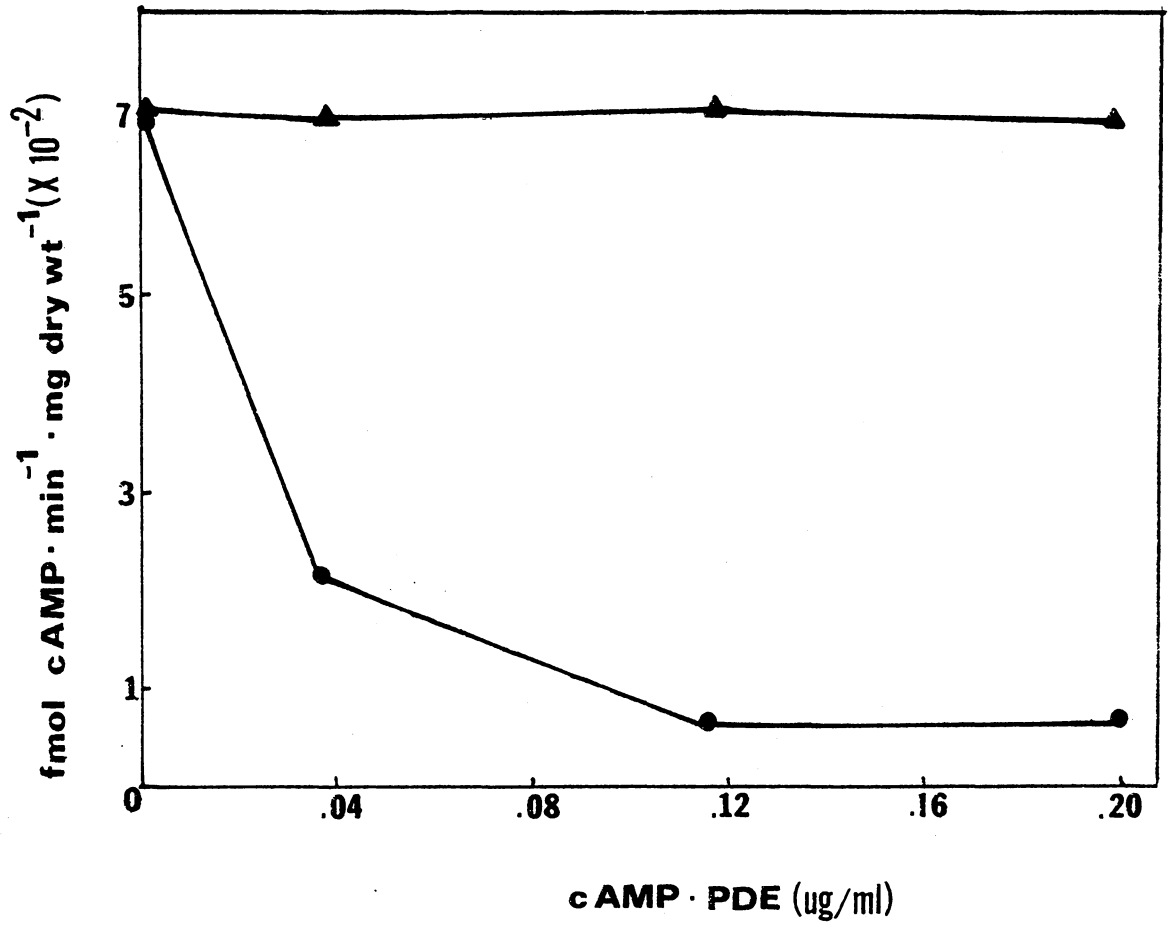


FIGURE 6

Cyclic AMP formation in the presence of increasing concentrations of active (•) and heat-inactivated (▲) venom cAMP-phosphodiesterase I.

TABLE 1

EFFECT OF INCREASING Mn-ATP CONCENTRATION ON TUMOR PARTICULATE ADENYLATE CYCLASE ACTIVITY[1]

ATP(mM)	Mn^{2+}(mM)	AC activity (units/mg dry wt)[2]
0.0	0.0	12
0.5	2.0	550
1.0	4.0	625
2.0	5.0	680
3.0	6.0	955
4.0	7.0	1030
5.0	8.0	1040

[1]Tumor particulate fraction represents the washed 3,000 x g pellet fraction from benign tumor FA-1700.

[2]Activity values based upon the mean of 5 replicates for each condition. One unit is defined as the amount of enzyme required to catalyze the production of one fmol cAMP per minute at 37°C.

Results indicated that Co^{2+} and Fe^{2+} at the 8.0 mM level could partially replace Mn^{2+}, giving activity levels at 18 to 20% that obtained with 8.0 mm Mn^{2+}. Maximum activity of AC occurred in the presence of Mn^{2+}. Addition of both Mg^{2+} and Mn^{2+} to the reaction mixture did not give a synergistic or additive affect.

3.1.2. Assay Parameters in the Presence of Mg ATP: It is thought that Mg ATP serves as the true physiological substrate for the cyclase reaction in most mammalian tissues. Therefore, studies of tumor-associated AC were conducted in the presence of Mg^{2+}. Cyclic AMP production in the presence of 8.0 mM Mg^{2+} was 10-20% of that obtained with 8.0 mm Mn^{2+}. The effects of Mg^{2+}/Mn^{2+} with increasing ATP concentration on particulate AC from tumor S-79-3784 is shown in Figure 7.

Modification of the assay conditions was made to optimize cAMP productionin the presence of Mg ATP. Although the optimum pH remained at 7.5, the reaction mixture was adjusted to contain a final concentration of 3.0 mM ATP and 6.0 mM Mg^{2+}. The incubation time was decreased to 15 minutes at 37°C (Figures 8A,B,C).

3.1.3. Comparison of Adenylate Cyclase Activity in Benign and Malignant Mammary Tumors: Several particulate preparations of the two classes of tumors were analyzed for AC activity using Mn ATP as the substrate. As shown in Table 2 and Figure 9, the potential for cAMP production by extracts from benign tumors was 5 to 6 times greater than in corresponding extracts from malignant tumors when activity

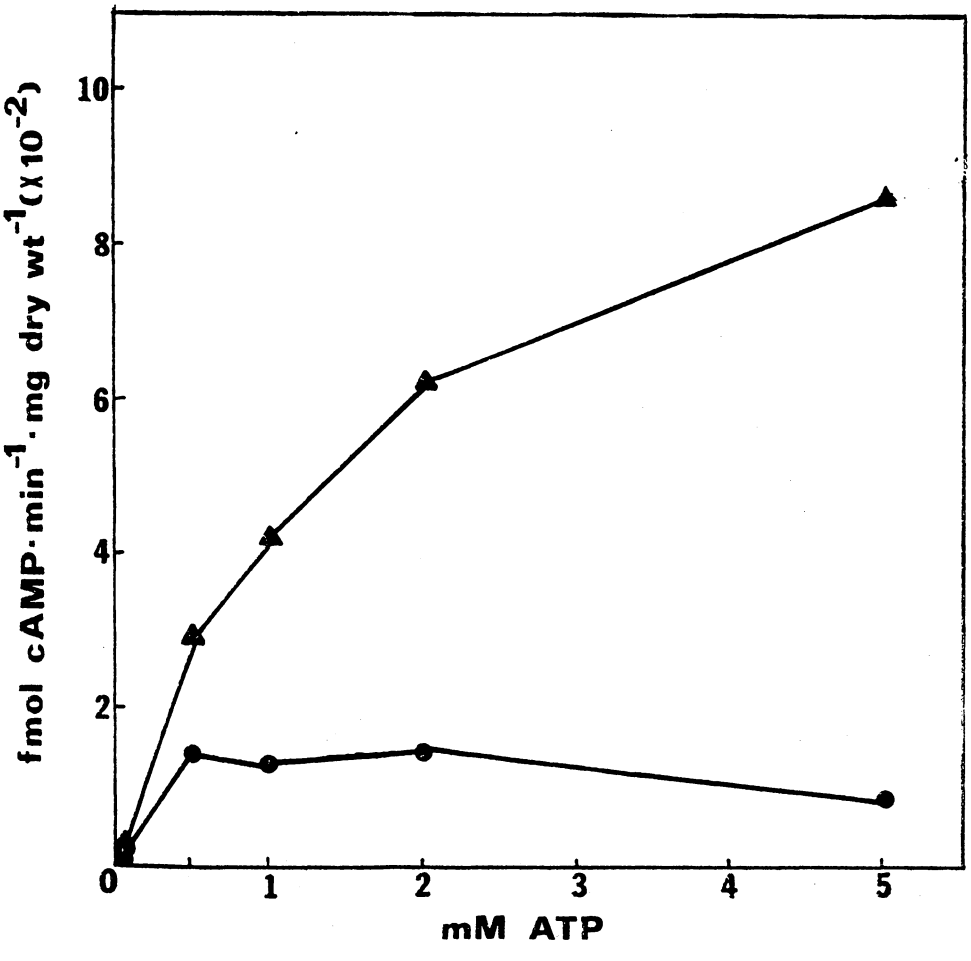


FIGURE 7

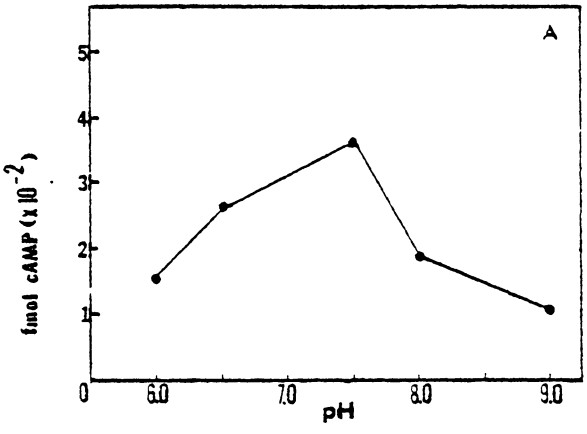
Cyclic AMP formation by particulate adenylate cyclase from malignant tumor IDC-3784 (65 µg/µl dry wt) incubated in 50 mM glycylglycine, pH 7.5, 1.0 mg/ml BSA, 10 mM theophylline, 8 mM MgCl (●) of 8 mM $MnCl_2$ (▲) in the presence of increasing ATP concentration.

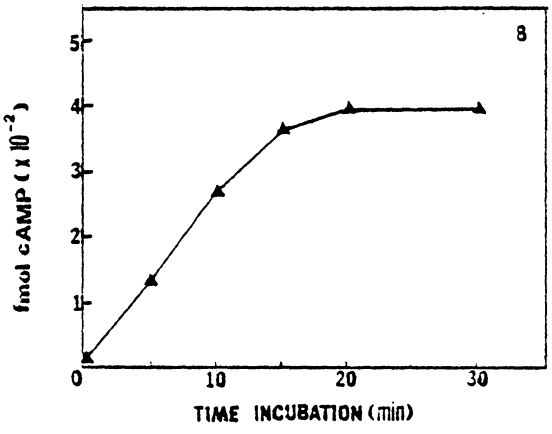
FIGURE 8

A. pH profile of adenylate cyclase activity associated with the particulate fraction from malignant tumor IDC-3669 (75 μg dry wt) in the presence of 5 mM ATP and 8 mM $MgCl_2$. Activity given as fmol cAMP produced (x 10^{-2}).

B. Linearity of cAMP production with time by the particulate fraction from IDC-3669 in the presence of 50 mM glycylglycine, pH 7.5, containing 1.0 mg/ml BSA, 5 mM ATP, 8 mM $MgCl_2$, and 10 mM theophylline.

C. Effect of increasing Mg-ATP concentration on cAMP production by the particulate fraction from IDC-3669 (•) and FA-4041 (▪).


A
fmol cAMP (x 10⁻²)
5
4
3
2
1
0
6.0
7.0
8.0
9.0
pH


B
fmol cAMP (x 10⁻²)
5
4
3
2
1
0
10
20
30
TIME INCUBATION (min)

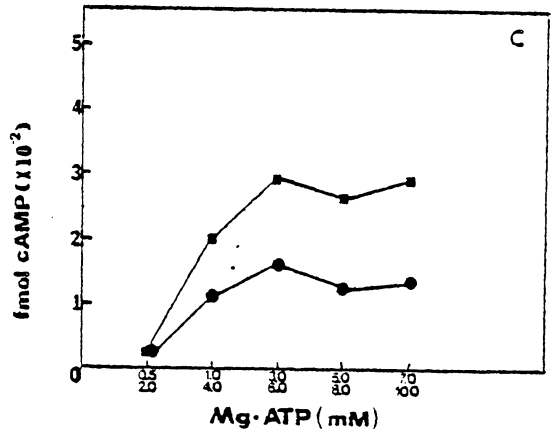

C
fmol cAMP (x10⁻²)
5
4
3
2
1
0
Mg·ATP (mM)

TABLE 2

COMPARISON OF ADENYLATE CYCLASE ACTIVITY ASSOCIATED WITH PARTICULATE FRACTIONS FROM BENIGN AND MALIGNANT MAMMARY TUMORS[1]

tumor type	AC activity (units/mg dry wt)[2]
Fibroadenoma:	
1709	770 ± 90
798	1470 ± 70
3070	2980 ± 460
1258	2070 ± 170
2732	2070 ± 190
1038	1940 ± 190
1700	1240 ± 70
Infiltrating Ductal Carcinoma:	
345	590 ± 40
1688	120 ± 20
3669	630 ± 60
4041	440 ± 40
3928	350 ± 30
147	180 ± 12
2760	230 ± 25

[1]Cyclic AMP formation was measured in the presence of 5 mM ATP and 8 mM $MnCl_2$.

[2]Specific activity values calculated as the mean of 3 replicate experiments for each tumor; given as mean ±SEM. One unit is defined as the amount of enzyme required to catalyze the production of one fmol cAMP per minute at 37°C.

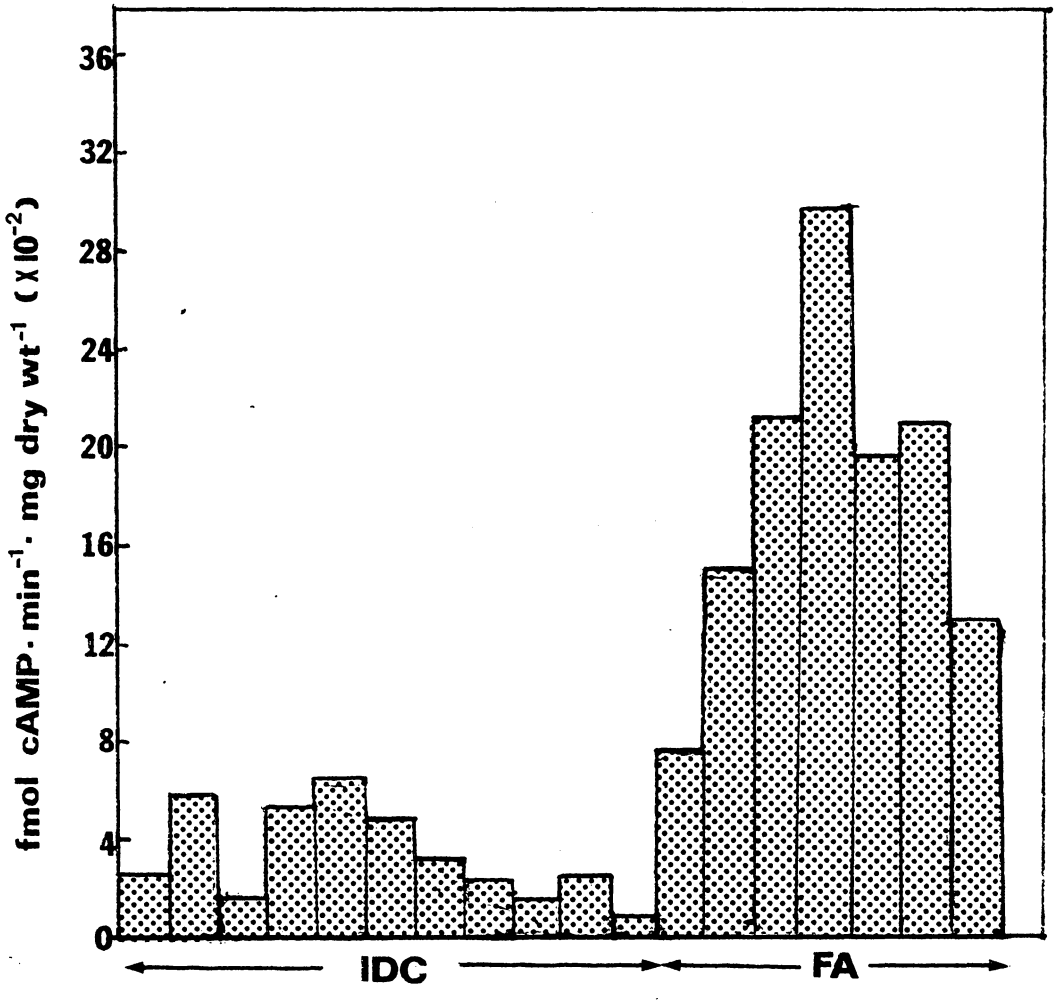


FIGURE 9

Levels of adenylate cyclase activity associated with the particulate fractions from a representative sampling of malignant and benign mammary tumors.

was expressed on the basis of dry tissue weight.

3.1.4. Calcium and Calmodulin Effects on Membrane-Bound Tumor Adenylate Cyclase: Addition of Ca^{2+} in micromolar amounts to the reaction caused a decrease in cAMP production, observable in both malignant and benign tumor preparations. Inhibition ranged from 20 to 50% of the basal activity in the presence of Mn ATP (Table 3). The effects of 50 µM Ca^{2+} on AC activity with increasing ATP concentration is given in Table 4. Simultaneous preincubation of the particulate AC with 50 to 250 µM EGTA and 50 µM Ca^{2+} did not restore levels of cAMP production to the basal level.

It was of interest to determine whether calmodulin (CDR, calcium-dependent regulator) was functional in activating mammary tumor AC. One source of calmodulin was prepared from rat cerebral tissue as described in methods and tested for its ability to activate rat cerebral particulate AC. Preincubation of rat AC (120 µg protein/ml) with the calmodulin preparation at (180 µg protein/ml) gave a 25 to 35% increase in cAMP production in the presence, but not absence of 50 µM Ca^{2+}. A similar preparation was obtained from extracts of malignant mammary tumors which was demonstrated to activate tumor-associated cAMP-PDE (E.L. Gutmann, personal communication). No Ca^{2+}-dependent activation of tumor-associated AC was observed in the presence of either calmodulin preparations at concentrations up to 1.0 µg/ml tumor protein using Mg ATP as the substrate. Likewise, as shown in Table 5, preincubation of washed particle fractions from malignant and benign tumors with 0.1 µg/µl or 1.0 µg/µl commercial

TABLE 3

TUMOR-ASSOCIATED ADENYLATE CYCLASE ACTIVITY IN THE PRESENCE OF Mn^{2+}, Mg^{2+}, AND Mn^{2+}-Ca^{2+}[1]

divalent cation	AC activity (units/mg dry wt)[2]	
	IDC-1688	FA-1258
8.0 mM Mg^{2+}	540	220
8.0 mM Mn^{2+}	2580	1050
8.0 mM Mn^{2+}, 50 μM Ca^{2+}	2010	820
8.0 mM Mn^{2+}, 100 μM Ca^{2+}	1980	800
8.0 mM Mn^{2+}, 500 μM Ca^{2+}	1860	760
8.0 mM Mn^{2+}, 1.0 mM Ca^{2+}	1470	600

[1]Enzyme analysis performed on 3,000 x g pellet fractions from the two tumors examined.

[2]Specific activities based upon the mean of 5 replicates for each condition. One unit is defined as amount of enzyme needed to produce one fmol cAMP per minute at 37°C.

TABLE 4

EFFECT OF Ca^{2+} AND INCREASING ATP CONCENTRATION ON TUMOR-ASSOCIATED ADENYLATE CYCLASE

	AC activity (units/mg dry wt)	
ATP (mM)	$-Ca^{2+}$	$+Ca^{2+}$
0.0	12	0
0.5	220	60
1.0	420	110
2.0	640	240
5.0	1030	510

Approximately 68 µg tissue (wet wt) from malignant tumor IDC-3784 was incubated in 50 mM glycylglycine buffer, pH 7.5, containing 8.0 mM $MnCl_2$, 1.0 mg/ml BSA, varying amounts of ATP, in the presence and absence of 50 µM $CaCl_2$. Incubation time was 20 minutes at 37°C. The reaction was terminated by the addition of 4.48 µl acetylation mixture. Activity values represent the mean of triplicate samples. One unit defined as amount of enzyme required to produce one fmol cAMP per minute at 37°C.

TABLE 5

EFFECT OF CALMODULIN ON PARTICULATE ADENYLATE CYCLASE FROM MALIGNANT AND BENIGN TUMORS

treatment[1]	AC activity (units/mg dry wt)[3]	
	IDC-147	FA-2760
control	660	1030
+ 50 μM Ca^2	590	850
+ 50 μM Ca^2 , 0.1 μg/μl CDR[2]	600	800
+ 50 μM Ca^2 , 1.0 μg/μl CDR	610	900

[1]Preincubation at 4°C for 30 minutes prior to assay.

[2]CDR = Calcium-Dependent Regulator (Sigma Chemical Co., St. Louis, MO).

[3]Specific activity values based upon the mean of 4 replicate samples One unit is defined as amount of enzyme required to produce one fmol cAMP per minute at 37°C.

CDR (Sigma Chemical Co.) in the presence of 50 μM Ca^{2+} for 30 minutes at 4°C did not result in stimulation of tumor AC activity, although similar concentrations did activate a tumor-associated cAMP-PDE that was prepared in this laboratory (E.L. Gutmann, personal communication).

3.1.5. Enzyme Activation by Guanyl Nucleotides: A critical step in activation of AC by hormones is the binding of guanyl nucleotides at the regulatory site. Guanosine triphosphate (GTP) activation of AC has been demonstrated, however the effect is transient (Cassel and Selinger, 1977). Activation of tumor-associated AC by GTP at final concentrations of 0.1 to 100 μM in the presence of Mn ATP was for the most part unsuccessful. It was speculated that GTP was being hydrolyzed by an endogenous tumor GTPase, or by the putative GTPase associated with the regulatory subunit of AC itself. However, stimulation of particulate AC from one benign tumor FA-3070 with 10 μM and 100 μM GTP gave a 1.5 and 2-fold increase in cAMP production over the basal activity level. Cholera toxin (CT) has been shown to catalyze ADP-ribosylation of the regulatory subunit of AC, consequently stimulating cAMP production by inhibiting the associated GTPase which is proposed to terminate the cyclase reaction.

The question arose as to whether cholera toxin could increase the GTP-stimulated AC activity from FA-3070. Cholera toxin (10 μg/ml) was first preactivated with 20 mM DTT for 10 minutes at 37°C, then NAD^+ was added (final concentration, 2.5 mM) and incubated for an additional 5 minutes at 4°C. One hundred microliters of particulate

AC from FA-3070 was incubated on a 1 to 1 volume ratio with activated cholera toxin in the presence of 100 μM GTP or 100 μM Gpp(NH)p. Addition of 100 μM GTP and 100 μM Gpp(NH)p gave a 67% and 93% increase in cAMP production over the basal level, respectively (Table 6). Incubation of the AC preparation with dithiothreitol-activated CT in the presence of Gpp(NH)p gave only slight stimulation of cAMP synthesis over that obtained with Gpp(NH)p alone, whereas incubation with activated CT and GTP gave over a four-fold stimulation in cAMP production over that obtained with GTP alone. This result indicated that CT was functional in modification of the regulatory subunit of AC so as to prevent GTP hydrolysis and termination of the cyclase reaction. In contrast, little change in activity was noted with Gpp(NH)p in the presence or absence of CT.

The nonhydrolyzable GTP analog, Gpp(NH)p, was used to determine the activation potential of tumor AC by guanine nucleotides. The potential for Gpp(NH)p activation in the presence of Mn ATP or Mg ATP (Table 7) was determined for a representative sampling of malignant and benign AC preparations. The activity ratio was expressed as the ratio of cAMP production in the absence of Gpp(NH)p to that obtained in the presence of 100 μM Gpp(NH)p. It was observed that the activation potential of AC was less in malignant than benign tumor preparations examined in the presence of both Mn^{2+} and Mg^{2+}.

Since GTP-stimulation of AC from tumor FA-3070 was observed, the potential for Gpp(NH)p activation was examined more closely. The

TABLE 6

EFFECT OF CHOLERA TOXIN ON MAMMARY TUMOR-ASSOCIATED PARTICULATE ADENYLATE CYCLASE ACTIVITY

treatment	AC activity (units/mg dry wt)[2]
control	440
+ 100 μM Gpp(NH)p	850
+ 100 μM GTP	730
+ 2.5 mM NAD^+	390
+ 2.5 mM NAD^+, 5.0 μg/ml CT[1]	460
+ 2.5 mM NAD^+, 5.0 μg/ml CT, 100 μM Gpp(NH)p	1000
+ 2.5 mM NAD^+, 5.0 μg/ml CT, 100 μM GTP	3180

[1]Preincubation of cholera toxin (10 μg/ml) with 20 mM DTT for 10 minutes at 37°C, followed by preincubation with 2.5 mM NAD^+ for 5 minutes at 4°C. A washed particle preparation from FA-3070 was incubated in a 1:1 ratio with the activated cholera toxin in the presence or absence of 100 μM Gpp(NH)p or GTP. Reaction time =20 minutes at 37°C.

[2]Activity determined from triplicate samples. One unit of enzyme will produce one fmol cAMP per minute at 37°C.

TABLE 7

ACTIVATION OF TUMOR-ASSOCIATED ADENYLATE CYCLASE BY GUANYL-5'-YL-IMIDODIPHOSPHATE IN THE PRESENCE OF Mn-ATP AND Mg-ATP[1]

tumor	source[2]	addition	activity ratio[3]
IDC:	1688	Mn^{2+}, Gpp(NH)p	0.72
	2278		0.91
	733		0.64
	345		0.80
FA:	1700	Mn^{2+}, Gpp(NH)p	0.28
	3070		0.56
	1038		0.36
	1258		0.57
IDC:	2760	Mg^{2+}, Gpp(NH)p	0.36
	3928		0.31
	147		0.30
	4041		0.58
	733		0.23
FA:	1709	Mg^{2+}, Gpp(NH)p	0.17
	798		0.14
	2732		0.14
	1038		0.13
	1700		0.12

[1]Enzyme was derived from washed 3,000 x g pellet fraction of the respective tumors. Reactions were run in the presence of 5 mM ATP, 8 mM $MgCl_2$ or $MnCl_2$, and 100 µM Gpp(NH)p.

[2]Protein addition equivalent to 5 to 30 µg dry wt.

[3]Activity ratio is expressed as the function:

$$\frac{\text{cAMP formation in absence of Gpp(NH)p}}{\text{cAMP formation in the presence of Gpp(NH)p}}$$

Specific activity values were calculated as the mean of three replicate experiments.

effect of increasing concentrations of Gpp(NH)p was determined for AC from FA-3070 in the presence of Mg ATP (Table 8), and indicated that maximum activation occurred with 10 μM Gpp(NH)p. Cyclic AMP production was also determined using 5.0 mM AMPP(NH)p, a non-hydrolyzable analog of ATP. In the latter case, a slight increase in cAMP synthesis observed suggested that some hydrolysis of ATP was occurring over the 15 minute reaction period. Analysis of Gpp(NH)p-stimulation of AC from a series of malignant and benign tumors indicated that the majority of the benign tumors examined showed higher cAMP production than malignant tumors, with a mean activity level 4 to 5 times greater than that from malignant tumors (Table 9). Variation in Gpp(NH)p-stimulated activity was evident within each tumor class.

3.1.6. Enzyme Activation by Fluoride: Adenylate cyclase activity from mammalian tissues is stimulated by fluoride ions supposedly mediated through the regulatory (G/F) subunit of the enzyme. In association with Gpp(NH)p activation of tumor-associated AC, the effect of NaF was also examined. Depending upon the tumor preparation, a 3 to 6-fold stimulation of cAMP production was seen in the presence of 10 mM NAF. As seen in Table 10, the activation ratio was similar for AC from malignant and benign tumors. A comparison of the rate increase of cAMP formation by AC from malignant tumor IDC-3784 in the presence of 100 μM Gpp(NH)p or 10 mM NaF was approximately two-fold and four-fold, respectively (Figure 10). Similar results were obtained with the benign tumor FA-1256; with basal, Gpp(NH)p-stimulated and NaF-

TABLE 8

GUANYL-5'-YL-IMIDODIPHOSPHATE-ACTIVATION OF ADENYLATE CYCLASE ASSOCIATED WITH A PARTICULATE FRACTION FROM BENIGN TUMOR FA-3070

reaction[1]	AC activity (units/mg dry wt)[3]
5.0 mM ATP	150
5.0 mM ATP, 1.0 μM Gpp(NH)p	2250
5.0 mM ATP, 10 μM Gpp(NH)p	2810
5.0 mM ATP, 100 μM Gpp(NH)p	2872
5.0 mM AMPP(NH)P[2]	300

[1] Reactions run in the presence of 8 mM $MgCl_2$; 8 to 17 μg dry wt tumor extract.

[2] AMPP(NH)P = adenyl-5'-yl-imidodiphosphate

[3] Specific activity values based upon 3 replicate samples. One unit of enzyme will catalyze the production of one fmol cAMP per minute at 37°C.

TABLE 9

COMPARISON OF ADENYLATE CYCLASE ACTIVITY IN PARTICULATE FRACTIONS FROM BENIGN AND MALIGNANT TUMORS IN THE PRESENCE OF GUANYL-5'-YL-IMIDODIPHOSPHATE[1]

tumor type	Gpp(NH)p-stimulated activity[2] (units/mg dry wt)
Malignant (IDC):	
2278	57 ± 7.0
733	273 ± 21.4
345	32 ± 5.3
1688	190 ± 16.2
457	338 ± 25.0
3669	200 ± 14.5
4041	158 ± 14.9
147	167 ± 17.5
2760	143 ± 9.7
3928	286 ± 13.6
Benign (FA):	
1709	220 ± 19.7
798	369 ± 26.3
2732	820 ± 20.1
3070	1480 ± 53.2
1038	1540 ± 69.5
1258	976 ± 35.4
1700	815 ± 21.1
2752	589 ± 30.5

[1] Cyclic AMP production determined in the presence of 5 mM ATP, 8 mM $MgCl_2$ and 10 µM Gpp(NH)p.

[2] Gpp(NH)p-stimulated activities based upon the average of six replicates for each tumor preparation. One unit of enzyme will produce one fmol cAMP per minute at 37°C. Mean activity levels for the groups of malignant and benign tumors examined were 185 and 852 units/mg dry wt, respectively.

TABLE 10

SODIUM FLUORIDE STIMULATION OF TUMOR-ASSOCIATED ADENYLATE CYCLASE ACTIVITY[1]

tumor source[2]		activity ratio[3]
IDC:	733	0.25
	345	0.31
	457	0.10
	2278	0.28
	1688	0.19
FA:	307	0.21
	1700	0.22
	1235	0.18
	1038	0.21
	1258	0.36

[1]Cyclic AMP production measured in the presence of 5 mM ATP and 8 mM $MgCl_2$.

[2]30 to 50 µg dry wt tumor extract added per reaction.

[3]Activity ratio represents the function:

$$\frac{\text{cAMP formation in the absence of 10 mM NaF}}{\text{cAMP formation in the presence of 10 mM NaF}}$$

Specific activity values were based upon the mean of triplicate samples.

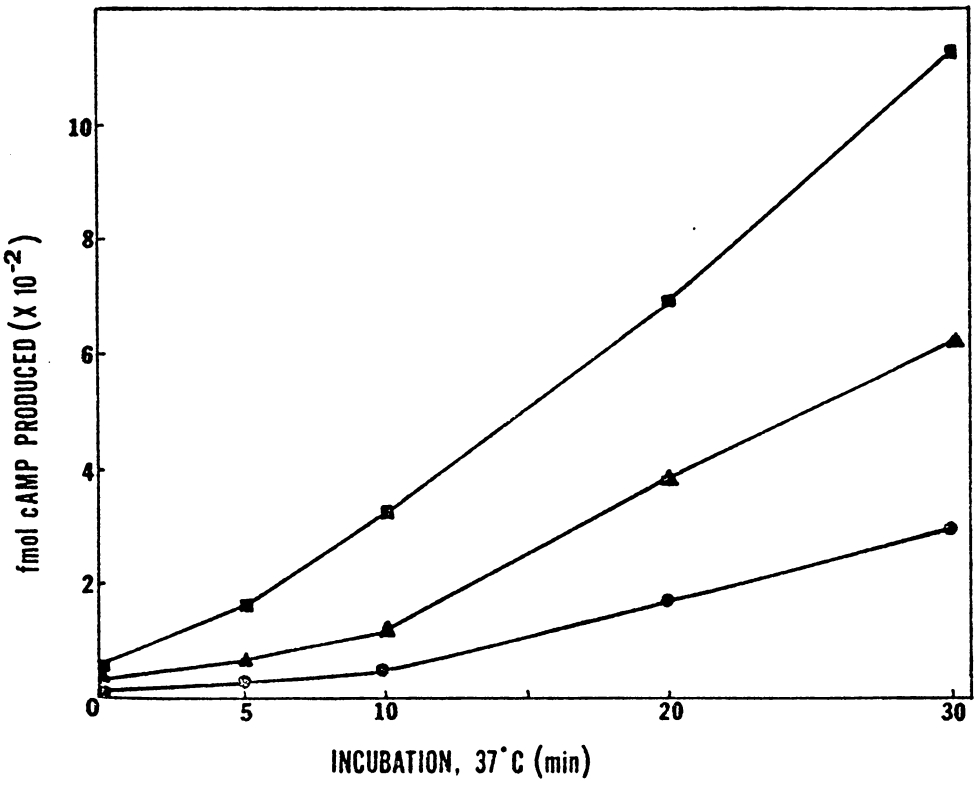


FIGURE 10

Activation of mammary tumor adenylate cyclase by Gpp(NH)p and NaF. The rate of cAMP formation by adenylate cyclase associated with the particulate preparation (11 μg/μl, wet wt) from malignant tumor IDC-3784 was measured with no addition (●). Activation occurred in the presence of 100 μM Gpp(NH)p (▲), and 10 mM NaF (■).

stimulated activity being 1038, 2700, and 4664 units per mg dry wt, respectively. As exemplified by malignant tumor IDC-3669 (45 µg dry tissue et/tube), the specific activity of the enzyme was higher following stimulation by NaF/Gpp(NH)p in the presence of Mn ATP than with Mg ATP, although the percent activation over basal activity levels were similar (Table 11).

3.1.7. Effect of Additional Substances on Tumor Adenylate Cyclase: Pyrophosphate, a product of the cyclase reaction, was found to be inhibitory to cAMP production at millimolar concentrations. Addition of 5.0 mM pyrophosphate resulted in 40-50% inhibition, whereas 10.0 mM pyrophosphate gave near total inhibition of cAMP synthesis. Phosphate had no effect from 10.0 µM to 5.0 mM. Addition of 10 to 100 µM adenosine, histamine, or lactate had no effect on the cyclase reaction.

3.1.8. Hormonal Responsiveness of Tumor Particle Adenylate Cyclase: A series of malignant and benign tumors were examined for hormone-responsiveness of particulate AC using the GTP analog in combination with various hormones. Based upon an analysis of glucagon-stimulated AC from a rat liver particle preparation, it was determined that maximum cAMP production was obtained using 10 µM Gpp(NH)p and 5 µM glucagon. The histograms presented in Figures 11, A-G summarize the observations found for various potential hormone effectors of membrane-associated AC. Considerable variation in hormone responsiveness was observed for the series of tumor preparations. Both positive

TABLE 11

SODIUM FLUORIDE AND GUANYL-5'-YL-IMIDODIPHOSPHATE-INDUCED STIMULATION OF ADENYLATE CYCLASE FROM MALIGNANT TUMOR IDC-3669[1]

cation	addition	AC activity[2] (units/mg dry wt)	% activation
Mn^{2+}	-	650	
	NaF	2220	70.8
	Gpp(NH)p	850	24.0
Mg^{2+}	-	210	
	NaF	1220	83.0
	Gpp(NH)p	270	22.0

[1]Tissue concentration, 7.33 mg/ml (1.2 mg/ml protein); 45 µg tissue dry weight per assay tube.

[2]Activity values determined as the mean of three replicates for each treatment. One unit enzyme will catalyze the production of one fmol per minute at 37°C.

FIGURE 11

Hormonal responsiveness of a series of malignant and benign human mammary tumors. Source of adenylate cyclase activity was a 3,000 x g particle fraction for each of the designated tissues used. Samples of 6.17 µl tumor extract were preincubated with 10 µM Gpp(NH)p and 5 µM concentration of various hormone for 15 minutes at 4°C prior to addition of Mg ATP. Cyclic AMP production was measured by RIA as previously described. Relative activity was determined by the function:

$$\frac{\text{cAMP synthesis in the presence of Gpp(NH)p/hormone}}{\text{basal cAMP synthesis}}$$

The data is given as the mean ±SEM of values from 4 replicates of each tumor particle fraction. TSH, thyrotropin-stimulating hormone; LH, luteinizing hormone; PGE_1, prostaglandin E_1; FSH, follicle-stimulating hormone; hCG, human chorionic gonadotropin.

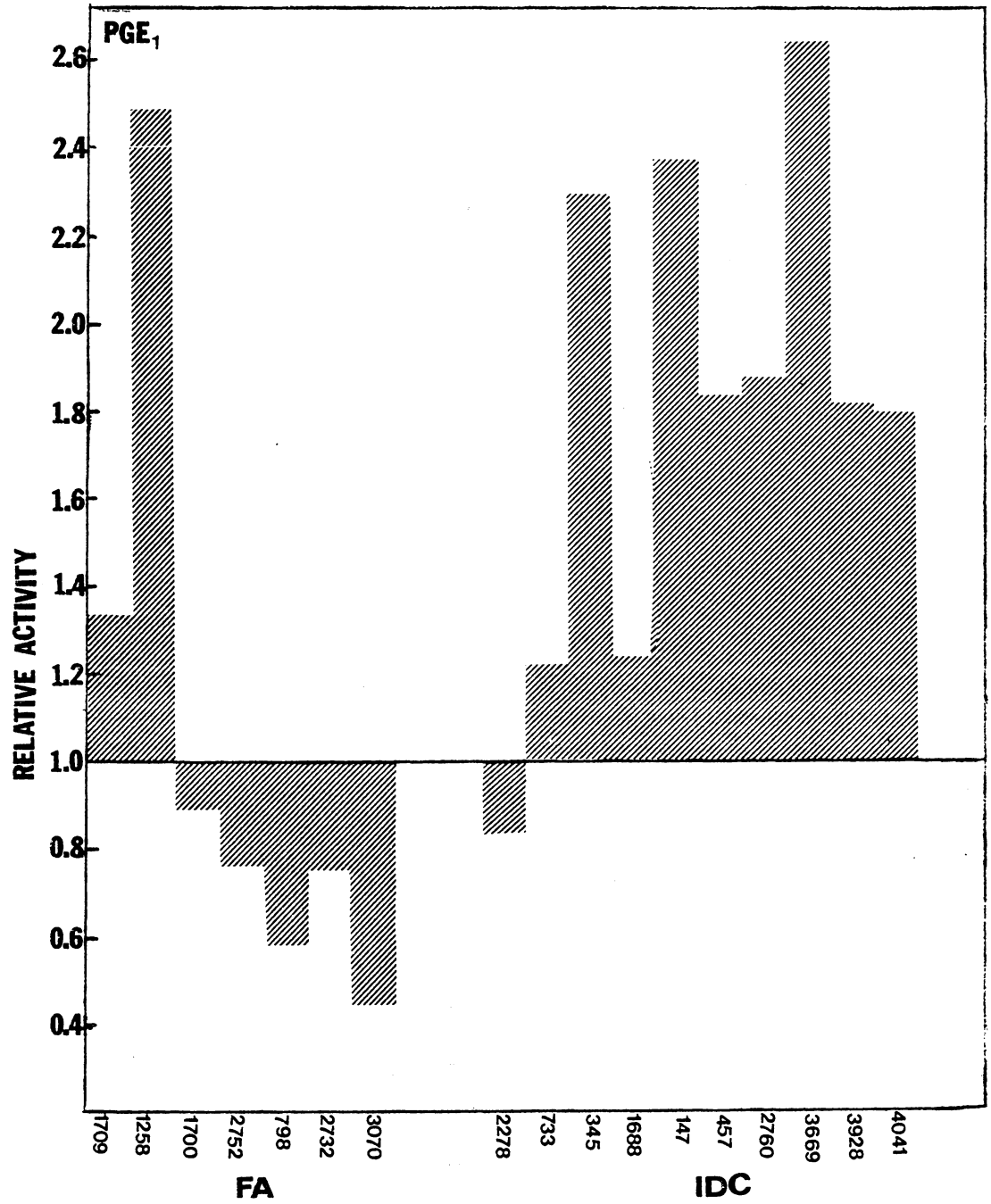


FIGURE 11A.

Adenylate cyclase activity in the presence of prostaglandin E_1.

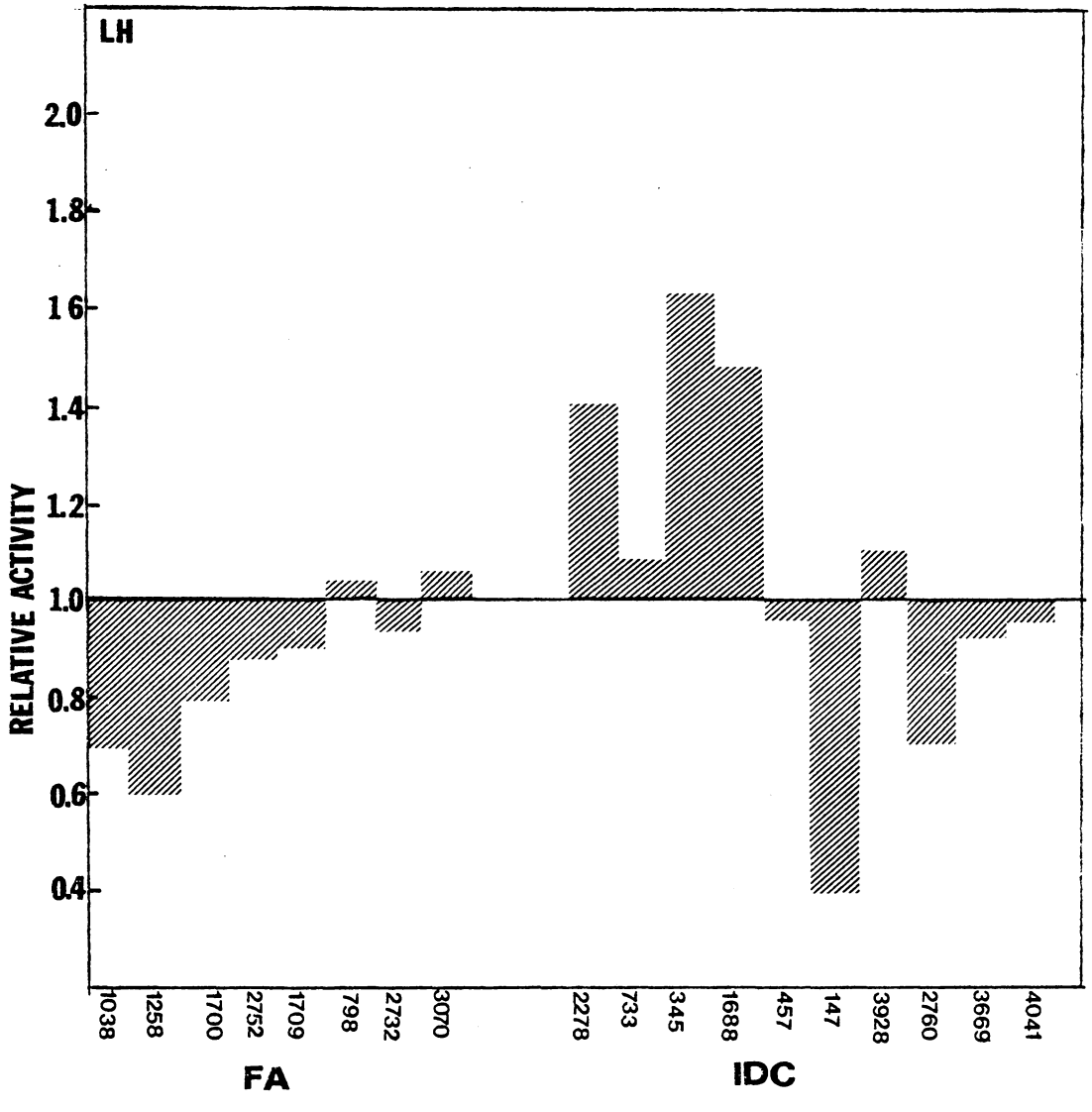


FIGURE 11B.

Adenylate cyclase activity in the presence of luteinizing hormone.

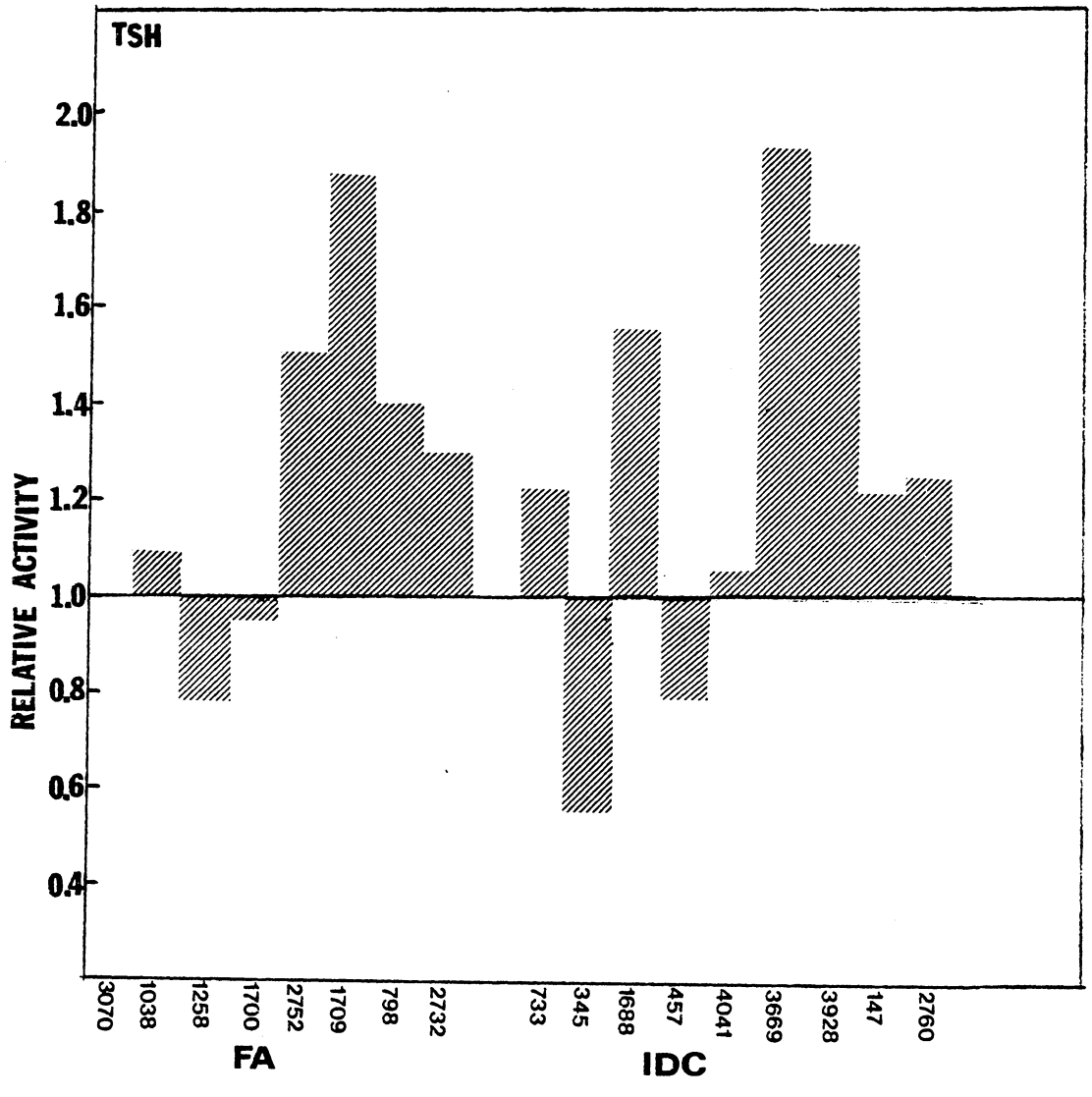


FIGURE 11C.

Adenylate cyclase activity in the presence of thyrotropin-stimulating hormone.

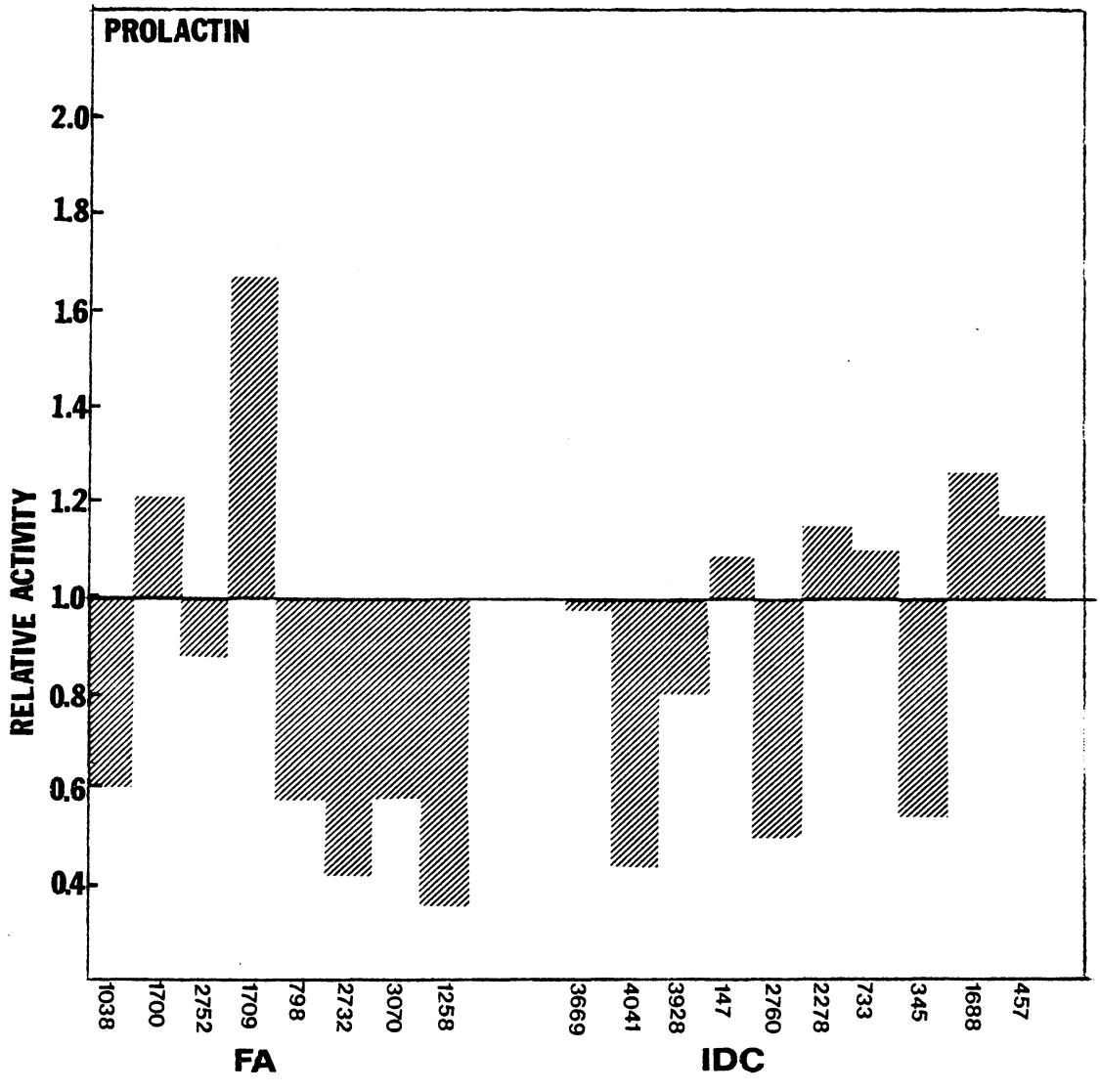


FIGURE 11D.

Adenylate cyclase activity in the presence of prolactin.

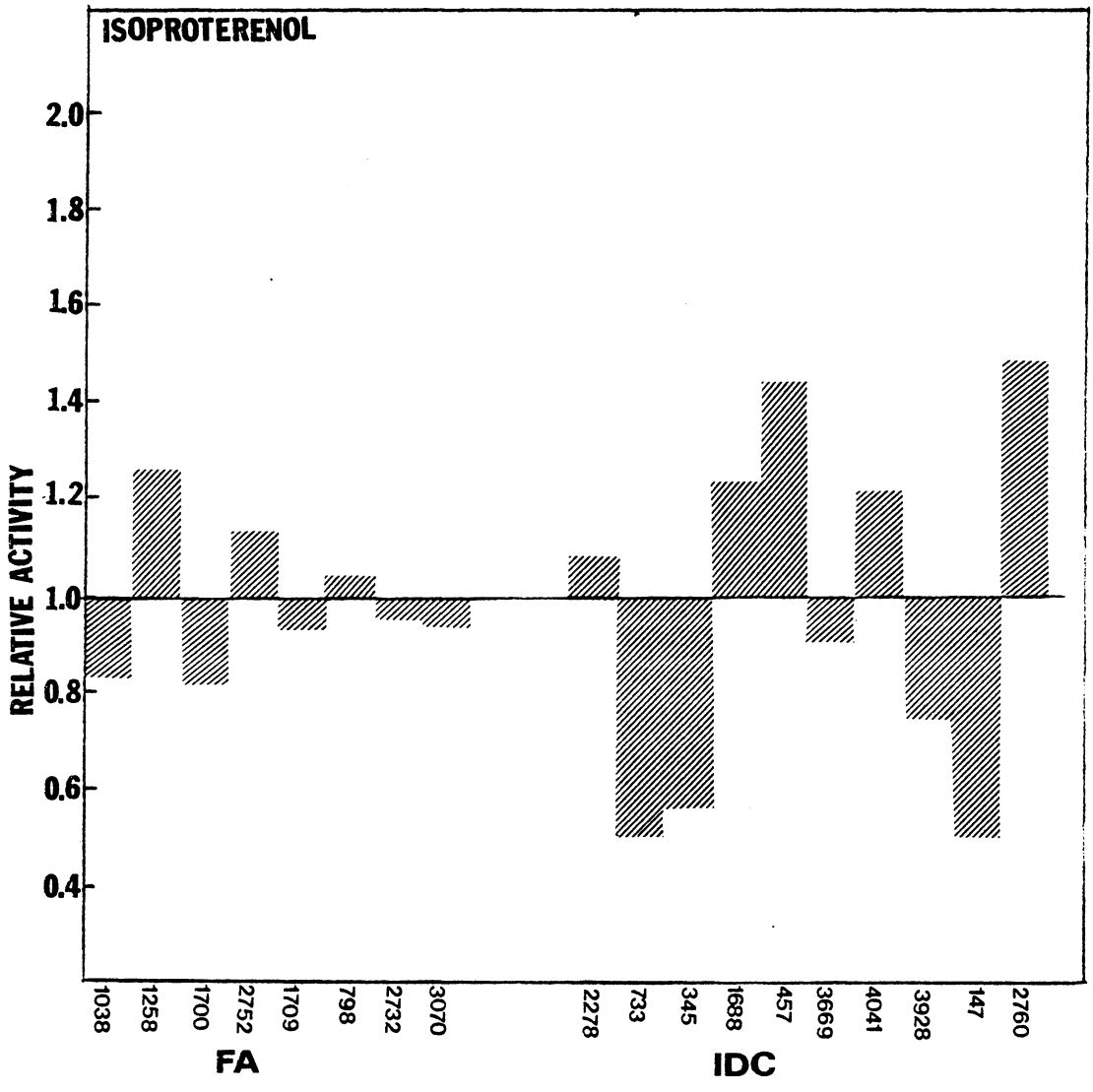


FIGURE 11E.

Adenylate cyclase activity in the presence of isoproterenol.

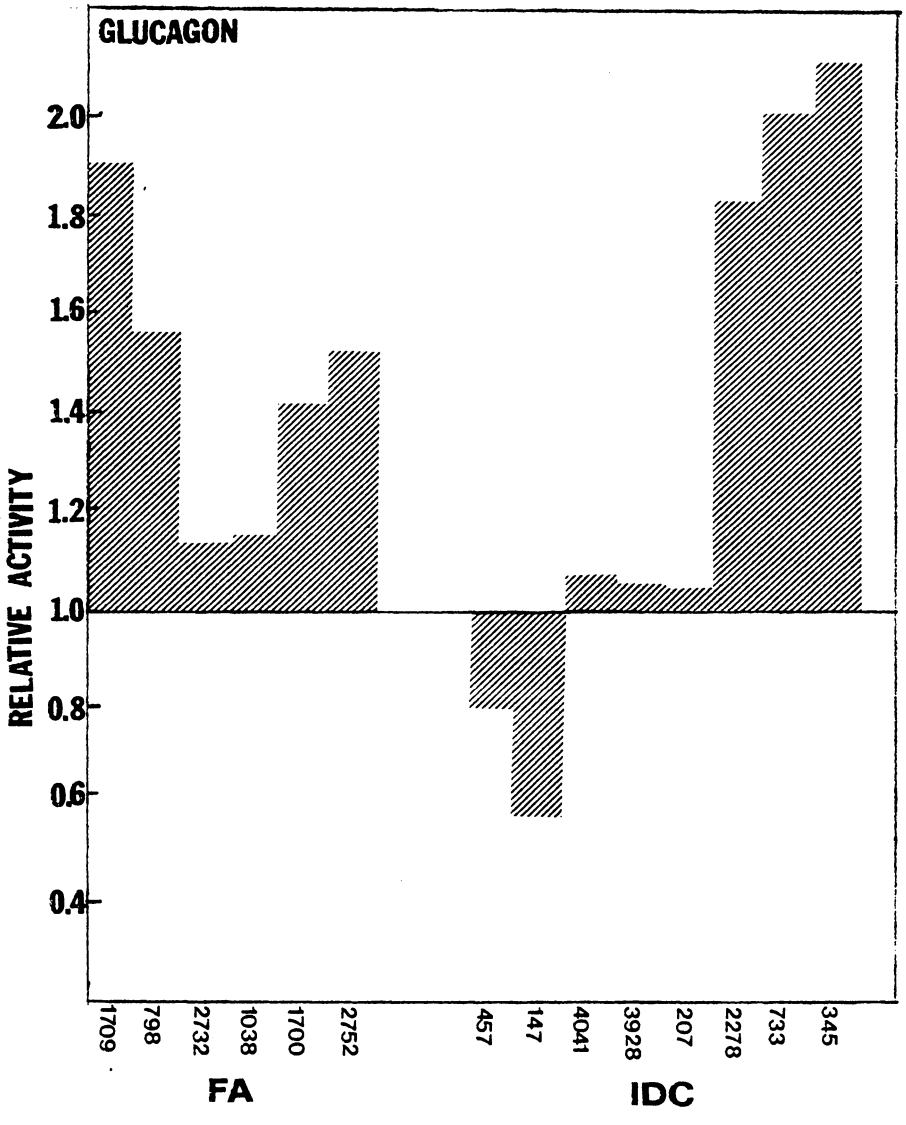


FIGURE 11F.

Adenylate cyclase activity in the presence of glucagon.

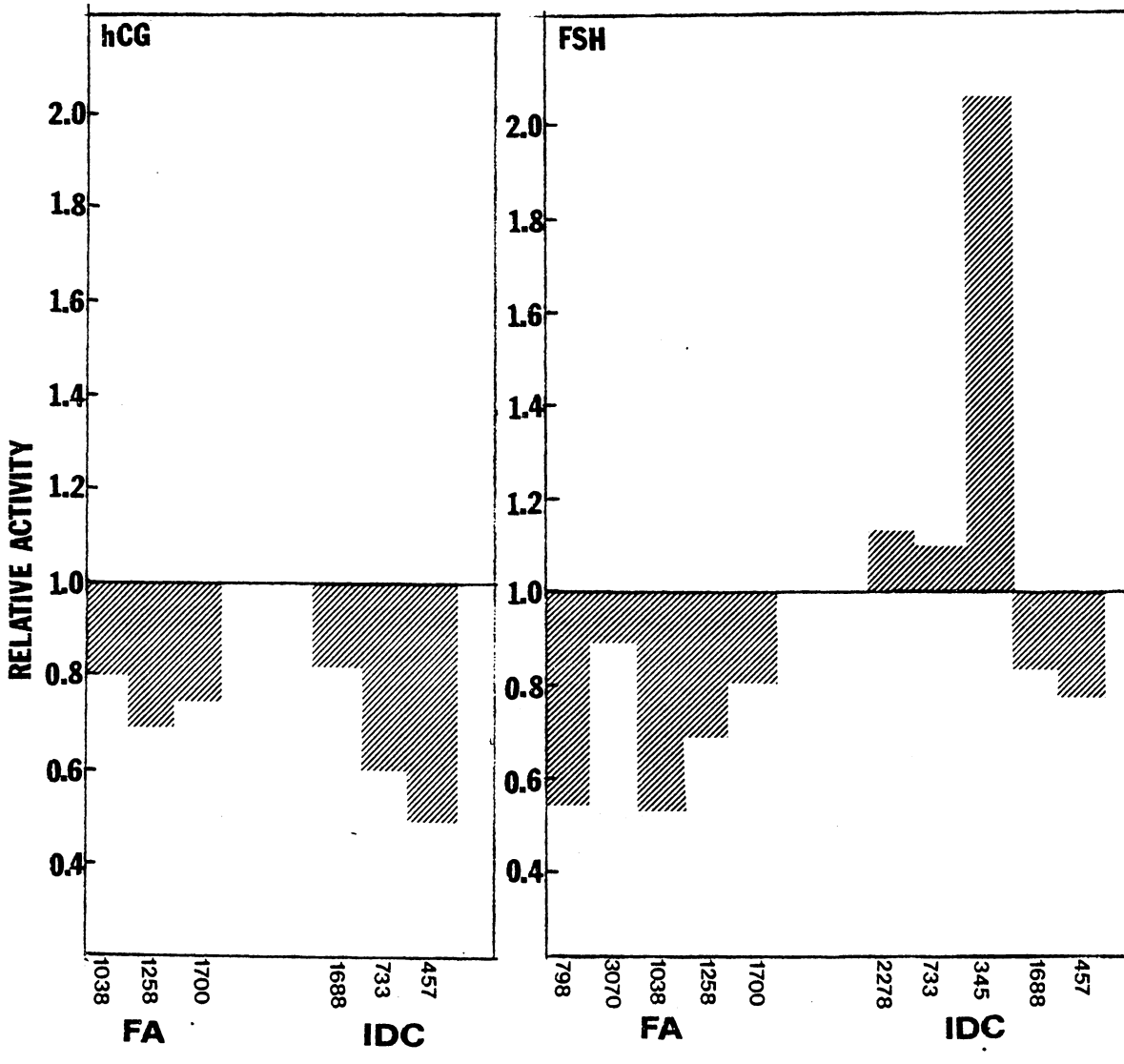


FIGURE 11G.

Adenylate cyclase activity in the presence of human chorionic gonadotropin and follicle stimulating hormone.

and negative responses were demonstrated in the presence of 5 μM isoproterenol, follicle-stimulating hormone (FSH) and luteinizing hormone (LH). The overall profile for the response of tumor AC to human chorionic gonadotropin (hCG) and prolactin seemed to indicate a negative response. Although a variable response to thyrotropin-stimulating hormone (TSH) and glucagon was obtained, the overall trend seemed to indicate these hormones were capable of activation of tumor AC in vitro. The most consistent hormonal response of tumor AC occurred with 5 μM PGE where eleven out of seventeen tumor preparations demonstrated a positive response, with activation in the presence of PGE_1/Gpp(NH)p being 3 to 7-fold over that obtained with Gpp(NH)p alone. A summary of various potential effectors on particulate AC for a benign (FA-1238) and malignant (IDC-3984) tumor is presented in Table 12.

3.1.9. Thermal Stability of Tumor Adenylate Cyclase: Thermal stability studies were conducted using particle preparations from tumors IDC-4041 and FA-3070. The enzyme was preincubated at 37°C in reaction buffer minus substrate for one to ten minutes in the presence of 10 mM NaF or 10 μM Gpp(NH)p prior to determination of cAMP production from Mn ATP. It was noted that AC from both tumor preparations showed a loss in activity after as little as one minute preincubation in the absence of NaF or Gpp(NH)p and a 50-70% loss in activity was obtained after 10 minutes preincubation (Figure 12). That NaF and Gpp(NH)p acted to stabilize the enzyme is shown in Figures 12 and 13. Preincubation with 10 mM NaF resulted in a 19% and 25% loss in cAMP

TABLE 12

INFLUENCE OF VARIOUS POTENTIAL EFFECTORS ON PARTICULATE ADENYLATE CYCLASE FROM TWO HUMAN MAMMARY TUMORS[1]

Effector	Concentration	Result	
		Malignant IDC-3984	Benign FA-1238
NaF	10 mM	4 to 5-fold stimulation	4 to 5-fold stimulation
Gpp(NH)p	100 µM	3-fold stimulation	3-fold stimulation
(Na) phosphate	10 µM-1.0 mM 10 mM	no effect 15% inhibition	no effect no effect
(Na) pyrophosphate	10 µM-1.0 mM 10 mM	no effect 100% inhibition	no effect 100% inhibition
Ca	100 µM	20-50% inhibition	20-30% inhibition
adenosine	10-100 µM	no effect	no effect
histamine	100 µM	no effect	no effect
isoproterenol	10 µM	2-fold stimulation	no effect
isoproterenol + Gpp(NH)p	10 µM, 100 µM	5-fold increase over Gpp(NH)p-stimulated activity	20% increase over Gpp(NH)p-stimulated activity
glucagon	10 µg/µl	no effect	no effect
glucagon + Gpp(NH)p	10 µg/µl, 100 µM	no effect over Gpp(NH)p-stimulated activity	no effect over Gpp(NH)p-stimulated activity
insulin	10 µg/µl	no effect	no effect
insulin + Gpp(NH)p	10 µg/µl, 10 µM	10-15% increase over Gpp(NH)p-stimulated activity	no effect over Gpp(NH)p-stimulated activity
PGE_1	10 µM	no effect	no effect
PGE_I + Gpp(NH)p	10 µm, 100 µm	50% increase over Gpp(NH)p-stimulated activity	20% increase over Gpp(NH)p-stimulated activity
17-β-estradiol[2]	saturated	no effect	no effect
progesterone[2]	saturated	no effect	no effect
prolactin	saturated	no effect	no effect
luteinizing[2] hormone	36 µg/µl	80-90% inhibition	50% inhibition

[1] Preincubation occurred for 20 minutes at 4°C prior to addition of Mg-ATP. Cyclic AMP formation was determined using the radioimmunoassay procedure.

[2] Solutions made in 2% ethanol.

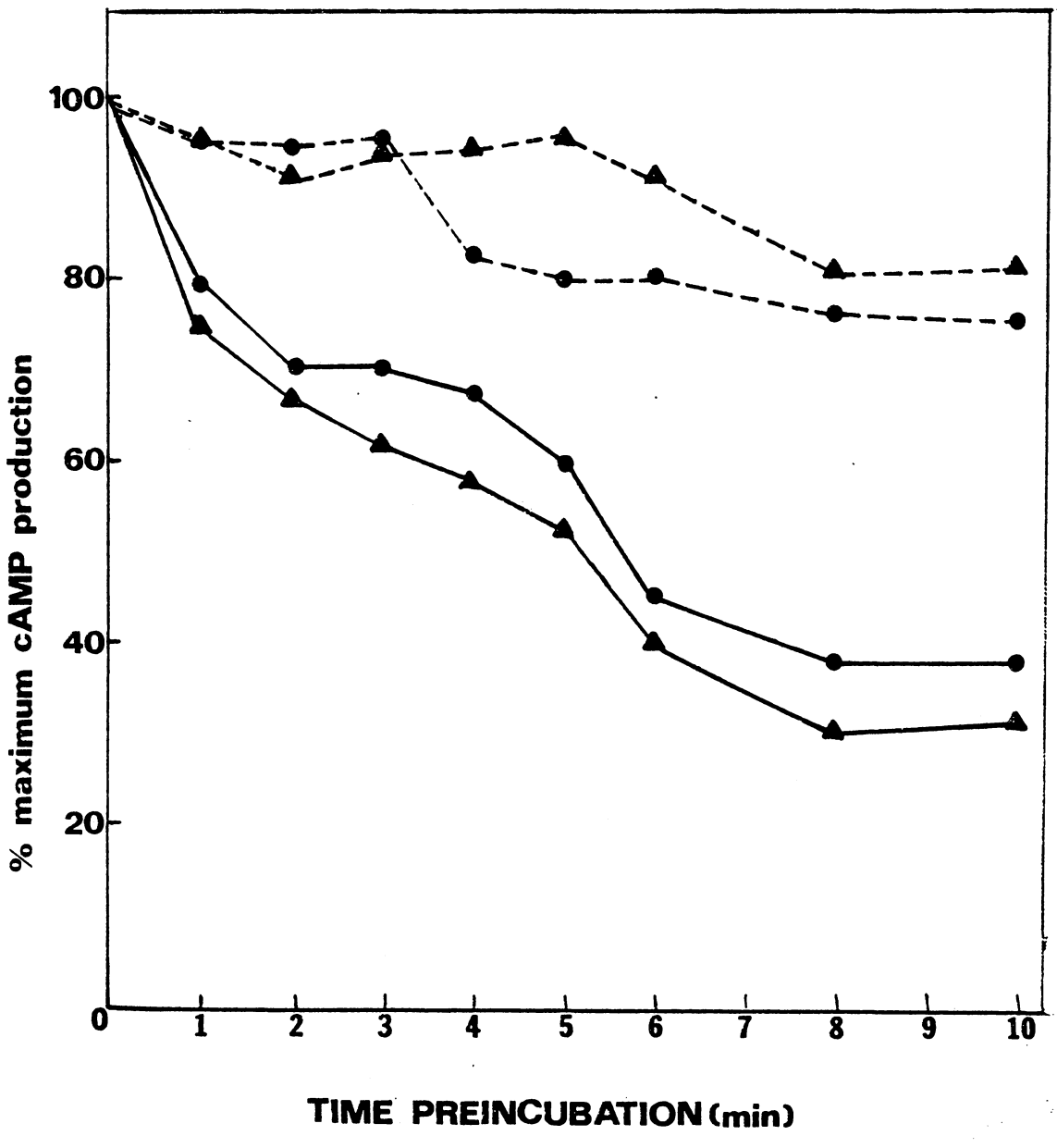


FIGURE 12

Thermal stability of adenylate cyclase from mammary tumors in the presence of sodium fluoride. Adenylate cyclase activity from malignant tumor IDC-4041 (—▲—) and benign tumor FA-1258 (—●—) was measured following preincubation in 50 mM glycylglycine, pH 7.5, at 37°C for 1 to 10 minutes prior to addition of the substrate, Mg-ATP. Preincubation of the particulate enzyme (11 µg/µl, wet wt) from the malignant (- ▲ -) and benign (- ● -) tissue with 10 mM NaF increased the thermal stability of both preparations.

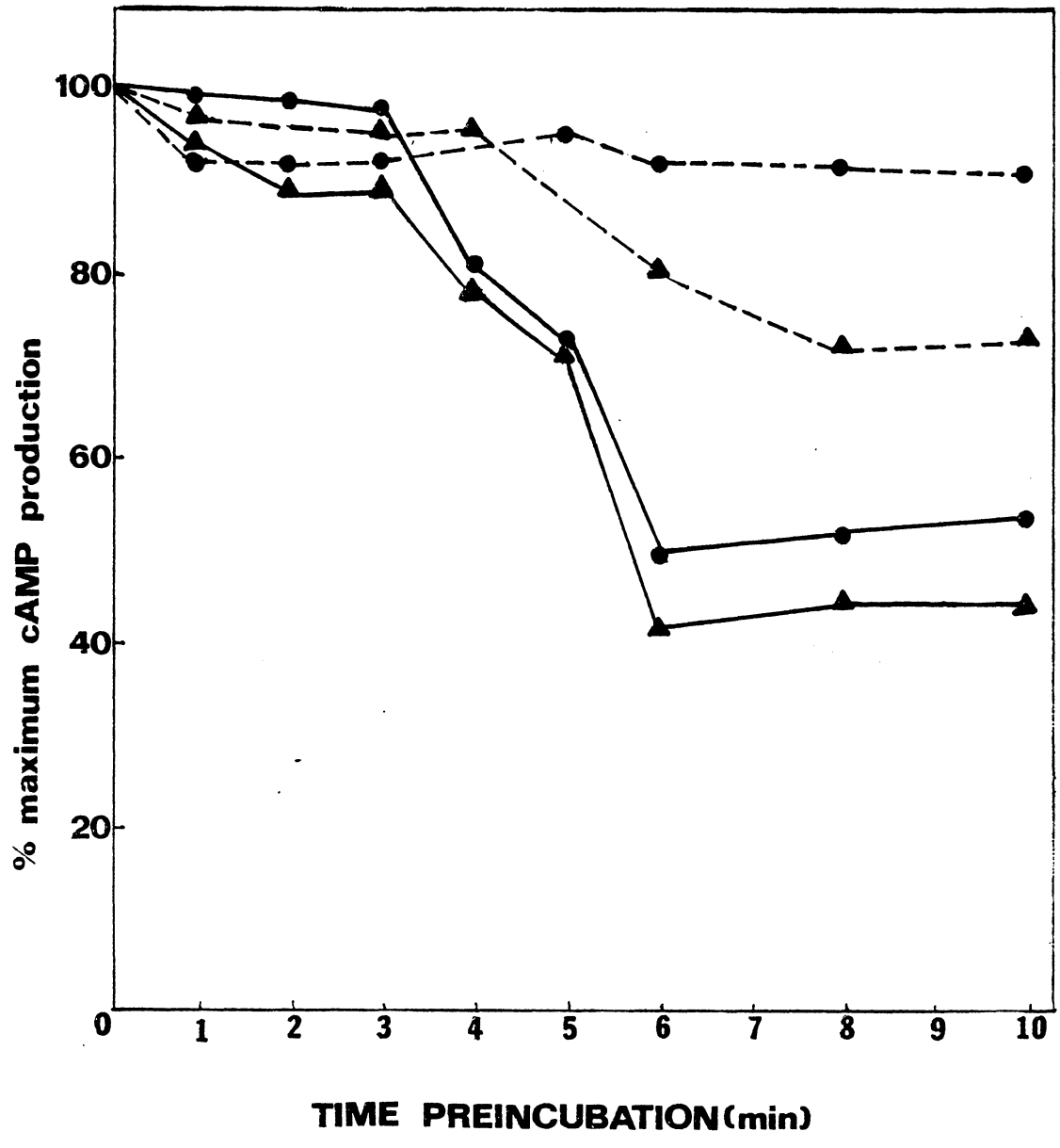


FIGURE 13

Thermal stability of adenylate cyclase from mammary tumors in the presence of guanyl-5'-yl-imidodiphosphate. Adenylate cyclase from malignant tumor IDC-4041 (—▲—) and benign tumor (—●—) was measured following preincubation in 50 mM blycylglycine, pH 7.5, at 37°C for 1 to 10 minutes prior to addition of Mg-ATP. Preincubation of the respective particulate fractions in the presence of 100 μM Gpp(NH)p resulted in stabilization of the malignant (- ▲ -) and benign (- ● -) derived enzyme activity.

production following the ten minute preincubation in the malignant and benign tumor preparations, respectively. The presence of 100 μM Gpp(NH)p resulted in a 10% and 28% loss in activity for the benign and malignant tumor preparations, respectively.

Attempts made to activate the unstabilized benign tumor AC after ten minutes preincubation at 37°C to activity levels achieved during preincubation with Gpp(NH)p or NaF were successful. Activation of the unstabilized malignant tumor enzyme to maximum levels attained during preincubation was successful in the case of NaF, and not Gpp(NH)p-activation (Figure 14). This observation suggests an increase in thermal lability of the GTP regulatory subunit in the malignant tumor AC, with respect to Gpp(NH)p-stimulation, although F^- stimulation of enzyme activity remained stable.

3.1.10. Solubilization of Membrane Bound Adenylate Cyclase: An attempt was made to solubilize AC activity from a particulate preparation of malignant tumor IDC-3784. The pellet fraction was preincubated with NaF and Gpp(NH)p prior to solubilization with 0.1% Lubrol PX in order to stabilize activity as described by Young and Stansfield (1978). The 105,000 x g supernatant fraction from the non-stabilized preparation gave the highest soluble enzyme activity when expressed as percent of the initial activity (Table 13). Although AC activity was greater from the NaF-stabilized preparation, this only represented 10.5% of the initial enzyme activity. The supernatant from the Gpp(NH)p-stabilized preparation allowed only a 7% recovery of enzyme activity. Recovery of enzyme activity was

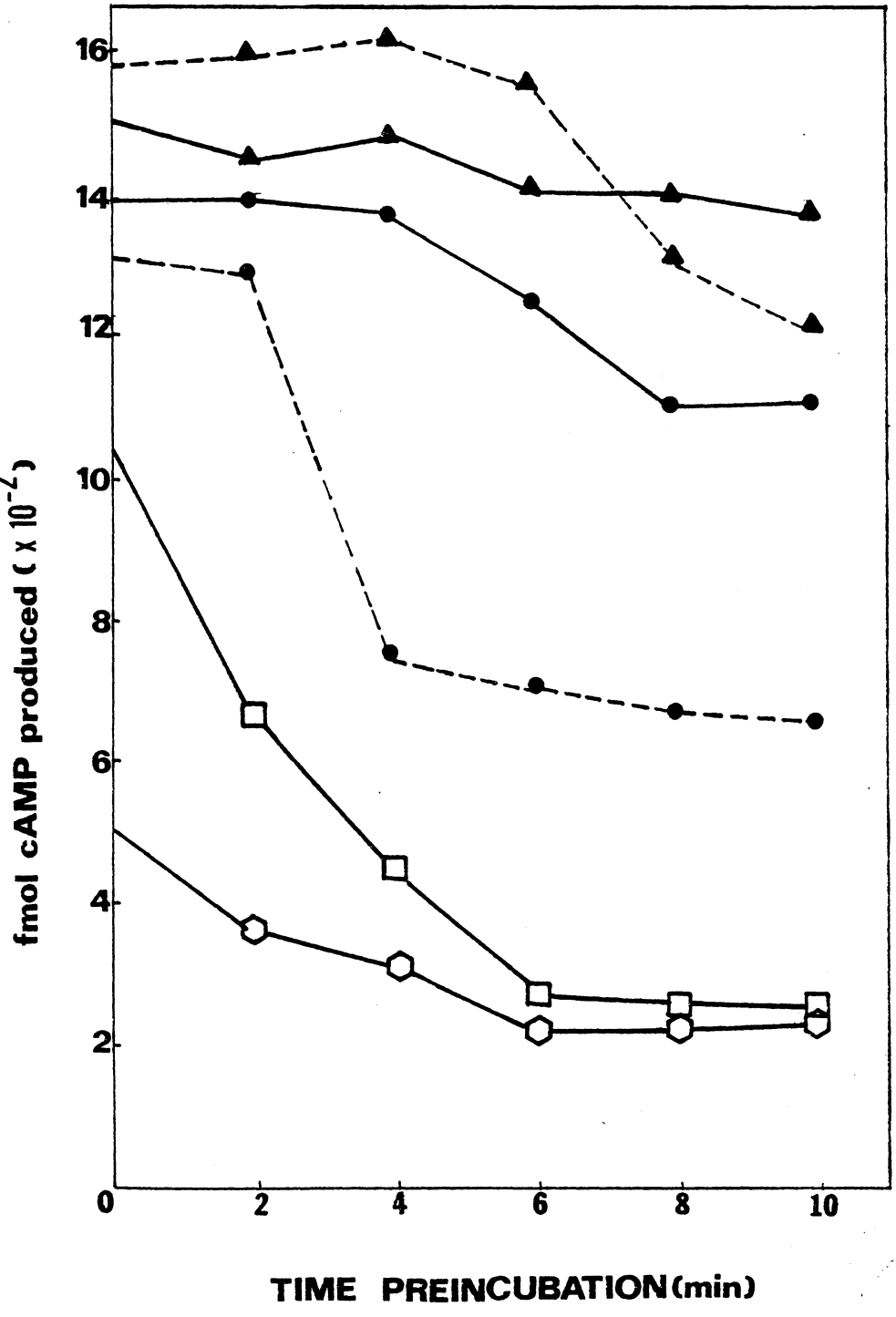


FIGURE 14

The production of cAMP by particulate adenylate cyclase from malignant tumor IDC-4041 was measured after 1 to 10 minutes pre-incubation in 50 mM glycylglycine, pH 7.5, at 37°C for the untreated enzyme (—□—), and enzyme in the presence of 8 mM $MnCl_2$ (—⬡—), 10 mM NaF (—▲—), and 100 µM guanyl-5'-yl-imidodiphosphate (—●—). This activity was compared to results obtained with unstabilized, heat-treated enzyme assayed in the presence of 10 mM NaF (- -▲- -) and 100 µM guanyl-5'-yl-imidodiphosphate (- -●- -). Tissue addition per assay tube was 50 µg, dry wt.

TABLE 13

SOLUBILIZATION OF ADENYLATE CYCLASE ACTIVITY ASSOCIATED WITH A PARTICULATE PREPARATION FROM MALIGNANT TUMOR IDC-3784

Fraction	AC activity (units/mg dry wt)[1]	% of particulate fraction
(a) Inactivated particulate	640	
(b) Particulate activated by NaF	2570	
(c) Particulate activated by Gpp(NH)p	1480	
(d) Supernatant from untreated particulate preparation	160	21.2
(e) Supernatant from NaF-activated particulate preparation	270	10.5
(f) Supernatant from Gpp(NH)p-activated particulate preparation	130	7.1

[1]Details of the solubilization procedure are given in "Methods". The particulate fractions (a-c) consisted of the 3,000 x g washed particle preparation subjected to centrifugation at 105,000 x g for one hour in the solubilization buffer minus 1% Lubrol PX, then activated during the assay procedure. Fractions (d-f) represent the 100,000 x g supernatant fractions from particulate preparations either inactivated or activated during solubilization with 1% Lubrol PX. One unit of enzyme will catalyze the production of one fmol cAMP per minute at 37°C.

comparable to that obtained following solubilization of AC in other mammalian tissues (Welton et al., 1978). Perhaps purification and characterization of soluble tumor-derived enzyme activity will be possible as it has been for other cyclase systems studied.

3.2. Adenylate Cyclase From Microsections of Mammary Tumors

3.2.1. Determination of Enzyme Activity: Lyophilized sections from malignant and benign mammary tumors were examined under a dissecting microscope and tumor specific cellular regions were cut from the sections, and further subdivided, to be used for assessment of AC activity. After several micro-sections were accumulated for each tumor, they were weighed on the quartz fiber balance, and pooled in triplicate for each determination, giving a total of 10-50 µg dry wt tissue/assay tube. Reaction conditions were redefined to give maximum cAMP synthetic activity. A comparison of conditions for the tumor washed particle preparations and tissue micro-sections as the source of enzyme is summarized in Table 14. Endogenous cAMP levels were estimated to range from 100 to 200 fmol based upon RIA substrate blanks. Final concentrations of Mg^{2+} and ATP were decreased to 1.5 mM and 4.0 mM, respectively, which gave maximum activity levels. Also included was an ATP regenerating system consisting of 5.0 mM creatine phosphate and 10 units creatine kinase, the presence of which gave an approximate 3-fold increase in cAMP production. It appeared that endogenous ATPase activity was significant enough to interfere with the cyclase reaction.

TABLE 14

COMPARISON OF REACTION CONDITIONS FOR ANALYSIS OF ADENYLATE CYCLASE ACTIVITY IN TUMOR PARTICLE PREPARATIONS AND TUMOR MICRO-SECTIONS

	Washed Particulate Preparation (3000 x g pellet)	Tissue Micro-sections
assay mixture	50 mM glycylglycine, pH 7.5 10 µg/ml BSA 5.0 mM ATP 8.0 mM MgCl total volume, 26 µl	50 mM glycylglycine, pH 7.5 10 µg/ml BSA 1.5 mM ATP 4.0 mM MgCl 5.0 mM creatine PO 10 units creatine phosphokinase 5.0 mM isobutylmethylxanthine total volume, 26 µl
specific activity	pmol cAMP/min/mg/dry wt	pmol cAMP/min/mg dry wt
range of basal activities	Mn^{2+} IDC: 0.10 - 0.80 FA: 0.70 - 3.00 Mg^{2+} IDC: 0.05 - 0.35 (Gpp(NH)p FA: 0.20 - 1.50	Mn^{2+} IDC: 5 - 7 FA: 6 - 9 Mg^{2+} IDC: 0.5 - 6 FA: 0.5 - 6
incubation conditions	37°C, 15/30 min	37°C, 15 min
tissue addition	homogenate: mg/ml protein; IDC: 1.0 - 2.3 FA: 1.4 - 11.5 mg/ml wet wt; IDC: 21 - 56 FA: 11 - 21 dry wt; 50 - 70 µg	dry wt: 10 - 50 µg
standard curve	50 - 1000 fmol cAMP	50 - 1000 fmol cAMP

Activity levels observed with tumor microsections in the presence of Mn ATP were 3-fold greater than that with Mg ATP, a value similar to that obtained with washed particle preparations. It was necessary to include a phosphodiesterase inhibitor to prevent the enzymatic degradation of cAMP when reactions were carried out in the presence of either Mn ATP or Mg ATP. In the case of FA-3070, the absence of 5.0 mM theophylline in the reaction mixture resulted in a 45% loss in AC activity. In some tumor micro-sections, up to a 90% loss in cAMP production occurred in the absence of a PDE inhibitor. Theophylline or 3-methyl-1-isobutyl xanthine at a final concentration of 5mM were equal in their ability to prevent cAMP degradation.

Under the redefined assay conditions, cAMP synthesis measured as pmol cAMP produced min^{-1}. mg dry wt-1 was found to be linear over approximately a 15 minute incubation period at 37°C (Figure 15). Adenylate cyclase activity also increased linearly with addition of 2 to 50 μg/dry wt tissue (Figure 16).

3.2.2. Inter- and Intra-Tumor Comparisons: Variation in AC activity was observed to occur in adjacent tumor regions as well as in areas from different locations with the tumor section. Table 15 illustrates the range in activity resolved from tumors IDC-733 and FA-3070. Consequently, ten to twelve pooled values were averaged to estimate the mean potential for cAMP production for the mammary tumors examined.

3.2.3. Influence of Divalent Cations on Adenylate Cyclase: Adenylate cyclase activity from tumor IDC-733 in the presence of Mn^{2+}/Mg^{2+} is

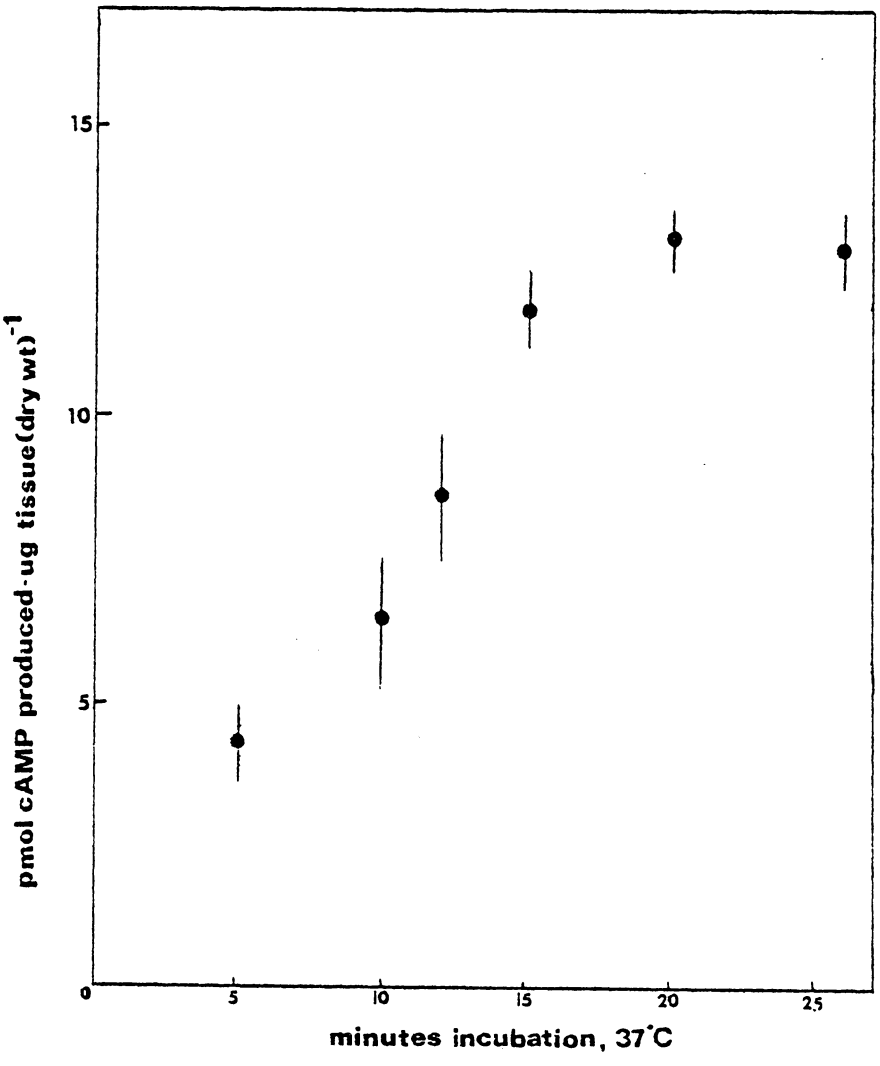


FIGURE 15

Cyclic AMP production as a function of time. A tumor-specific locus was micro-dissected from a lyophilized section from malignant tumor IDC-4041 and subdivided to yield 20 to 25 µg tissue dry wt per reaction tube. Cyclic AMP production was measured in the presence of 1.5 mM ATP and 4.0 mM $MnCl_2$, following incubation at 37°C for 15 minutes. Specific activity is expressed as pmol cAMP produced per microgram tissue dry wt. Activity values represent the mean of 5 replicates for each weight range. Bars indicate the standard error of the mean.

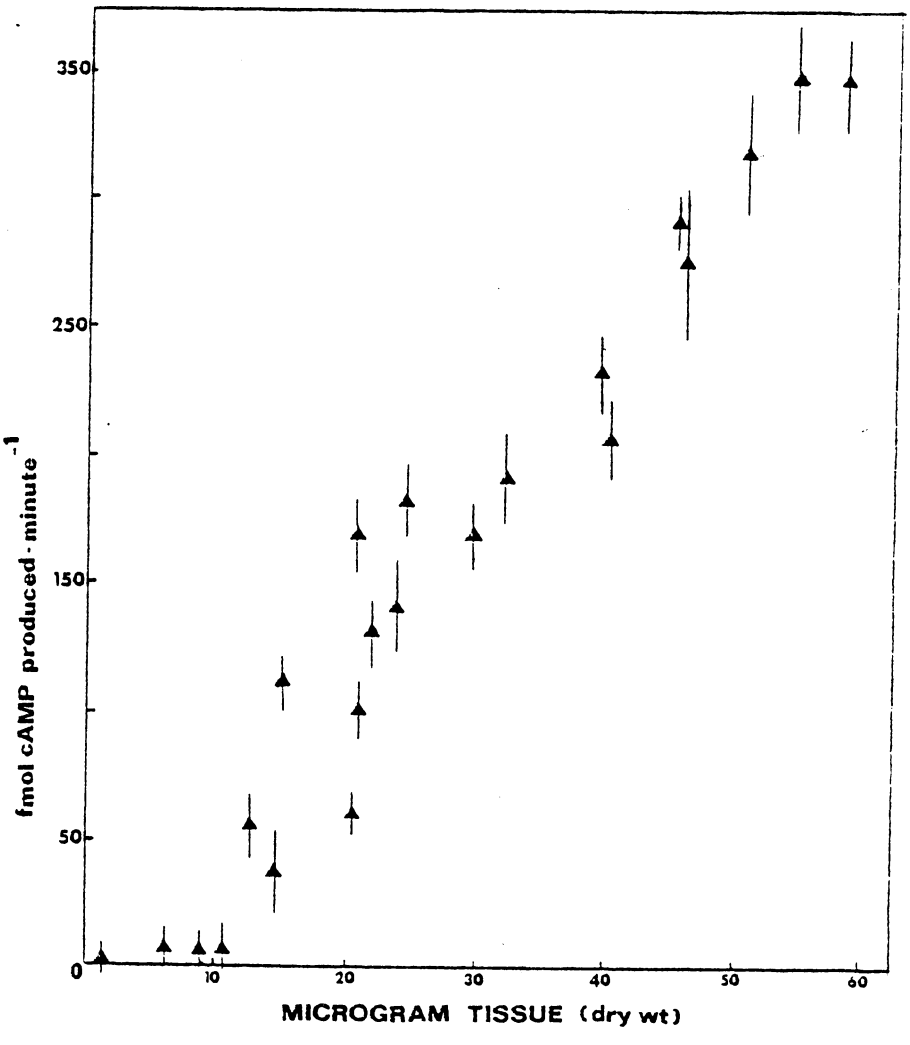


FIGURE 16

Cyclic AMP formation was measured as a linear function of tissue dry wt addition. Distinct loci were located and microdissected from several lyophilized sections from malignant tumor IDC-4041, and further subdivided to give 2 to 60 µg tissue dry wt per assay tube. Cyclic AMP production was measured in the presence of 1.5 mM ATP and 4.0 mM $MnCl_2$, following incubation at 37°C for 15 minutes. Specific activity is expressed as fmol cAMP produced per microgram tissue dry wt. Activity values represent the mean of triplicate samples taken from identical regions within the tumor tissue sections. Bars indicate standard error of the mean.

TABLE 15

VARIATION IN ADENYLATE CYCLASE ACTIVITY IN TISSUE MICRO-PIECES DISSECTED FROM LYOPHILIZED MALIGNANT AND BENIGN MAMMARY TUMOR SECTIONS

micro-section number	µg	specific activity[1] (units/mg dry wt)
IDC-733		
1	2.9	1.35
2	2.2	1.18
3	4.0	0.35
4	3.1	0.60
5	2.4	2.80
6	2.1	1.70
7	3.1	0.58
8	3.6	1.17
9	2.8	1.18
10	2.9	0.43
11	2.4	0.96
12	2.4	0.48
FA-3070		
1	3.1	2.80
2	2.9	1.44
3	4.3	0.67
4	4.5	0.39
5	2.1	1.19
6	2.4	1.73
7	2.1	1.10
8	2.1	1.20
9	2.9	1.32
10	2.9	2.07

[1]Cyclic AMP production was measured in the presence of 1.5 mM ATP and 4.0 mM $MgCl_2$. Mean activity ±SEM for IDC-733 and FA-3070 were 1.07 ± 0.20 and 1.38 ± 0.22 units/mg dry wt. One unit of enzyme will catalyze the production of one pmol cAMP per minute at 37°C.

shown in Table 16. It was noted that cAMP production was 2 to 3-fold higher with Mn ATP than with Mg ATP, and variation was greater between equivalent tissue samples with Mg ATP as the reaction substrate. Inclusion of 50 μM Ca^{2+} in the reaction mixture had variable effects on cAMP production (Table 17). Over 50% of the malignant tumor sections analyzed showed inhibition ranging from 7 to 76%. Likewise, over 60% of the benign tumor sections showed inhibition ranging from 8 to 57%. Alternatively, FA-3070 demonstrated a 2-fold increase in AC activity and FA-1038 showed a 5-fold increase in the presence of 50 μM Ca^{2+}.

3.2.4. Stimulation by Guanyl Nucleotides: Basal and Gpp(NH)p-stimulated AC activity determined from micro-sections for a series of malignant and benign tumors are given in Table 18 and 19. The mean basal activities for both tumor classes were similar, and calculated to be 1.6 pmol cAMP.min-1.mg dry wt-1 and 2.0 pmol cAMP.min-1.mg dry wt-1 for the benign and malignant tumor samples, respectively. Likewise, the range of activities was found to be similar with a range of 0.31 to 6.26 pmol cAMP.min-1.mg dry wt-1 for benign and 0.22 to 5.66 pmol cAMP.min-1 mg dry wt-1 for malignant. AC activity levels were determined to be higher in tissue microsections from both tumor types compared to tumor washed particle fractions.

Tumor micro-section derived AC was sensitive to activation by guanine nucleotides. The activity ratio determined in the presence of 100 μM Gpp(NH)p was determined to vary from 0.07 to 0.50 and 0.19 to 0.42 for the malignant and benign tumor sections examined.

TABLE 16

VARIATION IN ADENYLATE CYCLASE ACTIVITY FOUND IN ADJACENT TISSUE PIECES FROM MALIGNANT TUMOR IDC-733 IN THE PRESENCE OF Mn-ATP OR Mg-ATP

addition	micro-section[2] number	µg dry wt	units/mg dry wt
1.5 mM ATP/4.0 mM Mn^{2+}	1a	7.4	5.41
	1b	7.4	4.46
	2a	7.0	5.11
	2b	7.4	4.19
	3a	7.2	4.16
	3b	6.7	5.97
	4a	9.9	3.01
	4b	7.2	2.63
	5a	8.6	3.37
	5b	7.0	4.68
1.5 mM ATP/4.0 mM Mg^{2+}	1a	7.4	3.11
	1b	6.7	2.24
	2a	7.0	1.17
	2b	8.4	0.49
	3a	8.3	0.72
	3b	7.4	1.01
	4a	6.7	0.78
	4b	5.8	1.29
	5a	5.5	1.18
	5b	8.3	0.43

[1]Activity values based upon triplicate samples taken from two specific loci in each tumor section. One unit of enzyme will catalyze the production of one pmol cAMP per minute at 37°C.

[2]Two adjacent loci within an individual tumor section are designated a and b.

TABLE 17

INFLUENCE OF CALCIUM ON ADENYLATE CYCLASE ACTIVITY IN MALIGNANT AND BENIGN MAMMARY TUMOR SECTIONS

tumor	basal activity[1] (units/mg dry wt)	+50 μM Ca^{2+} (units/mg dry wt)
Malignant:		
2278	0.17	0.05
733	0.63	0.29
345	1.33	0.59
1688	1.46	0.35
147	1.57	2.09
2760	0.60	0.79
3669	2.60	2.57
3928	3.94	2.41
4041	7.89	8.46
Benign:		
798	1.65	1.51
1258	2.49	2.29
1700	3.15	3.53
2752	0.58	0.34
2732	1.23	0.88
3070	2.19	4.15
1709	0.44	0.19
1038	0.39	1.20

[1]Activity values based upon triplicate samples. Microsections were dissected from identical single loci within two lyophilized sections from each tumor examined. Cyclic AMP production was measured in the presence of 1.5 mM ATP and 4.0 mM $MgCl_2$. One unit of enzyme will catalyze the production of one pmol cAMP per minute at 37°C.

TABLE 18

BASAL AND GUANYL-5'-YL-IMIDODIPHOSPHATE-STIMULATED ADENYLATE CYCLASE ACTIVITY IN MALIGNANT MAMMARY TUMOR SECTIONS

tumor	basal activity[1]	Gpp(NH)p-stimulated[2] activity (units/mg dry wt)	Activation[3] Index
2760	0.76	10.00	0.08
3669	2.82	13.05	0.22
4041	5.66	11.40	0.50
733	2.57	8.40	0.30
2278	0.22	2.98	0.07
3928	1.35	4.48	0.30
345	2.04	4.89	0.42
147	0.67	3.08	0.22
1688	1.88	5.86	0.32

[1]Activity values represent the mean of triplicate experiments. Tumor microsections were dissected from single identical loci from three lyophilized sections for each tumor examined; total weight per reaction was 20-40 μg. One unit of enzyme will catalyze the production of one pmol cAMP per minute at 37°C.

[2]Activation occurred in the presence of 10 μM Gpp(NH)p.

[3]Activation index given as the function:

$$\frac{\text{cAMP formation in the absence of Gpp(NH)p}}{\text{cAMP formation in the presence of Gpp(NH)p}}$$

TABLE 19

BASAL AND GUANYL-5'-YL-IMIDODIPHOSPHATE-STIMULATED ADENYLATE CYCLASE ACTIVITY IN BENIGN MAMMARY TUMOR SECTIONS

tumor	Basal activity[1]	Gpp(NH)p-stimulated[2] activity (units/mg dry wt)	Activation[3] Index
798	1.28	4.01	0.31
1258	0.83	4.40	0.19
1700	0.92	2.19	0.42
2752	0.31	0.84	0.37
2732	2.18	11.77	0.19
3070	6.26	20.86	0.30
1709	0.60	2.98	0.20
1038	0.50	2.30	0.22

[1]Activity values represent the mean of triplicate experiments. Tumor microsections were dissected from single identical loci from three lyophilized sections for each tumor examined; total weight per reaction was 20-40 µg. One unit of enzyme will catalyze the production of one pmol cAMP per minute at 37°C.

[2]Activation occurred in the presence of 10 µM Gpp(NH)p.

[3]Activation index given as the function:

$$\frac{\text{cAMP formation in the absence of Gpp(NH)p}}{\text{cAMP formation in the presence of Gpp(NH)p}}$$

Although tumors IDC-2760 and IDC-2278 showed tremendous activation by Gpp(NH)p, it appeared that the activation potential was similar for the two classes of tumors giving a one to four-fold increase in cAMP production in the presence of 10 μM Gpp(NH)p.

3.2.5. Hormonal Responsiveness of Adenylate Cyclase: Hormonal stimulation of AC from tumor microsections was initially studied in malignant tumor microsections. Cyclic AMP synthesis in the presence of 10 μM Gpp(NH)p and 10 μM PGE_1 was examined and the specific activities calculated on a mg dry wt basis (Table 20). As was evident in the tumor washed particle preparations, increased AC activity occurred in the presence of the coupled guanine nucleotide/hormone system. Hormonal responsiveness in the presence of 10 μM Gpp(NH)p was extended to include the hormones prolactin, TSH, FSH, LH and hCG (Figure 17, A-E). Little response was observed with FSH and hCG. Variable response was observed from LH and TSH. Cyclic AMP production in three out of eight benign tumors and five out of nine malignant tumors was stimulated by TSH. Six out of eight benign tumors and six out of nine malignant tumors showed decreased cAMP synthesis in the presence of LH. Activation of tumor-associated AC occurred in the presence of prolactin and PGE1 in both classes of tumors. Six out of eight benign tumors and four out of nine malignant tumors showed increase cAMP with prolactin. Seven out of eight benign tumors and all nine malignant tumors gave a positive response with PGE1. These results indicate that hormone-responsiveness was demonstratable using

TABLE 20

EFFECT OF GUANYL-5'-YL-IMIDODIPHOSPHATE AND PROSTAGLANDIN E ON ADENYLATE CYCLASE ACTIVITY IN MALIGNANT MAMMARY TUMOR SECTIONS

tumor[1]	AC activity (units/mg dry wt)[2]		
	basal	+Gpp(NH)p	+PGE_1 /Gpp(NH)p
733	1.7	9.1	27.50
345	3.7	20.3	26.2
1688	2.3	15.0	18.9
3669	1.1	5.9	7.3
4041	2.0	3.3	6.3

[1]Tumor microsections were dissected from a single locus within the tumor.

[2]Reactions run in the presence of 10 μM Gpp(NH)p +/- 10 μM PGE_1. One unit of enzyme will catalyze the production of one pmol cAMP per minute at 37°C. Activity values represent the mean derived from triplicate experiments.

FIGURE 17

Hormonal responsiveness in a series of malignant and benign human mammary tumor microsections. Tissue dry weight samples ranging from 20 to 45 µg were assayed for cAMP production following preincubation with 10 µM Gpp(NH)p and 10 µM hormone for 15 minutes at 4°C. Cyclic AMP synthesis was measured by RIA. Relative activity was determined from the function;

$$\frac{\text{cAMP production in the presence of Gpp(NH)p/hormone}}{\text{basal cAMP production}}$$

TSH, thyrotropin-stimulating hormone; LH, luteinizing hormone; PGE_1, prostaglandin E_1; FSH, follicle-stimulating hormone; hCG, human chorionic gonadotropin.

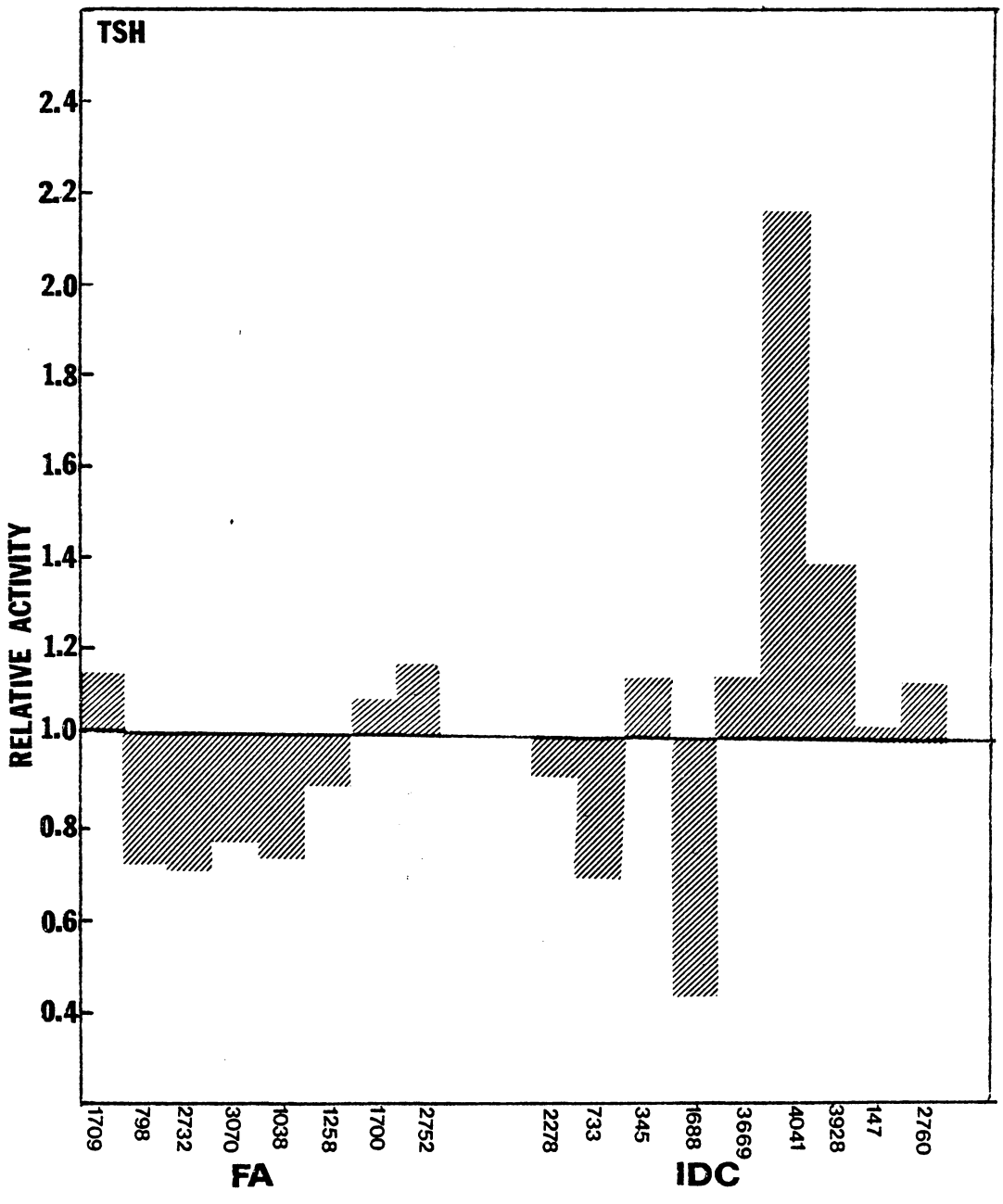


FIGURE 17A.

Adenylate cyclase activity in the presence of thyrotropin-stimulating hormone.

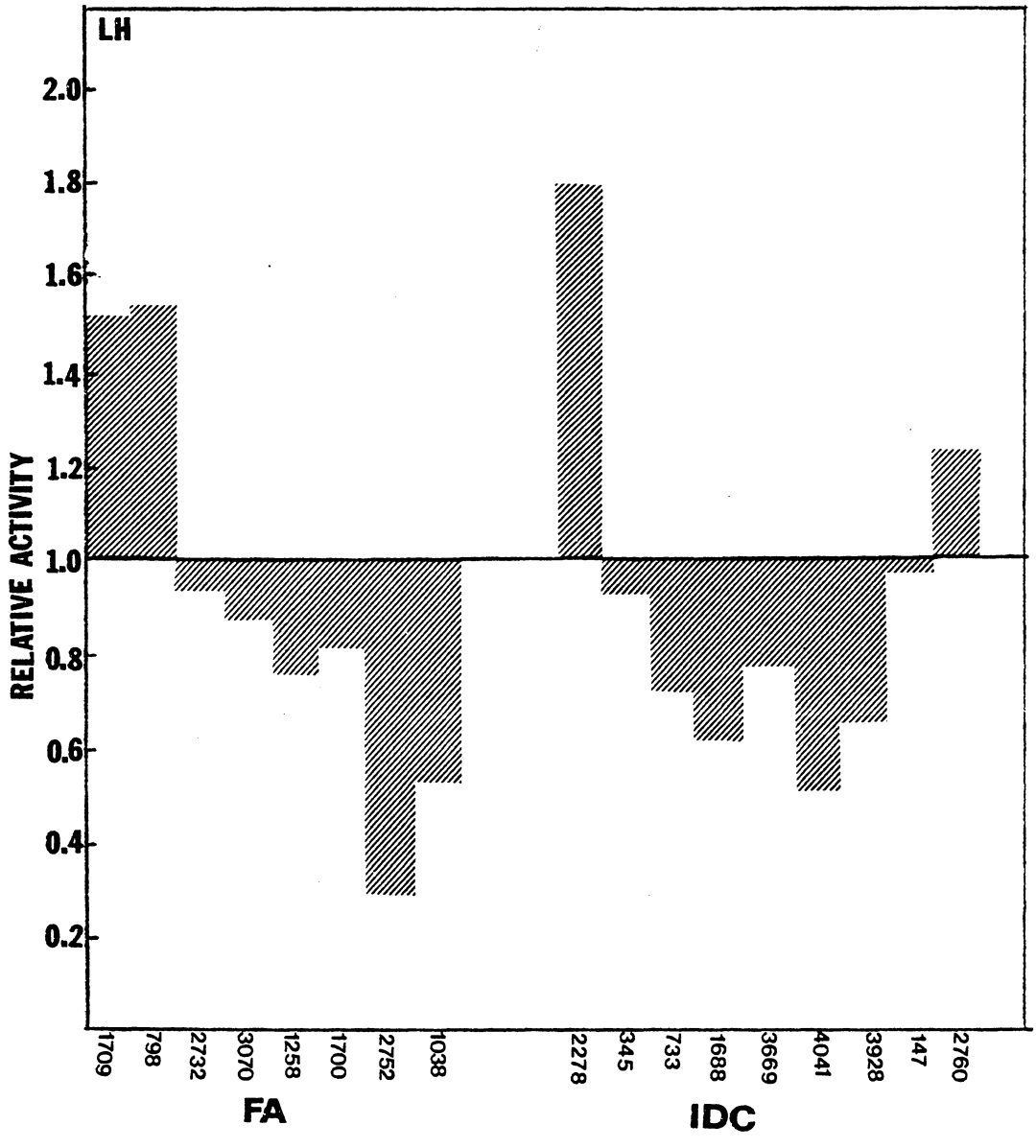


FIGURE 17B.

Adenylate cyclase activity in the presence of luteinizing hormone.

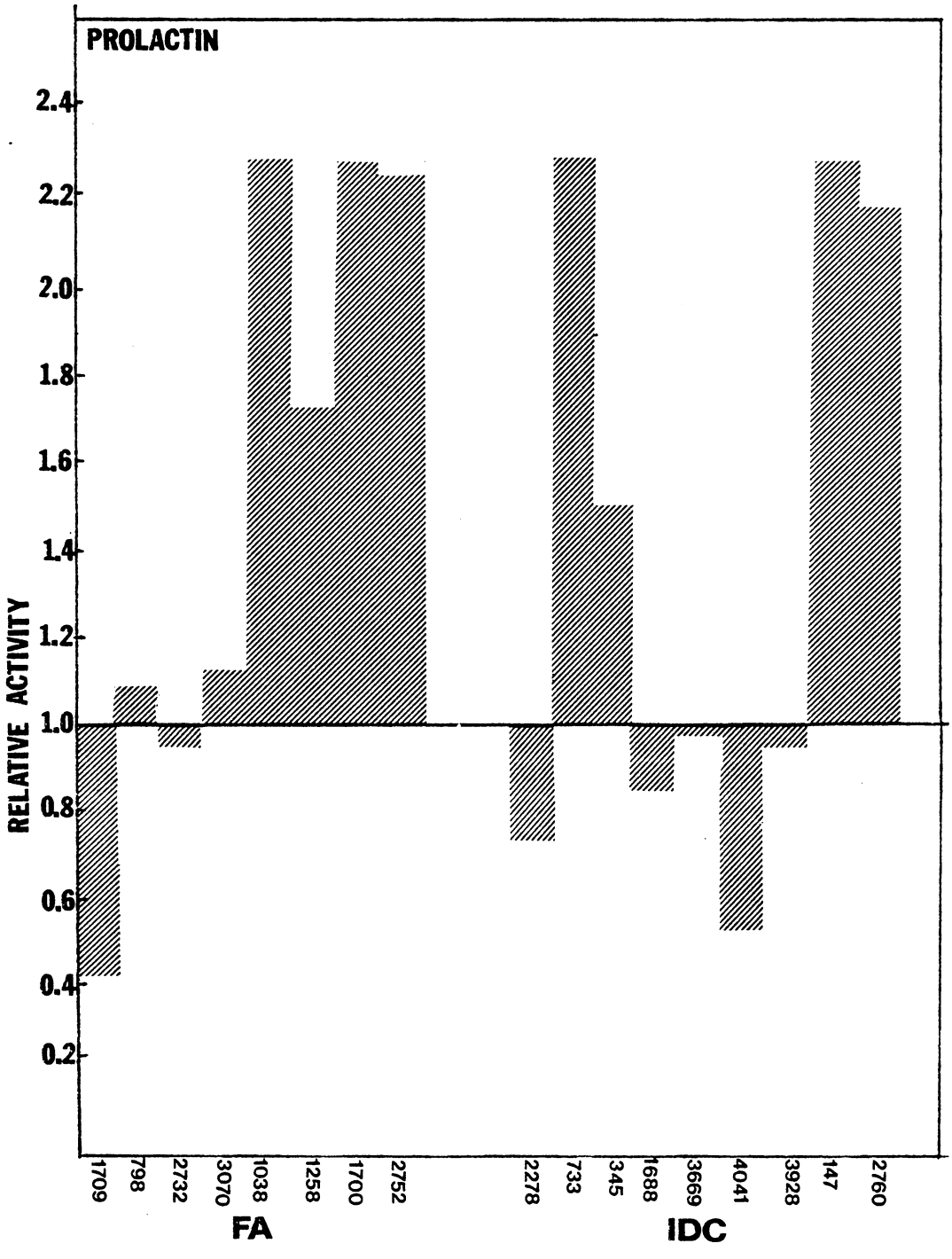


FIGURE 17C.

Adenylate cyclase activity in the presence of prolactin.

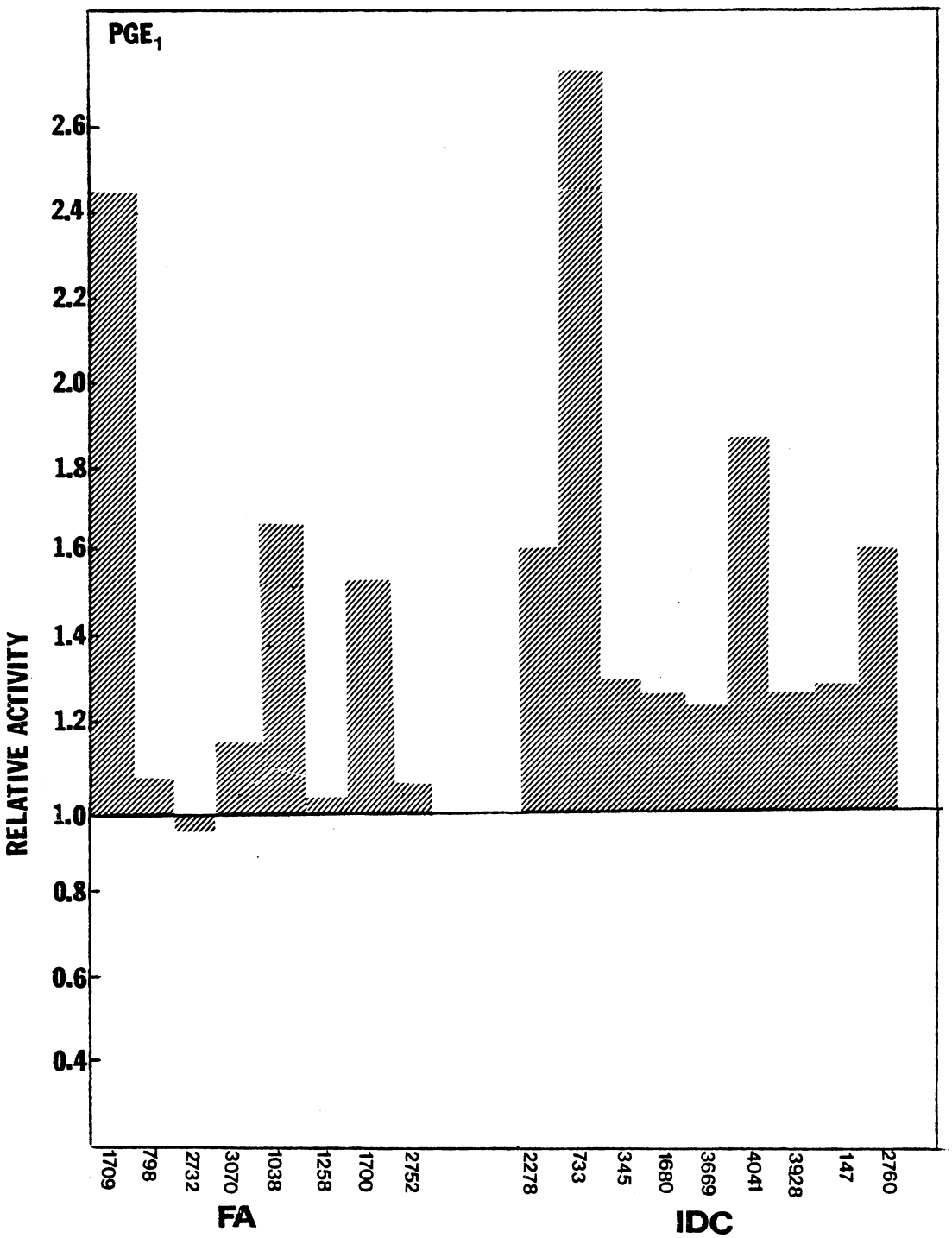


FIGURE 17D.

Adenylate cyclase activity in the presence of prostaglandin E_1.

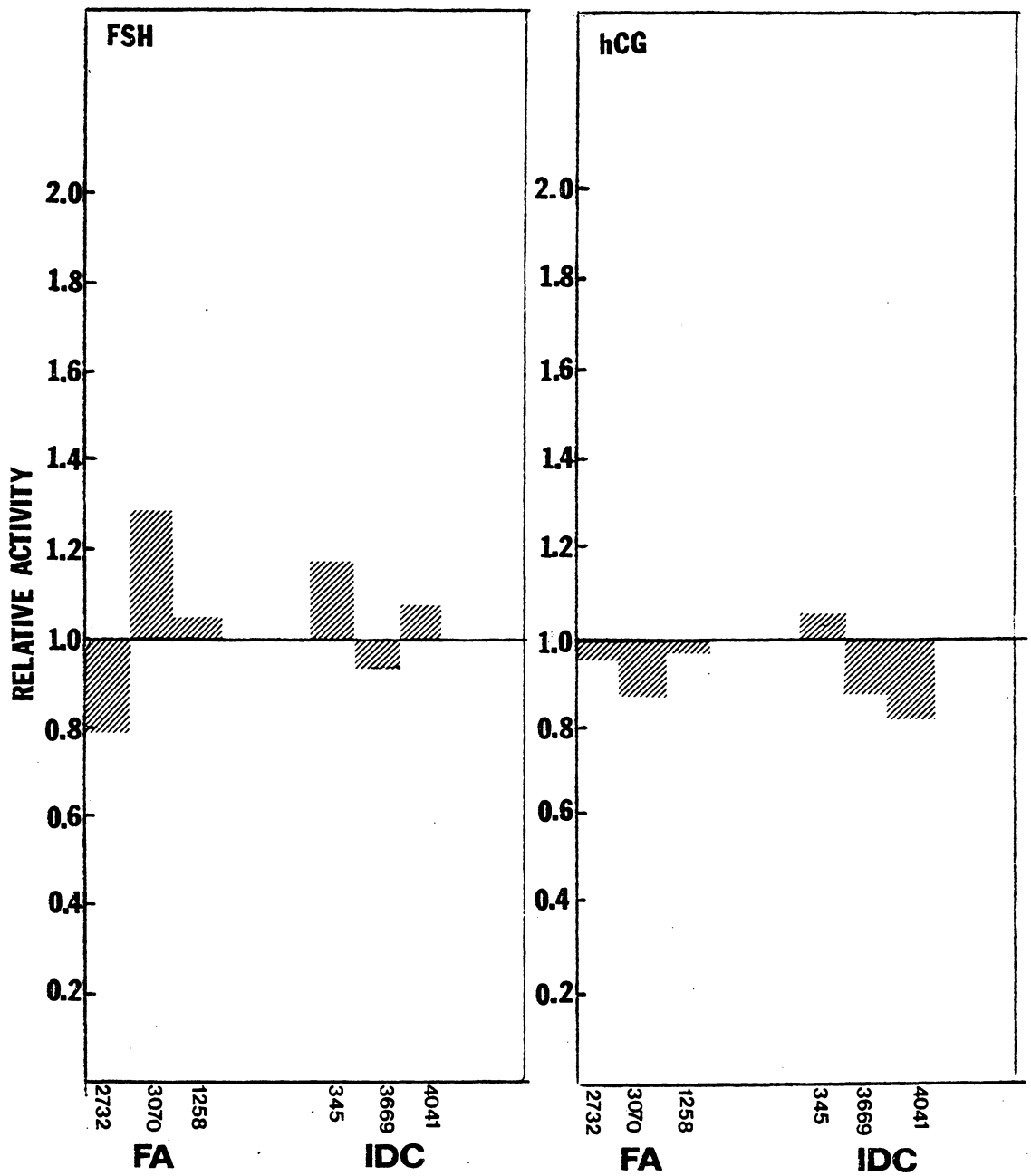


FIGURE 17E.

Adenylate cyclase activity in the presence of follicle-stimulating hormone and human chorionic gonadotropin.

confined loci of specifically neoplastic cells.

3.2.6. Thermal Stability of Tissue Associated Adenylate Cyclase: Since mammary tumor particle AC was temperature-sensitive, it was of interest to determine whether tumor microsection-derived AC showed the same character. The thermal stability of three malignant and three benign tumor microsections was examined. Tissue pieces were preincubated at 37°C for 0, 2, 5, 10 and 15 minutes in the presence or absence of 10 μM Gpp(NH)p, then cAMP production was determined after 15 minutes incubation with Mg ATP at 37°C. In all cases, pretreatment with the guanine nucleotide allowed for greater cyclase activity after maximum preincubation, and increased the stability of the enzyme from 10 to 60% (Figures 19 and 20). Basal activities declined 55 to 90% after 15 minutes preincubation at 37°C. These observations indicate that cAMP production in tumor microsections was temperature sensitive, but couldbe partially stabilized, similar to AC activity in tumor particle fractions.

3.2.7. Fractionation of Tissue Associated Adenylate Cyclase Activity: Tumor microsections from FA-3070 and IDC-3667 were cut and pooled to give a total dry wt accumulation of 5.3 mg and 4.0 mg, respectively. After brief homogenization at 4°C in 1.0 ml glycylglycine, pH 7.5 containing 10 g/ml BSA, and 5.0 mM IBMX, using a Radnoti #612 glass homogenizer (Radnoti Glass Technology, Arcadia, CA), the samples were centrifuged at 3,000 x g for 20 minutes, and the supernatant and pellet fractions assayed for AC activity. As indicated in

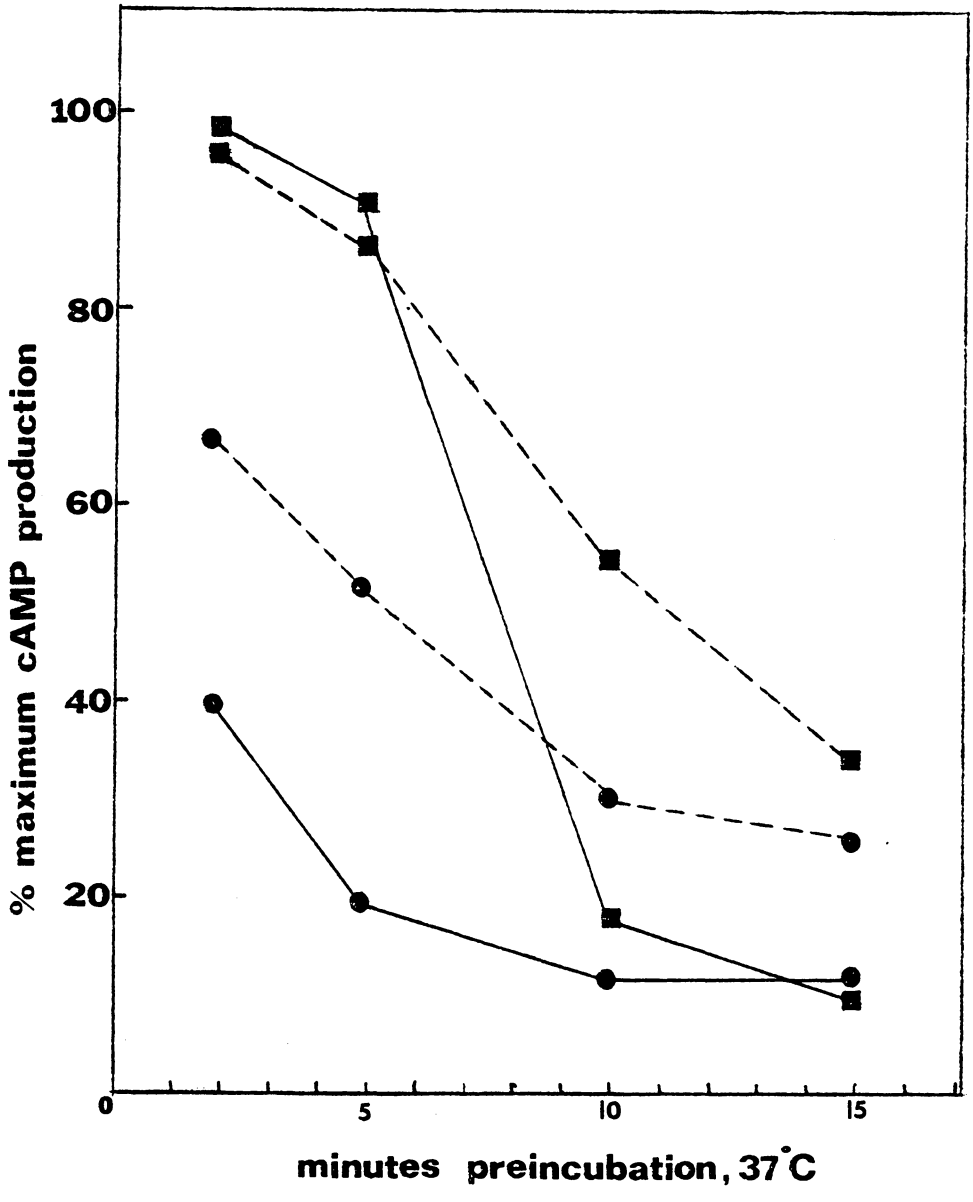


FIGURE 18

Thermal stability of cAMP formation by adenylate cyclase associated with tissue micro-sections from benign mammary tumors. Tissue pieces from specific loci were preincubated for 2, 5, 10, or 15 minutes at 37°C, then cAMP production from Mg-ATP was measured in the presence or absence of 10 μM guanyl-5'-yl-imidodiphosphate following 15 minutes incubation at 37°C. Activity is expressed as % maximum cAMP production using non-preincubated tissue as the control. Tissue addition per reaction was 20 to 35 μg dry wt. FA-1700, in the presence (-■-) or absence (—■—) of Gpp(NH)p; FA-1258, in the presence (-●-) or absence (—●—) of Gpp(NH)p.

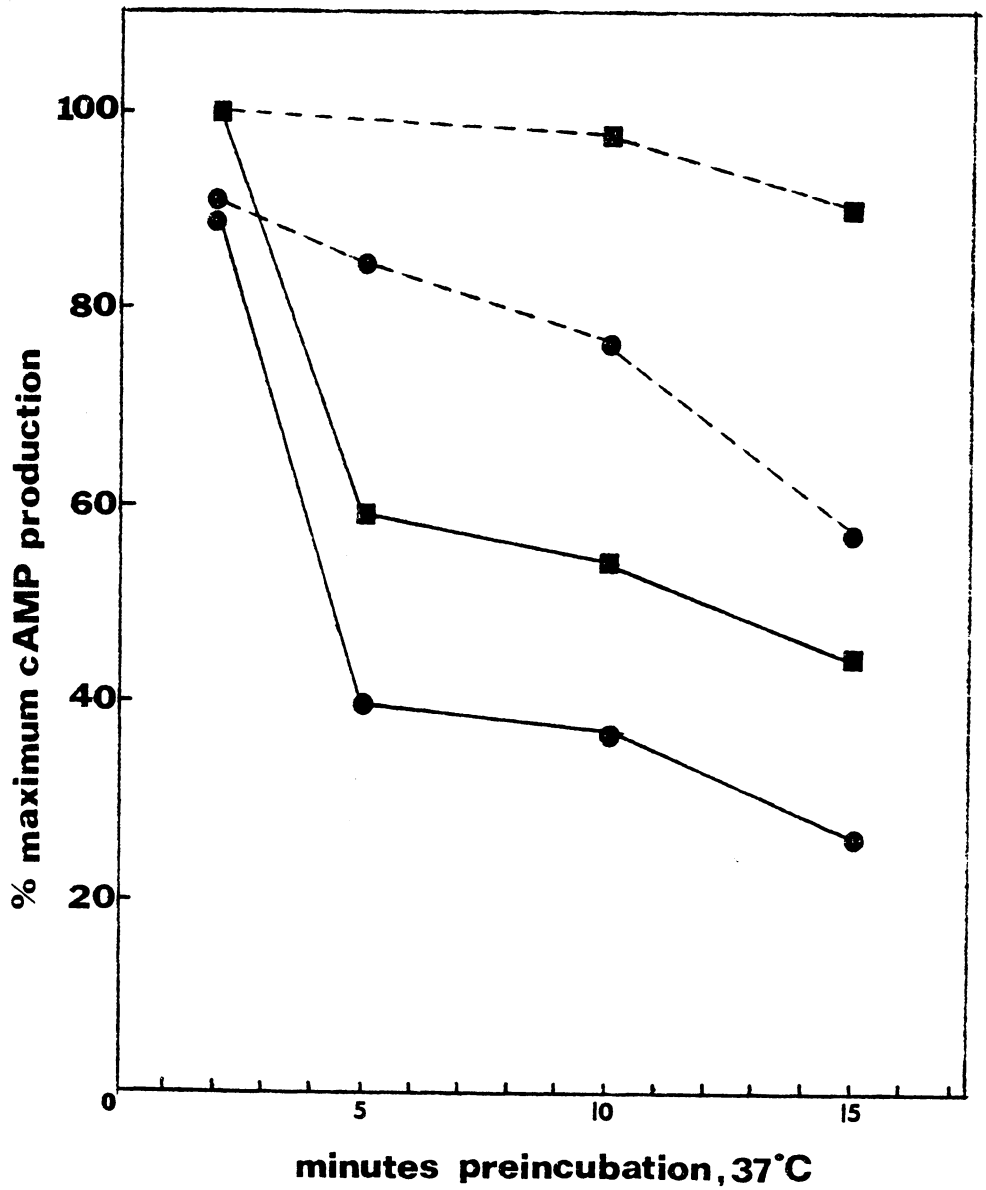


FIGURE 19

Thermal stability of cAMP formation by adenylate cyclase associated with tissue micro-sections from malignant mammary tumors. Tissue pieces from specific loci were preincubated for 2, 5, 10, or 15 minutes at 37°C, then cAMP production from Mg-ATP was measured in the presence of absence of 10 µM guanyl-5'-yl-imidodiphosphate following 15 minutes incubation at 37°C. Activity is expressed as % maximum cAMP production (using non-preincubated reactions as the control activity values). Tissue addition per reaction was 25 to 35 µg dry wt. IDC-3928, in the presence (—o—) and absence (-o-) of Gpp(NH)p; IDC-3669, in the presence (—■—) and absence (-■-) of Gpp(NH)p.

TABLE 21

PARTITIONING OF ADENYLATE CYCLASE ACTIVITY IN MICRO-SECTIONS FROM MALIGNANT AND BENIGN MAMMARY TUMORS[1]

tumor		AC activity (units/mg dry wt)	% pellet activity
FA-3070	3,000 x g supernatant	1.55	47
	3,000 x g pellet	3.29	
IDC-3669	3,000 x g supernatant	0.32	22
	3,000 x g pellet	1.44	

[1]Micro-sections from benign tumor FA-3070 (5.3 mg dry wt) and malignant tumor IDC-3669 (4.0 mg dry wt) were homogenized in 1.0 ml homogenization buffer using a Radnoti #612 homogenizer and centrifuged at 3,000 x g for 20 minutes at 4°C. Cyclic AMP formation was measured in the supernatent and pellet fractions using the radioimmunoassay procedure. Specific activity values were derived from the mean of 4 replicates for each tumor fraction, with microsections being dissected from a single locus within the lyphilized tumor section. One unit of enzyme will catalyze the production of one pmol cAMP per minute at 37 37°C.

Table 21, cyclase activity was higher in both the supernatant and pellet fractions from FA-3070 than IDC-3669. It was also noted that supernatant AC activity from the benign tissue represented 47% of that found in the pellet fraction compared to 22% found in the malignant tumor supernatant when expressed in terms of percent of the pellet (bound) AC activity.

3.3. Protein Kinase Associated with Malignant and Benign Human Mammary Tumors

3.3.1. Evaluation of Protein Kinase Assay Conditions: Cyclic AMP-dependent protein kinase was measured using a modification of the procedure described by Gill and Walton (1979). The experimental methods used with the tumor extracts were validated by measuring the activity of bovine heart cAMP-dPK preparations, from both a commercial source and prepared from fresh bovine heart tissue. Using 5 nmol units/ml purified cAMPdPK in a reaction volume of 30 µl specific activity (S.A. of [^{32}P]-ATP = 15.4 cpm/pmol), a maximum of 6.4 pmol min^{-1} phosphotransferase activity into histone IIA was observed following 10 minutes incubation at 30°C in the presence of 5 µM cAMP. The activity ratio was found to increase from 0.20 at 2 minutes incubation to 0.70 after 45 minutes incubation. A 30,000 x g supernatant fraction prepared from bovine heart tissue showed a ^{32}P incorporation rate of 1 to 2 pmol min^{-1} into histone IIA, as well as VIIS using a final tissue concentration of 0.6 mg/ml. The activity ratios in the presence of histone IIA and VIIS were determined to be 0.40 and 0.45, respectively, using a final concentration of 5 µM cAMP (S.A. [^{32}P]-

ATP = 15.4 cpm/pmol). The phosphorylation levels and activity obtained indicated that the assay procedure measuring TCA-precipitable, radiolabeled phosphoprotein would provide a valid assessment of protein kinase activity.

Similarly, addition of 2.3 µg protein from a 30,000 x g supernatant fraction prepared from a tissue homogenate of tumor FA-3070 gave an incorporation rate of 1.6 pmol min^{-1} using histone IIA in the presence of 5 µM cAMP (S.A. of [^{32}P]-ATP = 12.84 cpm/pmol). Pretreatment of this fraction by heating at 96°C for 10 minutes prior to assay abolished the phosphotransferase activity.

The protein kinase assay using malignant and benign mammary tumor tissue was conducted using several buffer systems, and pH values, including 25 mM sodium acetate, pH 6.2, 25 mM MES, pH 5.5; 25 mM MOPS, pH 7.2; 25 mM glycyglycine, pH 7.5, 25 mM Tris-HCl, pH 7.5, and 25 mM potassium phosphate, pH 7.0. All reactions were run in the presence of 2.5 mM DTT and 5.0 mM theophylline or IBMX. The reaction mixture components buffered with Tris-HCl gave maximum ^{32}P incorporation into histone.

3.3.2. Substrate Specificity, Substrate Affinity, and pH Optimum for Tumor-Derived Protein Kinase: The substrate specificity of protein kinase activity from FA-1035 was examined using six histone preparations, casein, and two forms of protamine, each at a final concentration of 2.0 mg/ml. As indicated in Figure 20, maximum ^{32}P incorporation from ATP occurred using histone VS as the

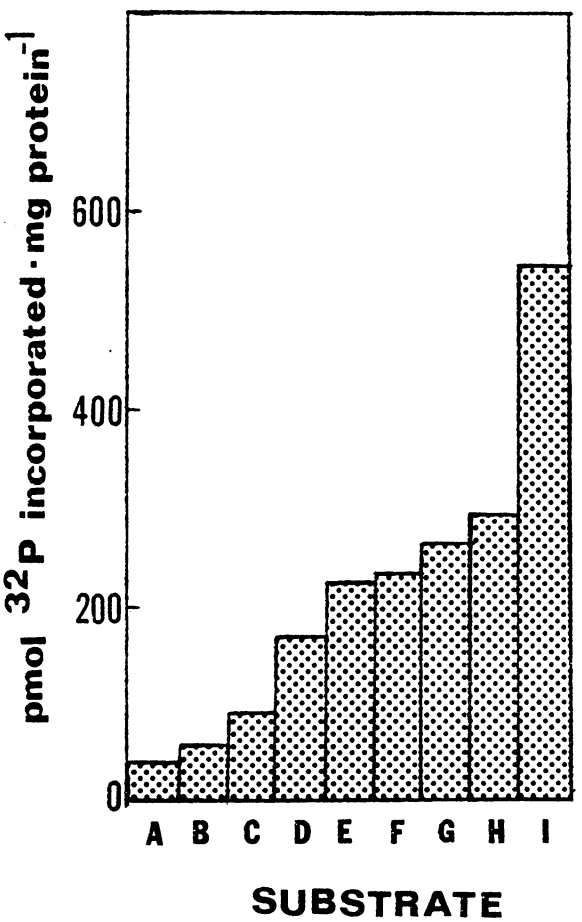


FIGURE 20

Comparison of substrate phosphorylation by protein kinase activity derived from a 30,000 x g supernatant fraction from benign tumor FA-3070. Final substrate concentration was 2.0 mg/ml. Specific activity of (^{32}P)-ATP = 23.3 cpm/pmol. Phosphorylation was measured in the presence of 5 µM cAMP. A, protamine phosphate; B, protamine sulfate; C, histone IIIS; D, histone VIIIS; E, histone IIA; F, histone VIS; G, histone VIIS; H, casein; I, histone VS.

substrate, being 2-fold increased over histone IIA and 10-fold over that obtained with protamine. The optimum pH for phosphotransfer to histone VS was determined by incubating tissue microsections from IDC-2760 in 25 mM Tris-HCl containing 30 µM ATP (S.A.[^{32}P]-ATP = 14.9 cpm/pmol) and 4 mg/ml histone VS at pH values adjusted from 6.5 to 9.0. Maximum incorporation of ^{32}P occurred at pH 8.0 both in the presence and absence of 5 µM cAMP (Figure 21). The pmole ^{32}P incorporated/mg protein of tumor supernatant fractions showed a linear increase with time up to 20 minutes incubation at 30°C (Figure 22). Linear incorporation of ^{32}P was also demonstrated with the addition of 10 to 40 µg protein of tumor supernatant (Figure 23).

The effect of increasing histone VS (0.1 mg/ml to 6.0 mg/ml) on ^{32}P incorporation is shown in Figure 24. The apparent Km for histone VS was approximately 2.0 mg/ml; Vmax for the reaction was approximately 0.5 pmol ^{32}P incorporated min^{-1}. Transfer of ^{32}P into histone VS increased with increasing concentrations of ATP, both in the presence and absence of cAMP. The apparent Km for ATP in the presence of 5 µM cAMP was estimated to be approximately 180 µM for cAMPdPK activity from malignant tumor IDC-3669 (Figure 25).

3.3.3. Activation of Tumor-Derived Protein Kinase by Cyclic 3':5' AMP and Cyclic 3':5' GMP: Activation of protein kinase catalytic subunit in the presence of 5 µM cAMP was demonstrated in the soluble fraction from FA-1038. An activity ratio from 0.14 to 0.16 was found up to 10 minutes incubation, after which the ratio increased to 0.42

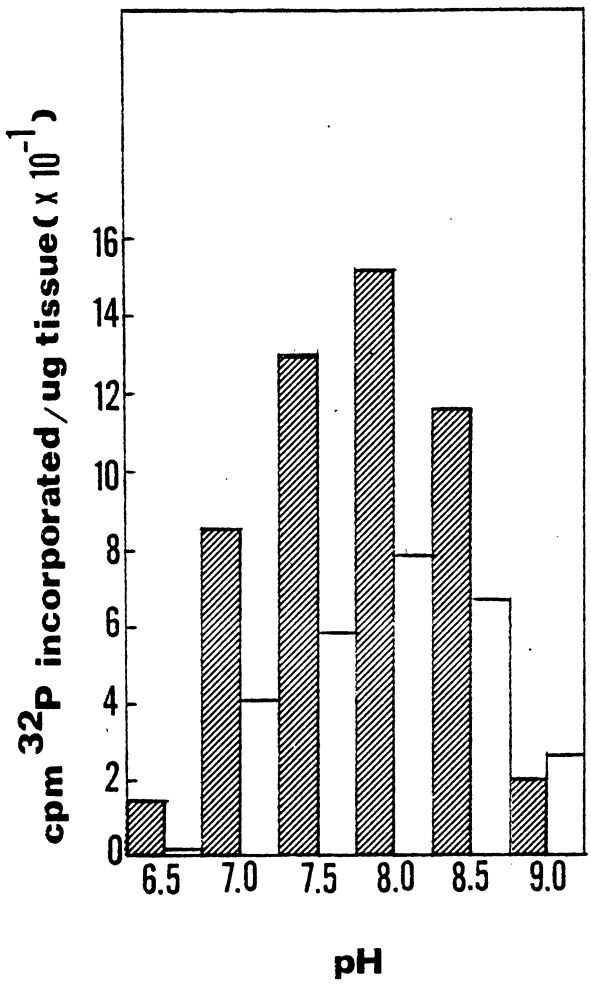


FIGURE 21

pH profile of protein kinase activity from malignant tumor IDC-2760. Phosphotransfer into histone VS (2.0 mg/ml) was measured in the presence (diagonal lines) or absence (unmarked) of 5.0 µM cAMP. Tissue addition per reaction ranged from 14 to 24 µg dry wt. Specific activity (^{32}P)-ATP = 25.9 cpm/pmol.

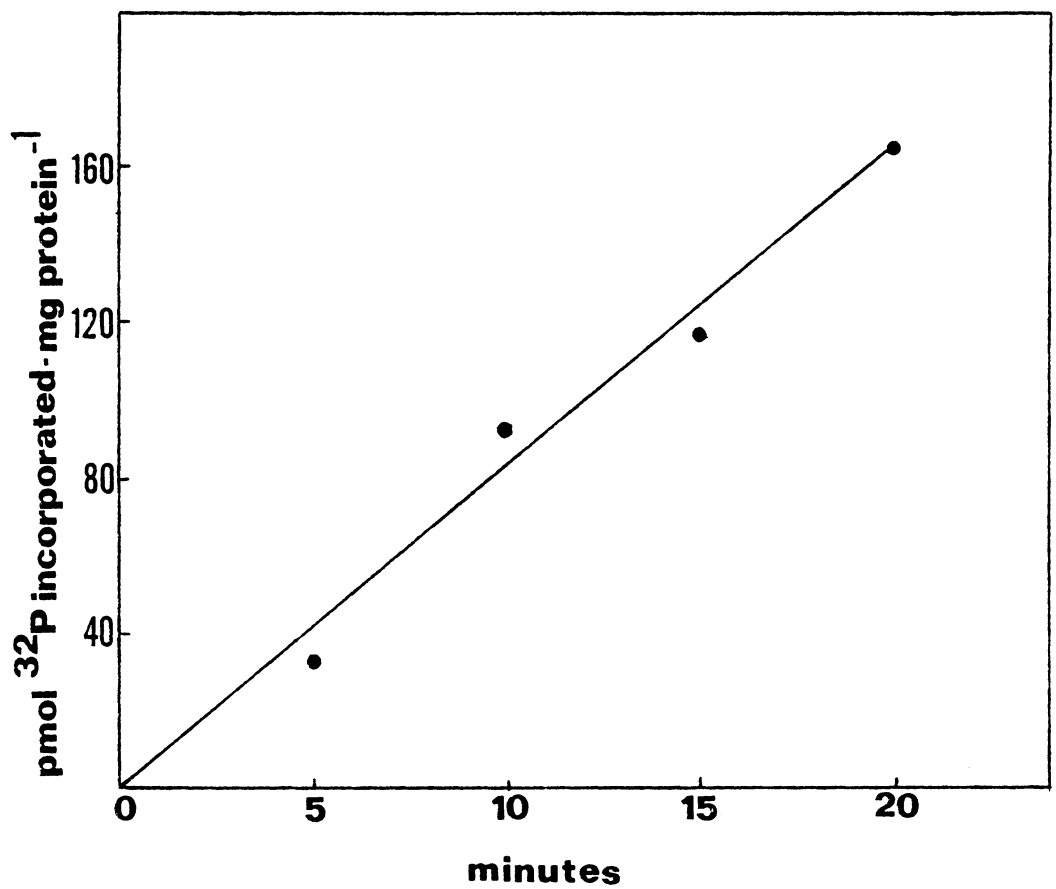


FIGURE 22

Incorporation of ^{32}P into histone VS (2.0 mg/ml) was measured as a function of time using a 30,000 x g supernatant fraction from benign tumor FA-1038 (protein addition, 42 µg). Specific activity (^{32}P)-ATP = 36.9 cpm/pmol.

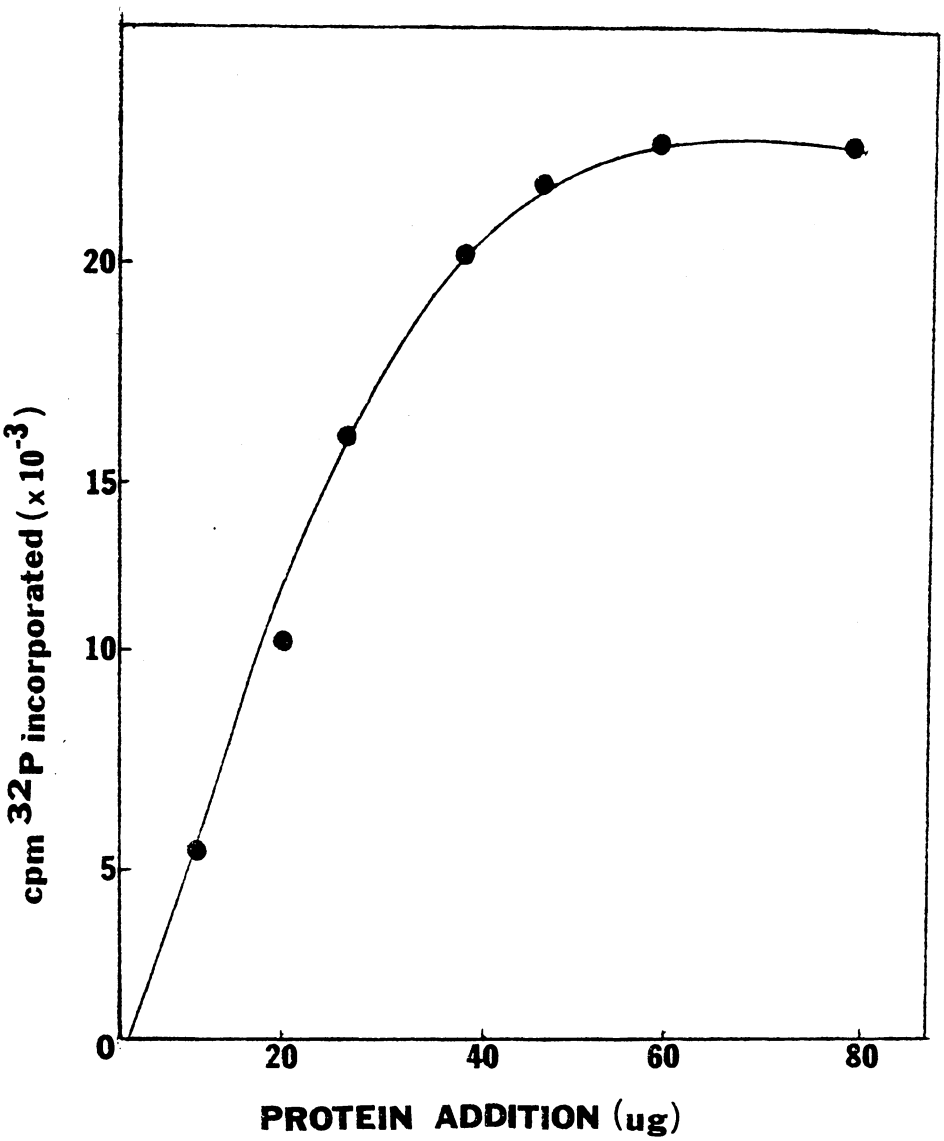


FIGURE 23

Incorporation of ^{32}P into histone VS with increasing addition of tumor soluble protein, using a 30,000 x g supernatant fraction from benign tumor FA-3070. Specific activity of (^{32}P)-ATP = 30.2 cpm/pmol.

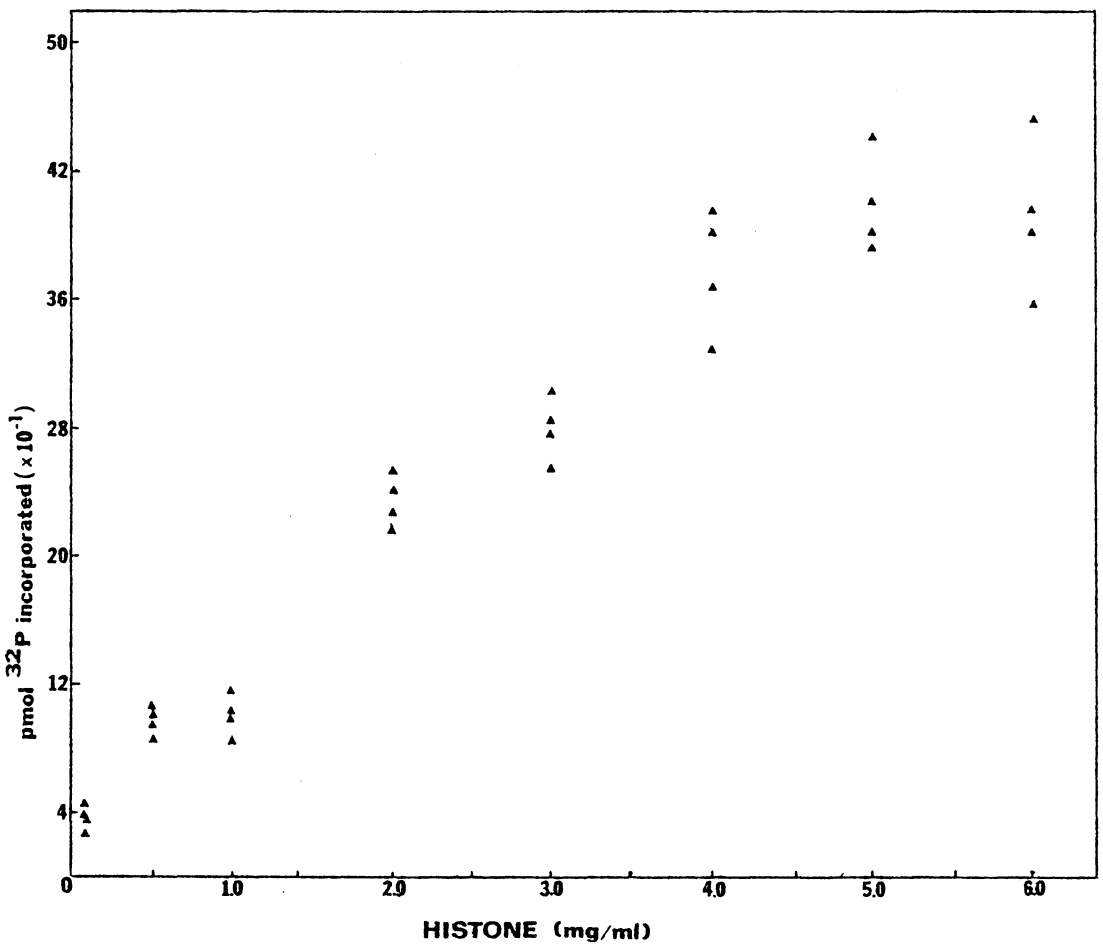


FIGURE 24

Incorporation of ^{32}P into histone VS at increasing concentration using a 30,000 x g supernatant fraction from benign tumor FA-3070 (protein addition, 40 µg). Phosphotransfer was measured using 0.1 to 6.0 mg/ml substrate in the reaction mixture. Specific activity (^{32}P)-ATP = 32.1 cpm/pmol.

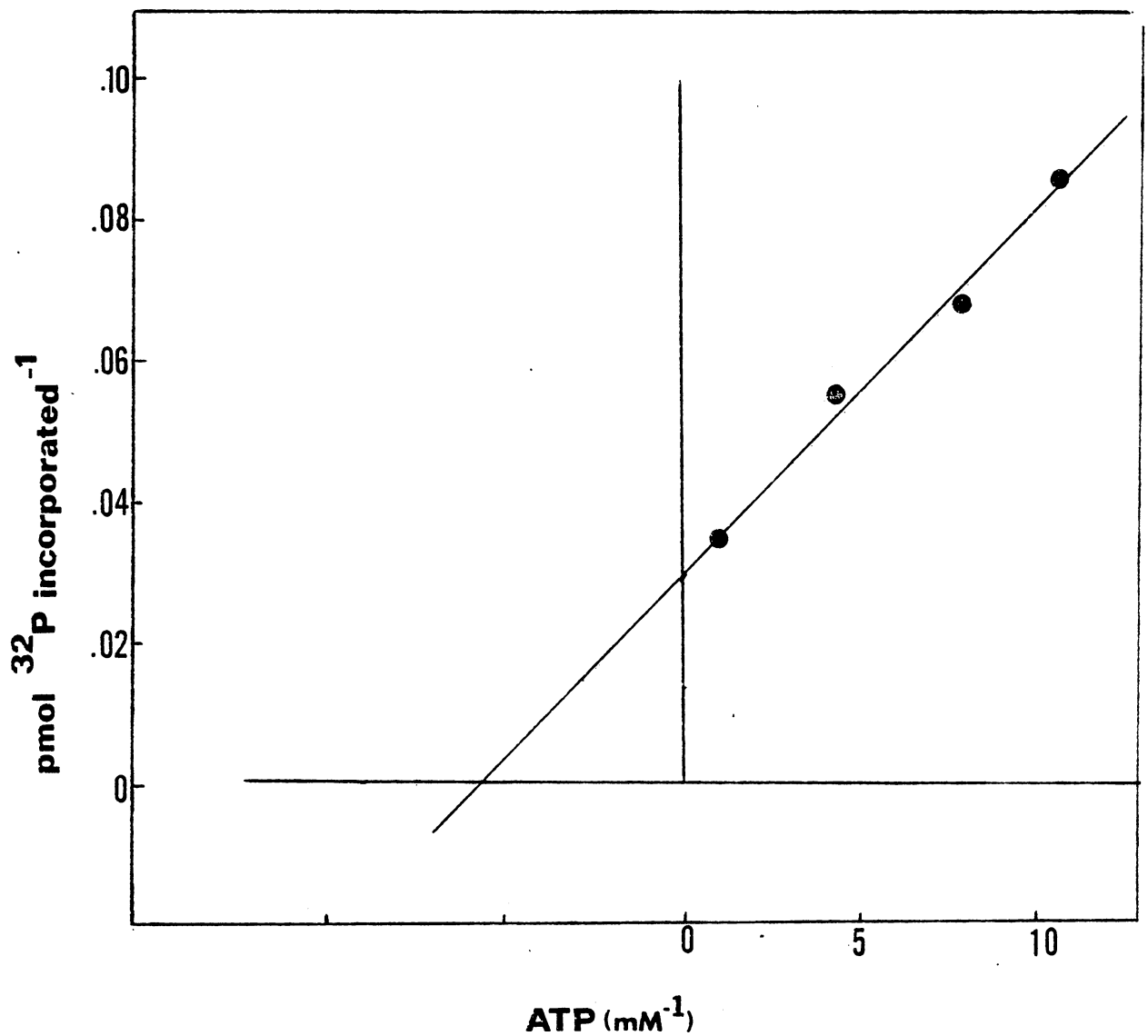


FIGURE 25

Lineweaver-Burke plot of incorporation of ^{32}P as a function of increasing ATP concentration from 50 µM to 1.0 mM in the presence of 5.0 µM cAMP using a 30,000 x g cytosolic fraction from malignant tumor IDC-3669 (protein addition/tube = 38 µg). Specific activity (^{32}P)-ATP = 30.5 cpm/pmol.

(Figure 26). The addition of 0.5 μM to 50 μM cAMP final concentration allowed for estimation of the enzyme affinity for the cyclic nucleotide in malignant tumor IDC-3669 (Table 22). The apparent Ka value for cAMP, based upon Michaelis-Menten kinetics, was approximately 1.25 μM.

The effect of increasing cAMP concentration on ^{32}P incorporation into histone VS and the stimulation of phosphotransfer by cGMP was examined. Table 23 shows that maximum activation of the catalytic subunit occurred with a final concentration of 5 μM cAMP. Addition of 10 μM cGMP gave approximately 60% the activity obtained with 5 μM cAMP. The tumor enzyme affinity for cAMP was estimated using a supernatant fraction from IDC-3669, and an apparent Ka value of 0.8 μM was determined (Figure 27).

3.3.4. Activation of Tumor-Derived Protein Kinase by Additional Cyclic Nucleotides: An attempt was made to further characterize soluble tumor protein kinase with respect to activation by additional cyclic nucleotides (Figure 28). Activation in the presence of 10 μM cGMP, cIMP and cCMP gave 60%, 85% and 76% that obtained with 10 μM cAMP. This could reflect a low specificity for cAMP by the soluble kinase, however possible contamination of these cyclic nucleotide preparations with cAMP was not determined.

3.3.5. Cyclic AMP-Dependent Protein Kinase Activity and Activity Ratios in a Series of Malignant and Benign Tumors: Cyclic AMP-dependent protein kinase activity and activity ratios in the presence

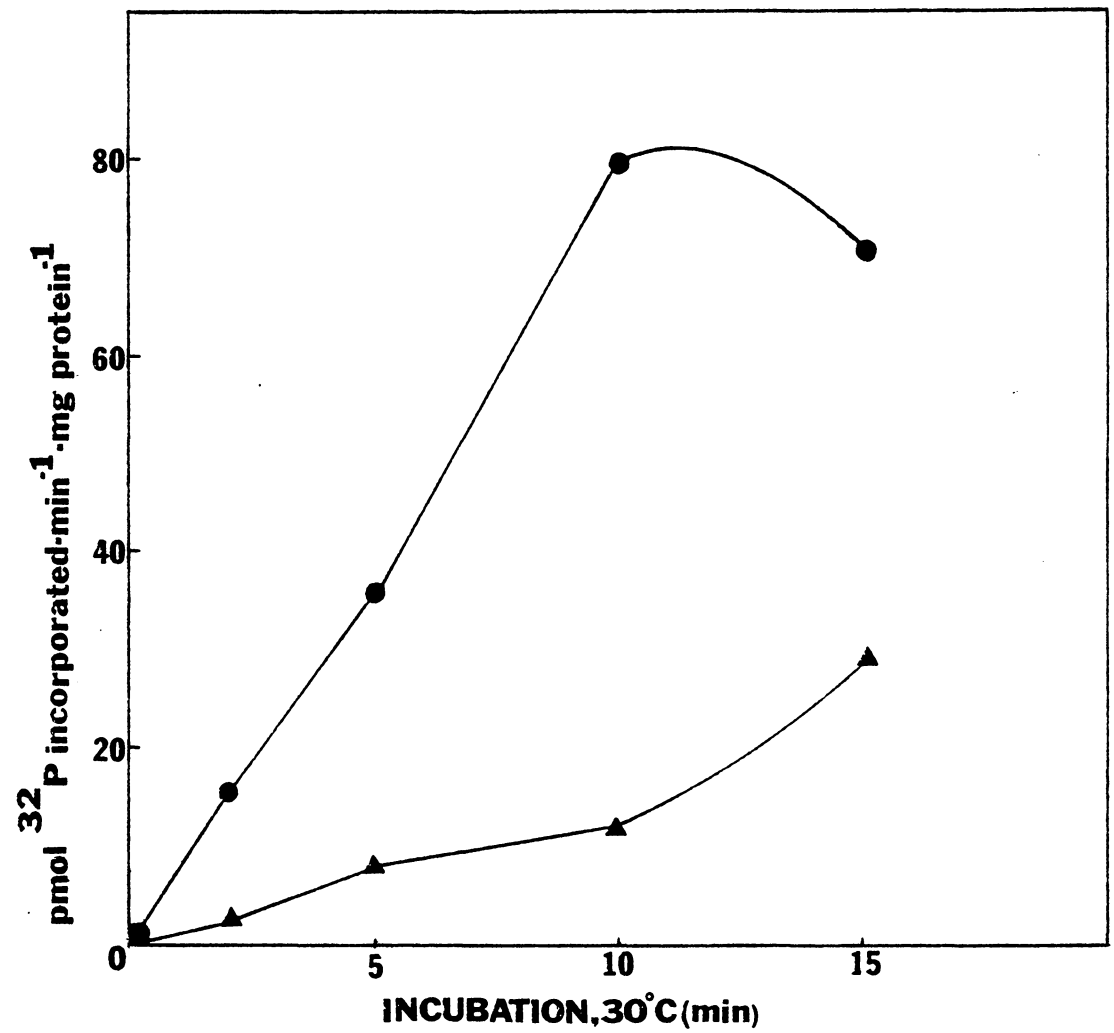


FIGURE 26

Cyclic AMP-activation of protein kinase from a 30,000 x g supernatant fraction from benign tumor FA-1038 was measured as a function of time. Reactions run in the presence (●) or absence (▲) of 5.0 µM cAMP were sampled at 0, 2, 5, 10, and 15 minutes incubation at 30°C in the presence of 30 µM (^{32}P)-ATP (specific activity = 28.9 cpm/pmol) and 2.0 mg/ml histone VS.

TABLE 22

ACTIVATION OF CYTOSOLIC PROTEIN KINASE FROM MALIGNANT TUMOR IDC-3669 BY INCREASING CONCENTRATIONS OF CYCLIC AMP[1]

cAMP, μM	PK activity (units/mg protein)[2]
0.0	12.1
0.5	40.5
1.0	61.6
2.5	78.2
5.0	85.8
10.0	78.4
25.0	71.3
50.0	76.6

[1]Source of tumor-associated protein kinase activity was the 30,000 x g supernatant fraction; protein addition per reaction tube = 38. μg. Incubation occurred at 30°C for 10 minutes in the presence of 2.0 mg/ml histone.

[2]Activity values represent the mean of 3 replicate samples for each treatment. Specific activity (^{32}P)-ATP = 35.8 cpm/pmol. One unit is defined as the amount of enzyme required to transfer one pmol of phosphate from (γ-^{32}P)-ATP to histone VS per minute at 30°C.

TABLE 23

ACTIVATION OF CYTOSOLIC PROTEIN KINASE FROM BENIGN TUMOR FA-3070 BY CYCLIC AMP AND CYCLIC GMP[1]

addition	PK activity (units/mg protein)[2]
100 nM cAMP	245
1.0 μM cAMP	320
5.0 μM cAMP	343
10.0 μM cAMP	367
50.0 μM cAMP	337
100 μM cAMP	338
500 μM cAMP	262
10.0 μM cGMP	202
100 μM cGMP	268

[1]Source of tumor-associated protein kinase activity was the 30,000 x g supernatant fraction; protein addition per reaction tube = 80 μg. Reaction tubes were incubated at 30°C for 10 minutes in the presence of 2.0 mg/ml histone VS.

[2]Activity values represent the mean of 3 replicate samples for each treatment. Specific activity (^{32}P)-ATP = 28.9 cpm/pmol. One unit is defined as the amount of enzyme required to transfer one pmol of phosphate from (γ-^{32}P)-ATP to histone VS per minute at 30°C.

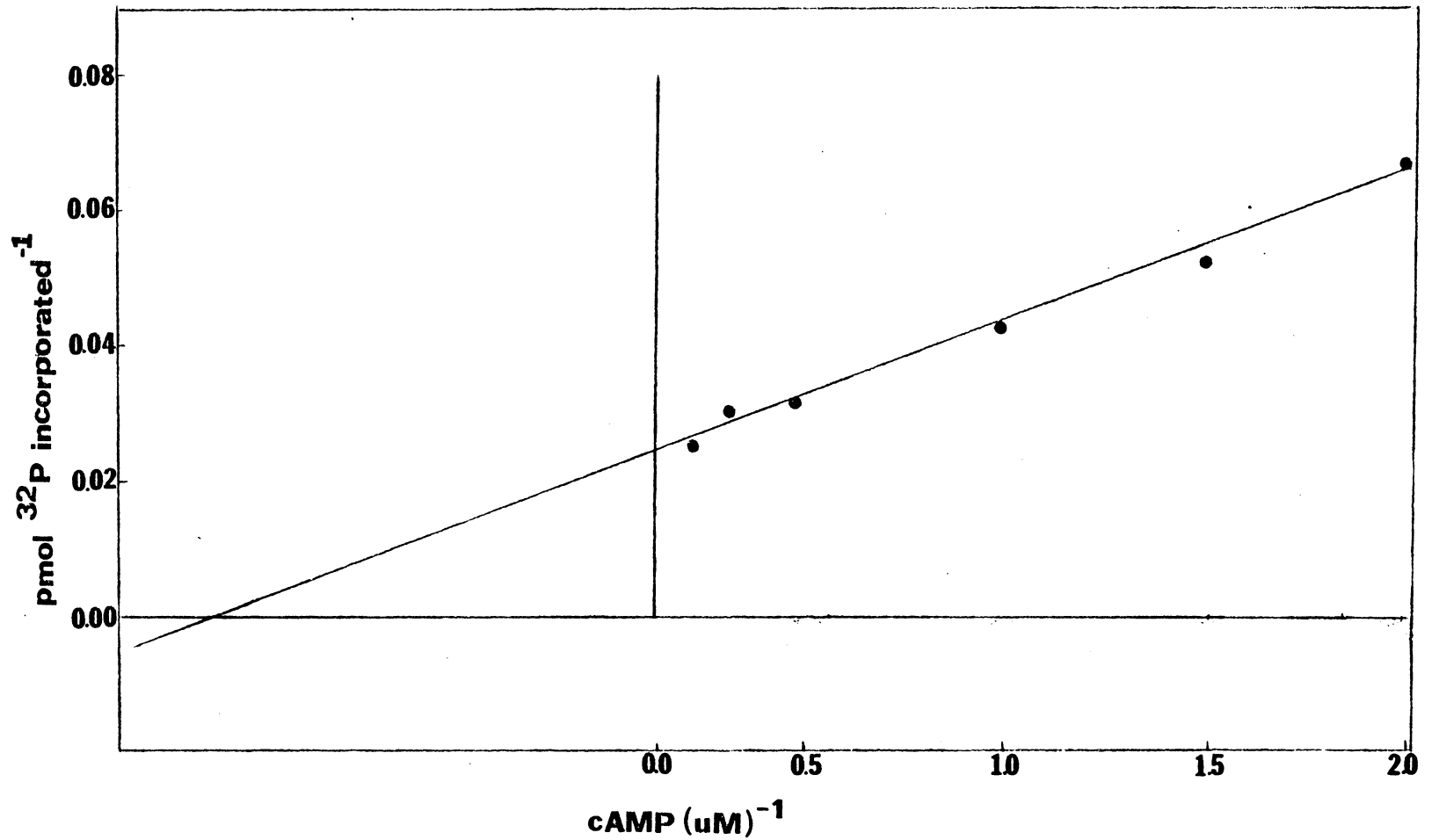


FIGURE 27

Lineweaver-Burke plot of incorporation of ^{32}P into histone VS as a function of increasing cAMP concentration using a 30,000 x g supernatant fraction from malignant tumor IDC-3669 (protein addition, 60 µg). Specific activity of (^{32}P)-ATP = 28.5 cpm/pmol.

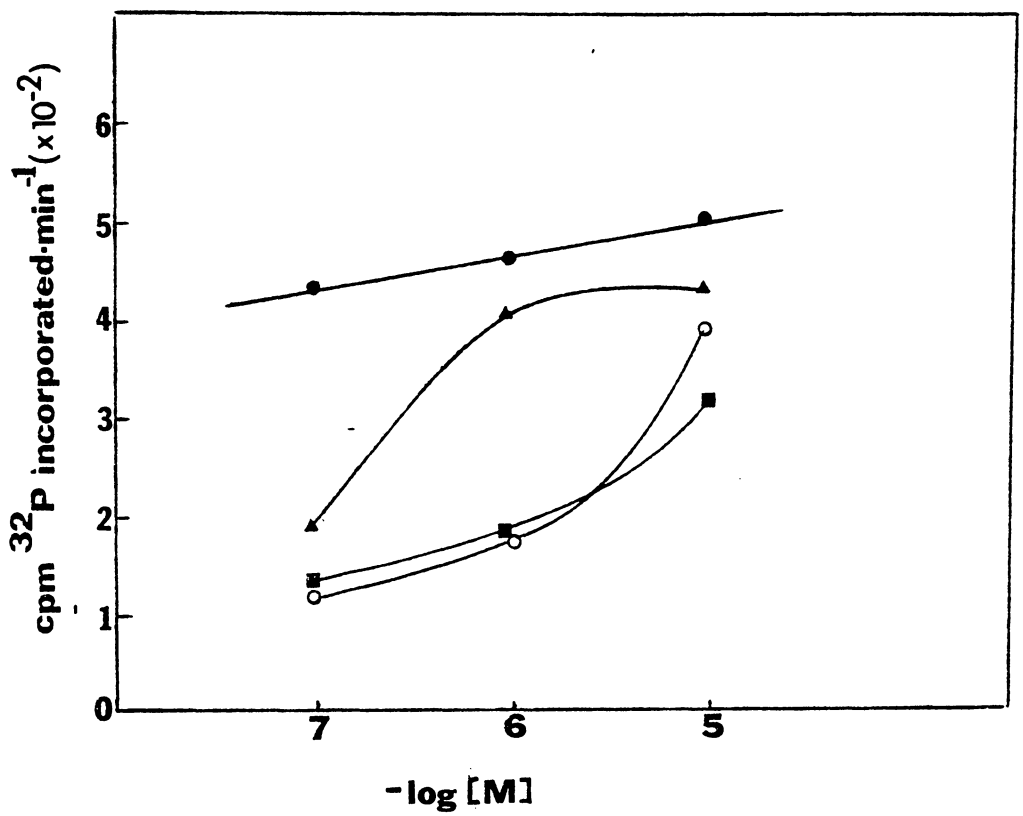


FIGURE 28

Activation of protein kinase from a 30,000 x g supernatant fraction from benign tumor FA-3070 by cyclic nucleotides as 10^{-5}, 10^{-6}, and 10^{-7} molar concentrations. Cyclic AMP (●), cIMP (▲), cCMP (○), and cGMP (■) were added to the reaction mixture prior to initiation of the reaction with 60 µg tumor soluble protein. Specific activity (^{32}P)-ATP = 21.3 cpm/pmol.

and absence of 5 μM cAMP were determined for a series of malignant and benign tumors using the 30,000 x g supernatant fraction. As shown in Table 24 and 25, specific activities from IDC tumors ranged from 165 to 467 pmol ^{32}P incorporated/min/mg protein with a mean of 294, while specific activities from FA tumors ranged from 65 to 403 pmol ^{32}P incorporated/min/mg protein, with a mean of 219. Activity ratios from malignant tumors ranged from 0.7 to 1.07, with a mean of 0.89, while ratios from benign tumors ranged from 0.42 to 0.87, with a mean of 0.65. Evaluation of this data using the student's "t" test indicated that there was no significant difference in activity levels between the tumor classes at the 5 percent probability level.

3.3.6. Cyclic AMP-Dependent Protein Kinase Activity in Tumor Microsections: Cyclic AMP-dependent protein kinase was also examined in mammary tumor tissue microsections. Phosphotransfer into histone VS was expressed as pmol ^{32}P incorporated/mg tissue dry weight, and was observed to be linear upon addition of 20 to 60 μg tissue. Activity levels associated with benign tumor tissue were approximately 2-fold higher than observed in malignant tumor tissue (Table 26). Activation by 5 to 25 μM levels of cAMP was only observed in a few instances. Cyclic AMP activation of PK from microsections of benign tumor FA-3070 is presented in Table 27. This observation indicated that cAMP activation of protein kinase can be demonstrated in certain tumors using microgram samples of tissue. Analyses of endogenous

TABLE 24

PROTEIN KINASE ACTIVITY FROM A SERIES OF MALIGNANT MAMMARY TUMORS IN THE PRESENCE AND ABSENCE OF CYCLIC AMP

tumor[1]	+/-cAMP[2]	units/mg protein[3]	activity ratio[4]
IDC 147	+	187	
	-	200	1.07
IDC 3669	+	312	
	-	218	0.70
IDC 733	+	467	
	-	424	0.91
IDC 3928	+	165	
	-	152	0.92
IDC 2760	+	370	
	-	276	0.75
IDC 207	+	322	
	-	278	0.86
IDC 4041	+	233	
	-	246	1.06

[1]Tumor source of protein kinase activity represents 30,000 x g supernatant fraction.

[2]Cyclic AMP final concentration, 5.0 μM.

[3]Incorporation measured using 2.0 mg/ml histone VS. One unit of enzyme will transfer one pmol phosphate from (γ-^{32}P)-ATP to histone VS per minute at 30°C.

[4]Activity ratio given as the function: $\frac{\text{incorporation - cAMP}}{\text{incorporation + cAMP}}$.

TABLE 25

PROTEIN KINASE ACTIVITY FROM A SERIES OF BENIGN MAMMARY TUMORS MEASURED IN THE PRESENCE AND ABSENCE OF CYCLIC AMP

tumor[1]	+/-cAMP[2]	units/mg protein[3]	activity ratio[4]
FA 1038	+	95	
	-	53	0.56
FA 1700	+	74	
	-	31	0.42
FA 1709	+	272	
	-	238	0.87
FA 1235	+	131	
	-	75	0.57
FA 1258	+	65	
	-	45	0.74
FA 2752	+	403	
	-	295	0.73
FA 3070	+	495	
	-	290	0.64

[1]Tumor source of protein kinase activity represents 30,000 x g supernatant fraction.

[2]Cyclic AMP final concentration, 5.0 μM.

[3]Incorporation measured using 2.0 mg/ml histone VS. One unit of enzyme will transfer one pmol phosphate from (γ-^{32}P)-ATP to histone VS per minute at 30°C.

[4]Activity ratio given as the function: $\frac{\text{incorporation - cAMP}}{\text{incorporation + cAMP}}$.

TABLE 26

CYCLIC AMP-DEPENDENT PROTEIN KINASE ACTIVITY ASSOCIATED WITH MALIGNANT AND BENIGN TUMOR MICROSECTIONS

tumor	µg dry wt	sample size	units/mg dry wt[1]
IDC-345	27.5-35.6	4	135 ± 33
IDC-733	26.5-31.9	6	152 ± 32
IDC-3669	21.5-35.4	6	164 ± 40
FA-1038	20.5-30.3	6	407 ± 46
FA-1258	23.2-41.3	6	335 ± 39
FA-1700	23.0-32.5	5	389 ± 45

[1]Specific activity (^{32}P)-ATP = 25.6 cpm/pmol. Incubation at 30°C for 10 minutes in the presence of 2.0 mg/ml histone VS. Activity values represent mean ±SEM. One unit of activity is the amount of enzyme required to transfer one pmol of phosphate from (γ-^{32}P)-ATP to histone VS per minute at 30°C.

TABLE 27

CYCLIC AMP ACTIVATION OF TUMOR MICROSECTION-DERIVED PROTEIN KINASE FROM BENIGN MAMMARY TUMOR FA-3070

equivalent tissue samples	+/-cAMP[2]	µg dry wt	cpm ^{32}P incorporated/ µg dry wt[3]
1	+	34.1	29.6
2	-	40.2	9.5
3	+	20.5	18.4
4	-	22.4	12.1
5	+	27.9	23.1
6	-	22.0	18.4
7	+	40.1	30.9
8	-	40.9	12.6
9	+	35.8	41.5
10	-	28.6	21.7
11	+	56.2	23.2
12	-	41.1	11.6

[1]Tumor microsections were dissected from a single specific locus within two tandem tissue sections.

[2]Cyclic AMP, final concentration = 50 µM.

[3]Specific activity (^{32}P)-ATP = 40.5 cpm/pmol.

cAMP levels in a random sampling of mammary tumor tissue indicated cAMP to be present at 0.43 to 2.65 fmol/µg tissue dry weight (Table 28). The contribution of endogenous cAMP in the PK reaction mixture would be between 0.4 and 1.0 µM when dilution effects of addition of the reaction mixture to the tissue is considered. Since the apparent K_a value for mammalian protein kinases is in the nanomolar range, endogenous levels of cAMP would allow for maximum activation of the enzyme. An attempt was made to preincubate tissue microsections with varying concentrations of cAMP-phosphodiesterase, followed by theophylline, prior to assay for cAMP activation. Difficulties were encountered due to contamination of the PDE preparation with protein kinase activity.

3.3.7. Effect of Known Inhibitors of Protein Kinase: The effect of ADP, 5'AMP, and adenosine,degradation products of ATP shown to be inhibitory to kinase activity,on phosphotransfer was studied using a soluble preparation from benign tumor FA-3070. All three compounds were inhibitory to the kinase reaction at 0.5 mM and 5 mM levels, particularly ADP, which gave 86% inhibition at the higher concentration level (Figure 29).

Inhibition of phosphotransfer by protein inhibitors of cAMP dependent protein kinase is one criteria for classification of enzyme activity as being cAMP-dependent. Incubation of FA-3070 soluble PK with 20 to 140 µg protein kinase inhibitor isolated from rabbit skeletal muscle resulted in a decrease in phosphotransferase activity,

TABLE 28

ENDOGENOUS CYCLIC AMP LEVELS IN A RANDOM SAMPLING OF MALIGNANT AND BENIGN MAMMARY TUMORS

tumor identification	type	fmol cAMP/µg dry wt[1]
IDC 3669	malignant	2.01
IDC 1688	malignant	2.52
FA 2760	benign	1.06
FA 1038	benign	2.65
FA 3070	benign	3.09
FA 1709	benign	0.43

[1]Data averaged from six samples for each tumor; micrograms dry tissue wt/sample = 10 to 40.

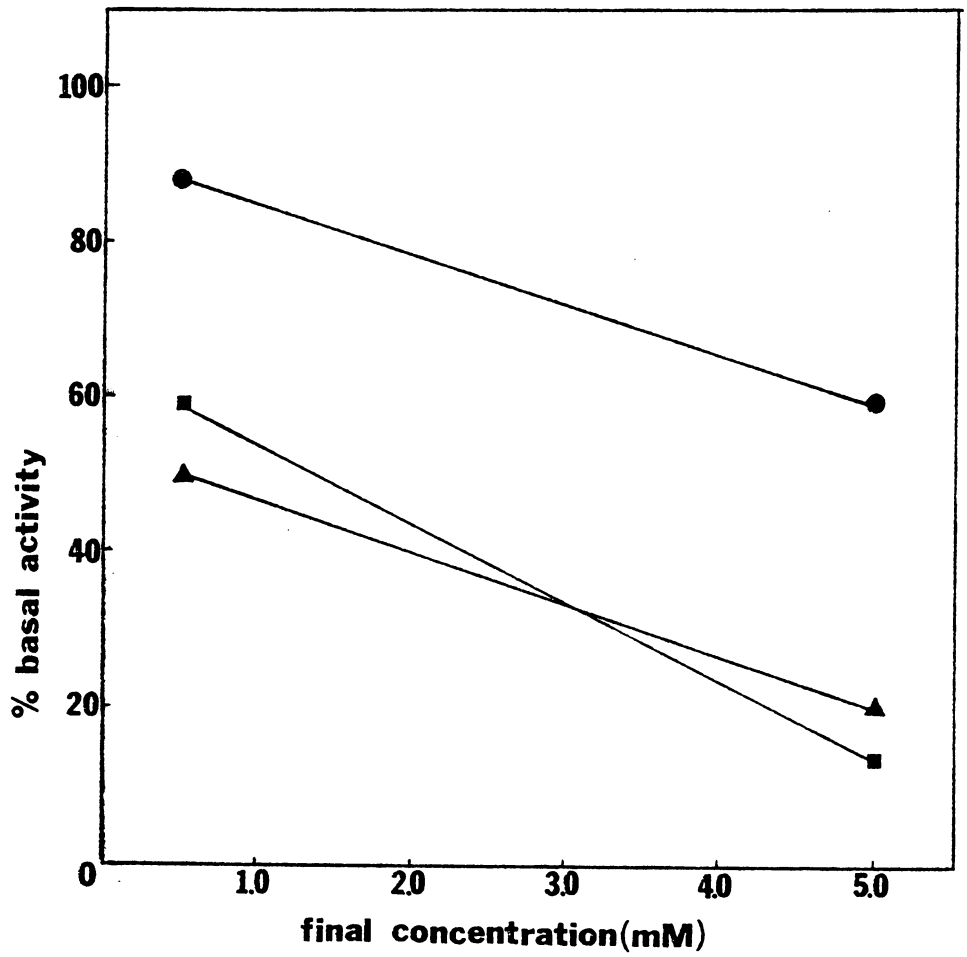


FIGURE 29

Inhibition of protein kinase activity from benign tumor FA-3070 by adenosine (●), 5'AMP (▲), and ADP (■) at 0.5 mM and 5.0 mM concentrations. Phosphorylation of histone VS was measured in the presence of 5.0 μM cAMP and 30 nM (^{32}P)-ATP (specific activity = 28.9 cpm/pmol).

with 87% inhibition observed in the presence of 140 µg PKI_1 (Figure 30). The effect of PKI_2 from bovine heart was similarly analyzed. Incubation with 140 µg PKI_2 gave 85% inhibition of tumor-associated cAMPdPK (Figure 31). These results further support the characterization of tumor histone kinase as being cAMP-dependent.

3.3.8. Thermal Stability of Tumor Protein Kinase: The thermal stability of tumor soluble PK activity was examined to determine whether increased temperature sensitivity occurred indicative of labile subunit activity. It was found the phosphotransferase activity into histone VS, both in the presence and absence of cAMP, was unaffected over a 15' preincubation at 30°C, therefore suggesting that cAMP binding to the regulatory subunit and cAMP-induced activation of the catalytic subunit was stable over the course of the reaction period (Table 29).

3.3.9. Subcellular Localization of Tumor Protein Kinase Activity: Subcellular fractionation of cAMPdPK from two malignant and two benign tumors indicated that the majority of phosphotransferase activity was soluble, and not pellet-bound (Table 30). A 6 to 28% enhancement of PK activity was observed in the 100,000 x g supernatant fraction, possibly indicating the presence of an endogenous inhibitor of tumor cAMPdPK. An attempt was made to isolate a heat-stable, TCA-precipitable inhibitor from both malignant and benign tumor preparations, however, no such effector was ever recovered. Separation of soluble and pellet-bound cAMPdPK activity was similar for both tumor classes.

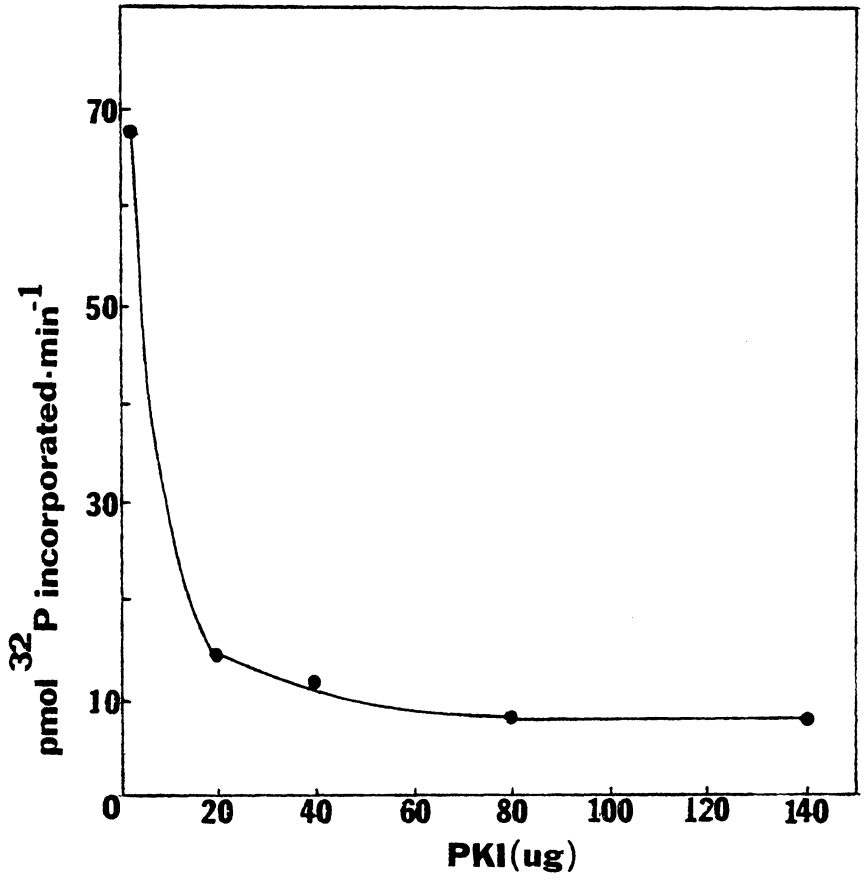


FIGURE 30

Effect of addition of 20 to 140 µg protein kinase inhibitor (PKI) isolated from rabbit skeletal muscle on phosphotransfer into histone VS by extracts from benign tumor FA-3070 (protein addition, 60 µg). Specific activity (^{32}P)-ATP = 13.4 cpm/pmol. Endogenous phosphorylation in the absence of added substrate was determined to be approximately 10.0 pmol ^{32}P incorporated per minute.

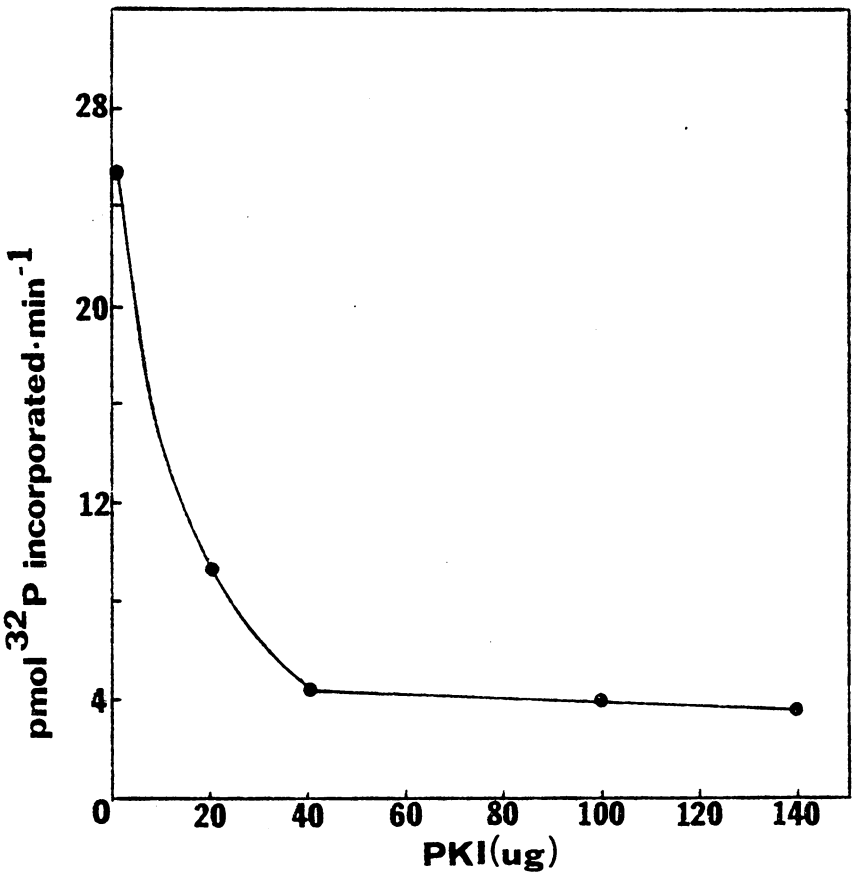


FIGURE 31

Effect of addition of 20 to 140 µg protein kinase inhibitor (PKI) isolated from bovine heart on phosphotransfer into histone VS by extracts from benign tumor FA-3070 (protein addition, 60 µg). Specific activity (^{32}P)-ATP = 35.6 cpm/pmol. Endogenous phosphorylation in the absence of added substrate was determined to be 3.8 pmol ^{32}P incorporated per minute.

TABLE 29

EFFECT OF PREINCUBATION AT 30°C ON STABILITY OF PROTEIN KINASE ACTIVITY AND CYCLIC AMP-DEPENDENCY IN BENIGN TUMOR FA-1258[1]

time preincubation (min)	+/-cAMP[2]	mean cpm ^{32}P incorporated[3] ($x10^3$)
0	+	1.67
	-	0.82(0.49)
2	+	1.72
	-	0.61(0.36)
5	+	1.59
	-	0.69(0.44)
10	+	1.80
	-	0.67(0.37)
15	+	1.77
	-	0.78(0.44)

[1]Source of tumor-associated protein kinase activity was the 30,000 x g supernatant fraction; protein addition per reaction tube = 132 µg.

[2]Following preincubation, phosphotransfer into histone VS was measured in the presence and absence of 5 µM cAMP, using an incubation at 30°C for 10 minutes. Specific activity (^{32}P)-ATP = 43.7 cpm/pmol.

[3]Values for cpm ^{32}P incorporated represent the mean for 3 replicate samples for each time period and treatment, corrected for substrate blanks. Numbers in parentheses represent the activity ratio.

TABLE 30

SUBCELLULAR FRACTIONATION OF TUMOR-ASSOCIATED CYCLIC AMP-DEPENDENT PROTEIN KINASE ACTIVITY

fraction	PK activity (units/mg protein)[1]			
	IDC		FA	
	3982	514	1700	2307
27,000 x g supernatant	109	114	183	188
27,000 x g pellet	34 (0.31)	46 (0.41)	44 (0.24)	29 (0.16)
100,000 x g supernatant	135 (1.22)	145 (1.28)	219 (1.19)	199 (1.06)
100,000 x g pellet	27 (0.24)	21 (0.19)	100 (0.06)	28 (0.15)

[1]Specific activity values represent the mean of 3 replicate samples. One unit of enzyme activity will catalyze the transfer of one pmol phosphate from (γ-^{32}P)-ATP to histone VS per minute at 30°C. Values in parentheses represent fraction of the 27,000 x g supernatant activity.

3.4. Mammary Tumor-Derived cAMP Binding Protein

3.4.1. Evaluation of cAMP Binding Assay: The capacity for cAMP binding to tumor cAMPdPK was determined using 3H-cAMP and separation of the radiolabeled ligand-enzyme regulatory subunit complex by either the charcoal method or the filter binding method (see Materials and Methods). Since reports of cAMP-binding activity at both acidic and neutral pH has been observed in several tissues studied, an attempt was made to optimize cAMP binding conditions. Cyclic AMP binding was measured in 50 mM sodium acetate, pH 4.5; 50 mM potassium phosphate, pH 6.5, 50 mM MES, pH 7.0, and 50 mM Tris-HCl, pH 7.5. The latter two buffers gave maximum cpm ^{3}H-cAMP bound for the charcoal and filter binding methods. The specificity of ^{3}H-cAMP binding was demonstrated in experiments with a supernatant fraction from tumor FA-3070 where the reaction mixture was supplemented with increasing concentrations of unlabeled cAMP or 5'AMP. As indicated in Table 31, addition of increasing amounts of unlabeled cAMP, but not 5'AMP, competed with ^{3}H-cAMP for binding sites on the binding protein.

3.4.2. Analysis of cAMP Binding Capacity in Human Mammary Tumors: Several malignant and benign tumors were homogenized and supernatant fractions obtained for analysis of ^{3}H-cAMP binding activity (Table 30). Using a 20 minute incubation at 4°C, the range in ^{3}H-cAMP binding for malignant tumor preparations were 33.0 to 81.7 nmol ^{3}H-cAMP bound/mg protein, with a mean of 63.6 binding activity for benign tumor preparations ranged from 21.5 to 65.9 nmol ^{3}H-cAMP bound/mg protein,

TABLE 31

SPECIFICITY OF CYTOSOLIC ^{3}H-cAMP-BINDING PROTEIN FROM BENIGN TUMOR FA-3070

addition	cpm ^{3}H cAMP bound[1]
control reaction	3052
+ 3.0 µM cAMP	122
+ 7.5 µM cAMP	95
+ 15 µM cAMP	72
+ 3.0 µM 5'AMP	3103
+ 7.5 µM 5'AMP	4318
+ 15 µM 5'AMP	4075

[1]Cyclic AMP-binding was measured as described in Materials and Methods. Values for cpm bound represent the mean of 3 replicates, corrected for nonspecific background activity.

[2]Source of tumor-associated binding protein was the 30,000 x g supernatant fraction; protein addition per reaction tube = 80 µg.

TABLE 32

CYCLIC AMP-BINDING ACTIVITY IN SOLUBLE FRACTIONS FROM MALIGNANT AND BENIGN HUMAN MAMMARY TUMORS[1]

tumor		nmol ^{3}H-cAMP bound/mg protein
IDC:	3669	80.6
	4041	49.7
	147	33.0
	733	80.8
	3928	75.8
	2760	81.7
FA:	1258	21.5
	1700	26.0
	1038	22.8
	2752	67.9
	1709	59.3
	1235	25.9

[1]Source of binding activity was the 30,000 x g cytosolic fraction prepared for each mammary tumor examined. Activity values represent the mean of five replicates for each tumor.

with a mean of 36.9. Evaluation of this data using the students "t" test indicated that the difference in binding capacity for the two tumor classes was significant ($p < 0.025$).

3.4.3. Estimation of Binding Affinity for cAMP: Addition of increasing amounts of ^{3}H-cAMP (14 to 210 nmoles) to the binding reaction mixture allowed for the estimation of apparent K_b values for some tumor preparations. An example of the Scatchard plot analysis used to determine ^{3}H-cAMP affinity constants is presented in Figure 32, using a partially-purified holoenzyme preparation from malignant tumor 3669 isolated by DEAE-Sephacel chromatography: The slope of the line represents the negative reciprocal of the K_b value when a plot of bound/free cAMP vs bound cAMP is made. The apparent K_b values for cAMP in benign tumor holoenzyme preparations examined ranged from 15 to 50 nM, with a mean value of 34 nM. Similarly, apparent K_b values for cAMP in malignant tumor holoenzyme preparations ranged from 27 to 56 nM, with a mean value of 45 nM (Table 33).

3.5. Partial Purification of Protein Kinase Holoenzyme

3.5.1. DEAE-Sephacel Chromatography of Tumor Supernatant Fractions: Samples from mammary tumor 30,000 x g supernatant fractions, either precipitated with ammonium sulfate or streptomycin sulfate, were applied to DEAE-Sephacel columns which were equilibrated with 20 mM Tris-HCl, pH 7.5, containing 1 mM EDTA and 2 mM 2-mercaptoethanol. After an initial buffer wash to remove nonabsorbed material, the columns were eluted with a two hour salt gradient from 0.0 to 0.3 M

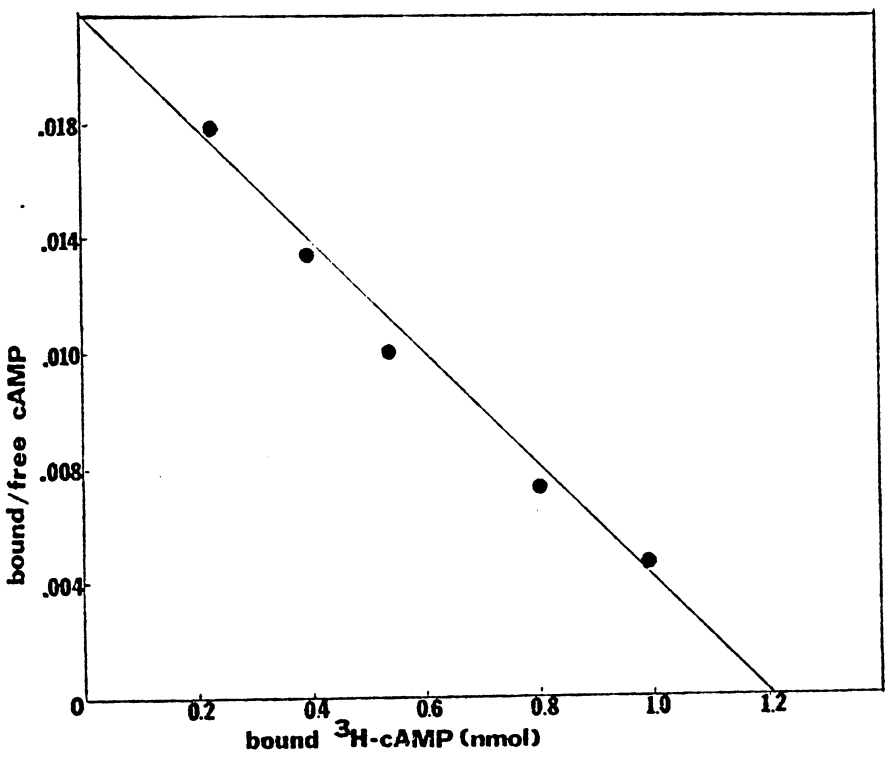


FIGURE 32

Scatchard plot of ^{3}H-cAMP binding activity of protein kinase activity from malignant tumor IDC-3669 resolved by DEAE-Sephacel chromatography. Cyclic AMP binding was measured using 14 to 210 nmol cAMP in the assay mixture.

TABLE 33

APPARENT CYCLIC AMP ASSOCIATION CONSTANTS DETERMINED FOR A SAMPLING OF MALIGNANT AND BENIGN MAMMARY TUMORS

tumor		K_b cAMP[1]
IDC:	147	56.0
	733	43.2
	4041	28.9
	375	55.1
FA:	1258	50.0
	1700	39.6
	2752	32.1
	3070	15.8

[1]Cyclic AMP binding activity was assayed under the described conditions. Apparent K_b values were calculated from the linear relationship obtained in double reciprocal plots of binding activity against cyclic nucleotide concentration, and represent the concentration of cAMP required to occupy 50% of the total cAMP-binding sites at 0°C.

NaCl. Fractions were collected at 2 minute intervals at a flow rate of 40 ml/hr for the flow-through elution and at 80 ml/hr for the salt gradient. Absorbance at 280 nm was monitored using an ISCO UV monitor.

Initial work using preparations from benign tumor FA-2307 and malignant tumor IDC-3928 (0.33 g/ml tissue wet wt.) indicated that two peaks of protein kinase activity were detectable using histone VS as the substrate for the phosphotransferase reaction (not shown). The phosphotransferase activity was measured after pooling and concentrating every three fractions to a 1.0 ml final volume prior to assay. The first peak of activity was eluted with approximately 0.06 to 0.08 M NaCl, representative of a type I holoenzyme, whereas the second peak of activity eluted with approximately 0.15 to 0.20 M NaCl, representative of a type II holoenzyme. The absence of protein kinase activity in the flow-through fractions was noted. It was not possible to estimate the ratios of the typeI/type II kinase activity from these two tumor preparations due to low activity levels. Holoenzyme preparations recovered from the columns were stable for 72 to 96 hours at 4°C. Freezing resulted in a 50% loss in kinase activity, but had no affect on the activity ratio. Benzamidine at a 10 mM final concentration was used to inhibit proteolytic degradation of the enzyme. Representative mammary tumors were fractionated as described in Materials and Methods. Descriptions of the chromatographic and dissociation properties of these mammary tumor holoenzyme preparations will herein be given, beginning with

the malignant tumor cytosolic fractions, followed by the benign tumor cytosolic fractions.

The column elution profile for a streptomycin sulfate-treated 30,000 x g supernatant preparation from malignant tumor IDC-375 is shown in Figure 33. PKII eluted near 0.16 M NaCl and represented about 75% of the total activity. Activity ratios for PKI and PKII were determined to be 0.60 and 0.25, respectively. Protein kinase type I and type II holoenzymes from a similar preparation from malignant tumor IDC-3669 represented 10% and 90% of the total kinase activity, with activity ratios of 0.56 and 0.23, respectively.

Protein kinase isoenzyme profiles from benign mammary tumors were also examined and compared to those obtained from malignant breast lesions. Protein kinase type I and type II holoenzymes were separated by DEAE-Sephacel chromatography for a benign tumor FA-1038, and were shown to represent approximately 45% and 55% of the kinase activity, respectively (not shown). The activity ratio determined in the presence and absence of 5 μM cAMP was determined to be 0.18 for the type I holoenzyme and 0.20 for the type II holoenzyme. Analysis of the holoenzyme constituents of FA-3070 kinase activity revealed that greater than 80% of the phosphotransferase activity was classified as type II based upon the salt elution profile. The partial purification scheme is presented in Table 34. The remainder of the kinase activity eluted as type I. Cyclic AMP binding activity exactly corresponded to fractions containing protein kinase activity (Figure 34). Fraction 32, which demonstrated high

FIGURE 33

DEAE-Sephacel chromatography of protein kinase in a streptomycin sulfate-treated 30,000 x g supernatant fraction from malignant tumor IDC-375. Tumor extracts were prepared in 20 mM Tris-HCl, pH 7.8, containing 3 mM mercaptoethanol and 1 mM EDTA (3 ml per g). DEAE-Sephacel column (1.6 x 6 cm) was equilibrated with the buffer and 6 ml of the treated supernatant fraction were applied, washing the column with the equilibration buffer (flow rate, 40 ml/hr), followed by a two hr linear salt gradient using 0.01 to 0.3 M NaCl (flow rate, 80 ml/hr). Column fraction (approximately 2.7 ml) were assayed for phosphotransferase activity into histone VS in the presence of 5 μM cAMP. The recovery of enzyme activity was approximately 85%. The arrow indicates initiation of the salt gradient.

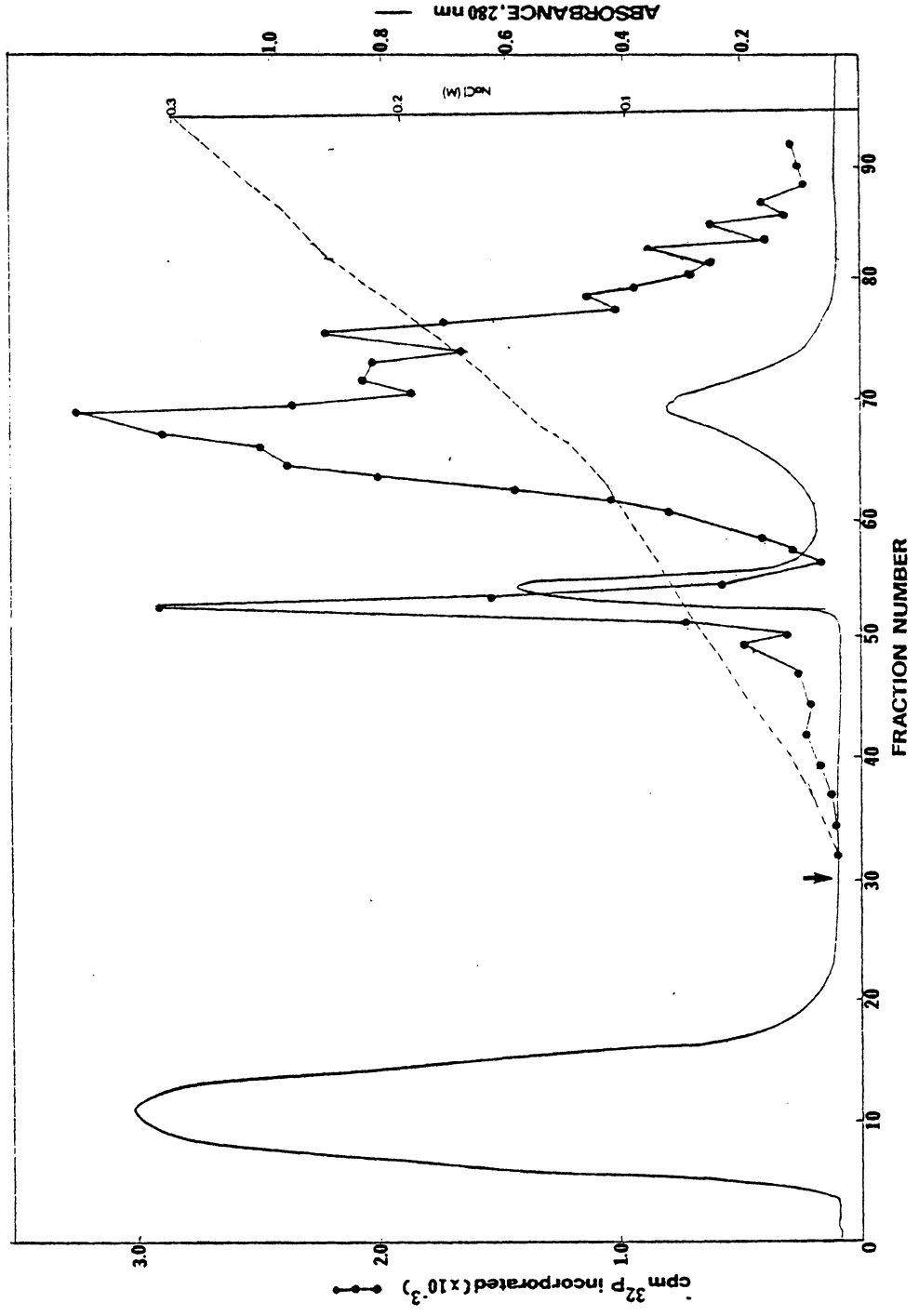

cpm ^{32}P incorporated ($\times 10^{-3}$)
ABSORBANCE, 280 nm
NaCl (M)
FRACTION NUMBER

TABLE 34

PARTIAL PURIFICATION OF HISTONE KINASE FROM
MALIGNANT MAMMARY TUMOR IDC-3070

step	total protein (mg)	total activity[1] (units)	specific activity (units/mg protein)	enrichment factor	yield
30,000 x g sup	38.0	4.81	0.13		100
30-50% NH_2SO_4 precipitate	12.5	5.07	0.41	3.2	105
DEAE-Sephacel					
peak I	0.56	8.00	14.2	34.6	17
peak II	0.82	26.9	32.8	80.0	56

[1]One unit is defined as nmol ^{32}P incorporated/min in the described assay using histone VS as the phosphate acceptor. Activity was determined in the presence of 5 µM cAMP. Specific activity (^{32}P)-ATP = 40.5 cpm/pmol.

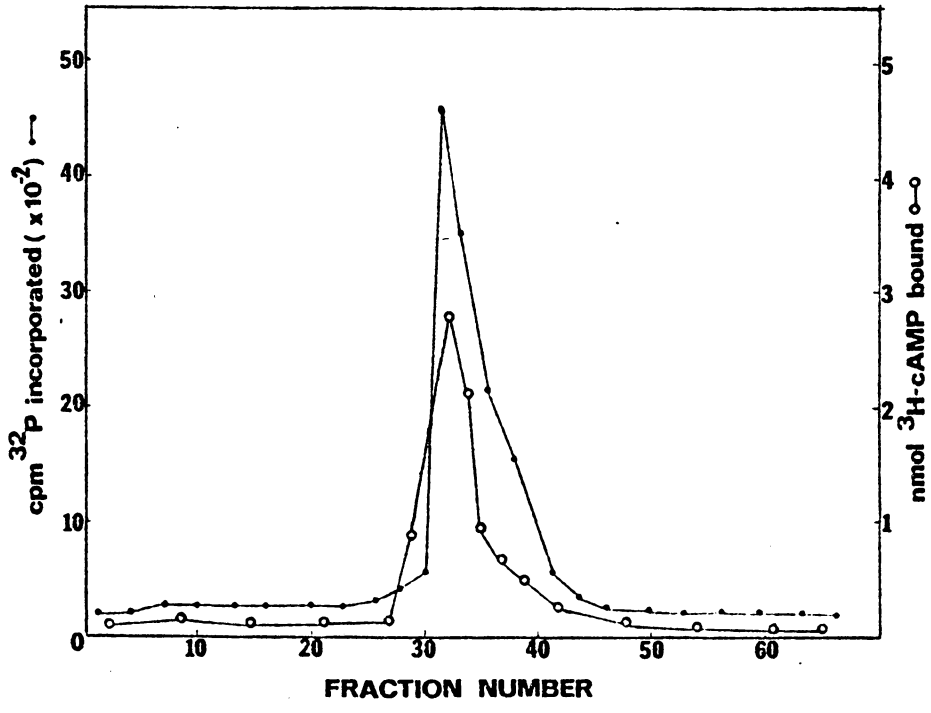


FIGURE 34

DEAE-Sephacel chromatography of a streptomycin sulfate-treated 30,000 x g supernatant fraction from benign tumor FA-3070 showing the elution profile during application of a linear salt gradient using 0.0 to 0.3 M NaCl. Maximum phosphotransferase activity into histone VS (•—•) was demonstrated to co-elute with ^{3}H-cAMP binding activity (o—o).

phosphotransferase activity into histone VS (14 pmol ^{32}P incorporated/min) was examined for its response to dissociation in the presence of 0.5 M NaCl. Figure 35 shows that the activity ratio of this enzyme preparation remained constant at approximately 0.31 over a 20 minute period in the absence of NaCl, while the amount of ^{32}P incorporated into histone VS was decreased by approximately 30% in the presence of 0.5 M NaCl. The activity ratios measured over a 20 minute period increased only slightly. This data suggested that some enzyme inactivation may have occurred in the presence of high salt, but dissociation of the holoenzyme appeared to proceed slowly.

An extensive characterization of kinase activity was made from a cytosolic preparation from benign tumor FA-137. Figure 36 indicates the elution profile of cAMP-dependent protein kinase from FA-127. PKI eluted with 0.04 to 0.06 M NaCl and represented approximately 2% of the total activity, whereas PKII eluted as a broad peak at 0.12 to 0.20 M NaCl, representing about 98% of the total kinase activity. The activity ratios for PKI and PKII were 0.73 and 0.20, respectively. Studies of the dissociation of PKI in the presence of 0.5 M NaCl revealed that the activity ratio increased over a 20 minute period in the absence of NaCl indicative of enzyme instability (Figure 37). In the presence of 0.5 M NaCl, an immediate increase in activity ratio to greater than 1.0 reflected the rapid loss of cAMP activation and rapid dissociation of the holoenzyme. In the case of PKII, kinase activity decreased in the presence of 0.5 M NaCl, however, the activity ratio remained relatively stable over a 20

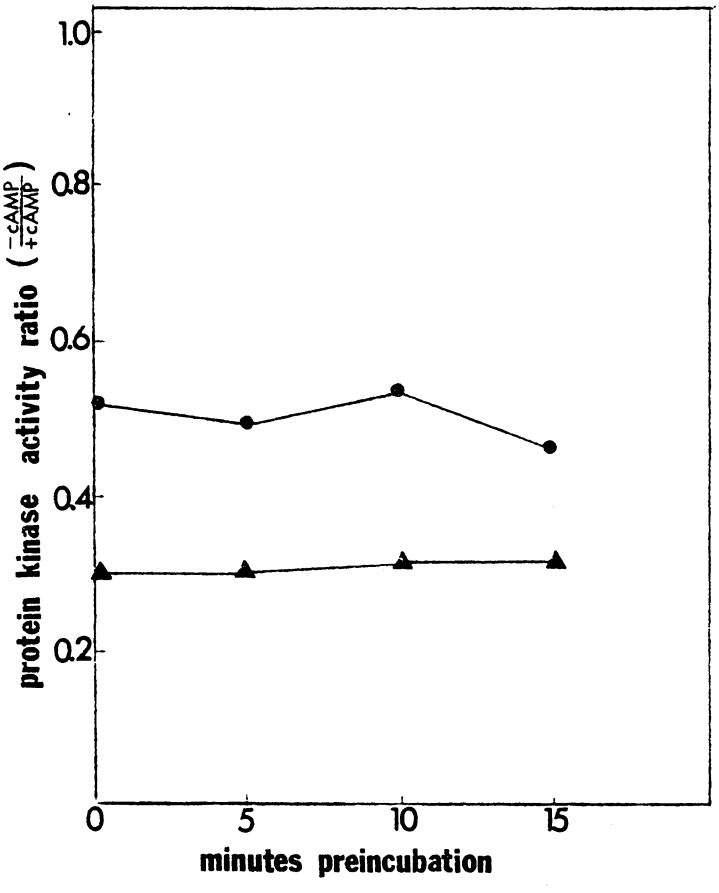
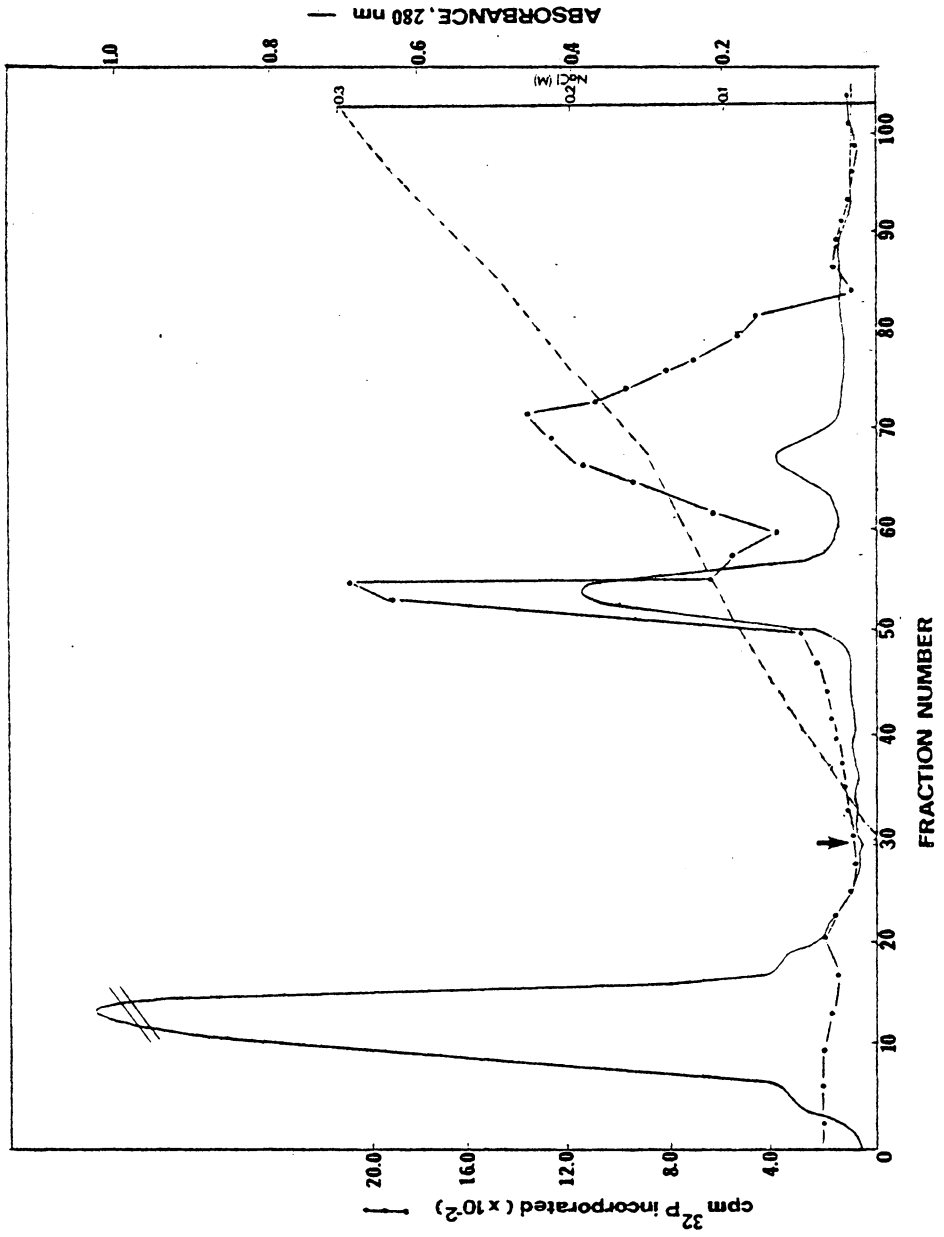


FIGURE 35

Effect of prior incubation with 0.5 M NaCl on the protein kinase activity ratio for type II cAMP-dependent protein kinase from benign tumor FA-3070, fraction #32 from the DEAE-Sephacel column (FIGURE 33). Following concentration and dialysis over night against 1000 ml 20 mM Tris-HCl, pH 7.8, containing 2 mM mercaptoethanol and 5 mM benzamidine at 4°C. At zero time, 50 µl of H_2O or 3.0 M NaCl were added to 250 µl enzyme (1.2 mg/ml protein), and samples were incubated at 30°C for the designated times. Ten microliter aliquots were removed for assay (15 minutes, 37°C) of phosphotransferase activity into histone VS (2.0 mg/ml) in the presence and absence of 5 µM cAMP. Specific activity (^{32}P)-ATP = 22.9 cpm/pmol. Tumor protein addition per reaction was 15 µg. Activity ratio, +0.5 M NaCl (●); -0.5 M NaCl (▲).

FIGURE 36

DEAE-Sephacel chromatography of protein kinase in a streptomycin sulfate-treated 30,000 x g supernatant fraction from benign tumor FA-137. Tumor extracts were prepared in 20 mM Tris-HCl, pH 7.8, containing 2 mM mercaptoethanol and 1 mM EDTA (3 ml per g). The DEAE-Sephacel column (1.6 x 6 cm) was equilibrated with the same buffer and 3 ml of the treated supernatant fraction were applied, washing the column with the equilibration buffer (flow rate, 40 ml/hr), followed by a linear salt gradient (arrow) using 0.0 to 0.3 M NaCl (flow rate, 80 ml/hr). The column fractions (approximately 2.7 ml) were assayed for phosphotransferase activity into histone VS in the presence of 5 μM cAMP. The recovery of enzyme activity was approximately 80%.


cpm ^{32}P incorporated (x 10^{-2})
20.0
16.0
12.0
8.0
4.0
0
10
20
30
40
50
60
70
80
90
100
FRACTION NUMBER
ABSORBANCE, 280 nm
1.0
0.8
0.6
0.4
0.2
NaCl (M)
0.3
0.2
0.1

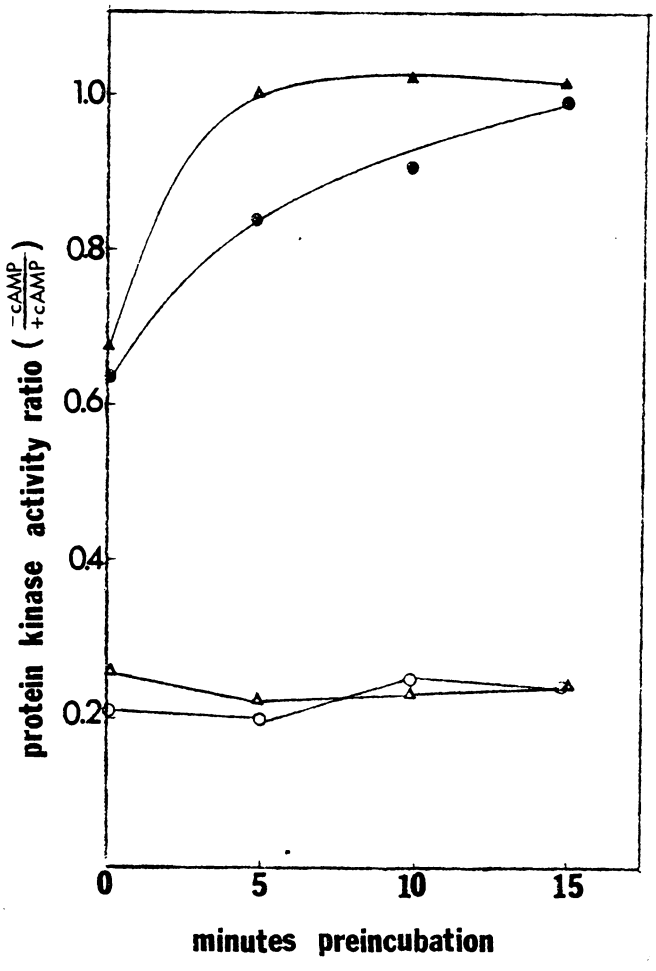


FIGURE 37

Effect of prior incubation with 0.5 M NaCl on the protein kinase activity ratio for type I and type II cAMP-dependent protein kinase from benign tumor FA-137. Partially-purified holoenzyme preparations were resolved by DEAE-Sephacel chromatography, concentrated, and dialyzed against 20 mM Tris-HCl, pH 7.8, containing 2 mM mercaptoethanol and 5 mM benzamidine over night at 4°C. At zero time, 50 µl of H_2O or 3.0 M NaCl were added to 250 µl enzyme, and samples were incubated at 30°C for the indicated times. Ten microliter aliquots were removed for assay (15 minutes) of phosphotransferase activity into histone VS (2.0 mg/ml) in the presence and absence of 5 µM cAMP. Specific activity (^{32}P)-ATP = 34.5 cpm/pmol. Tumor protein addition per reaction was 15 µg and 22 µg for type I and II protein kinase, respectively. Type I kinase (+0.5 M NaCl, ▲; -0.5 M Nacl, ●); Type II kinase (+0.5 M NaCl, Δ; -0.5 mM o).

minute incubation period both in the presence and absence of salt. Cyclic AMP binding activity was similar for the type I and II holoenzyme preparations from FA-137. Approximately 80 cpm ^{3}H-cAMP were bound per minute using a 20 incubation at 4°C. Apparent K_a values for PKI and PKII were determined to be 37 nM and 48 nM, respectively.

3.5.2. Separation of Catalytic and Regulatory Subunits by cAMP-Affinity Chromatography: Initial attempts to isolate subunits from protein kinase holoenzyme preparations from malignant and benign human mammary tumors involved a modification of the DEAE-Sephacel chromatography procedure. First, after application of the tumor supernatant fraction to the DEAE column followed by a washing step with Tris-EDTA-ME, elution of the catalytic subunit was attempted using the same buffer containing 0.009 M NaCl and 3 mM cAMP. Elution of the column and the post-cAMP wash was monitored by the change in absorption at 280 nm. Fractions collected were assayed for kinase activity in the presence and absence of cAMP, then pooled, concentrated to 1/10 the original volume using an Amicon PM-10 filter, then reassayed for activity. Following elution with cAMP, a salt gradient was applied using 0.01 to 0.3 M NaCl over a two hour period to remove the bound regulatory subunit. Individual fractions were tested for cAMP binding activity, then pooled, concentrated and reassayed. Such a protocol proved to be unsuccessful in the separation of tumor-associated catalytic and regulatory subunits.

A second attempt involved preincubation of the tumor supernatant fractions with 10 μM cAMP at 4°C for 60 minutes, followed by 30°C for 4 minutes prior to separation by DEAE-Sephacel chromatography. Again, after the initial buffer wash, the column was eluted with Tris-EDTA-ME, containing 1 μM cAMP, to remove the catalytic subunit, followed by a two hour salt gradient using 0.01 to 0.3 M NaCl to remove the regulatory subunit. Again, this technique proved unsatisfactory in the separation of the enzyme subunits.

The third attempt to resolve the catalytic and regulatory subunits was by the use of cAMP-agarose affinity chromatography, as detailed in the methods section. Following the application of a concentrated holoenzyme preparation, the catalytic subunit was removed by washing the affinity column with 5 mM MES buffer, pH 6.5, containing 0.1 M EDTA, 100 mM NaCl, and 15 mM 2-mercaptoethanol (Buffer A). An additional salt wash with the same buffer, containing 750 mM NaCl was used to remove nonspecifically-absorbed proteins from the resin.

Partially-purified protein kinase type I and II holoenzyme preparations were concentrated, dialyzed overnight at 4°C, then applied to cAMP-agarose affinity columns for separation of the catalytic and regulatory subunits. The elution profiles from the initial buffer and salt washes for cAMP agarose affinity chromatography of the type II holoenzyme preparation from IDC-375 is shown in Figure 38. Elution patterns after application of type I and type II holoenzyme preparations to the affinity columns were similar. Catalytic activity was

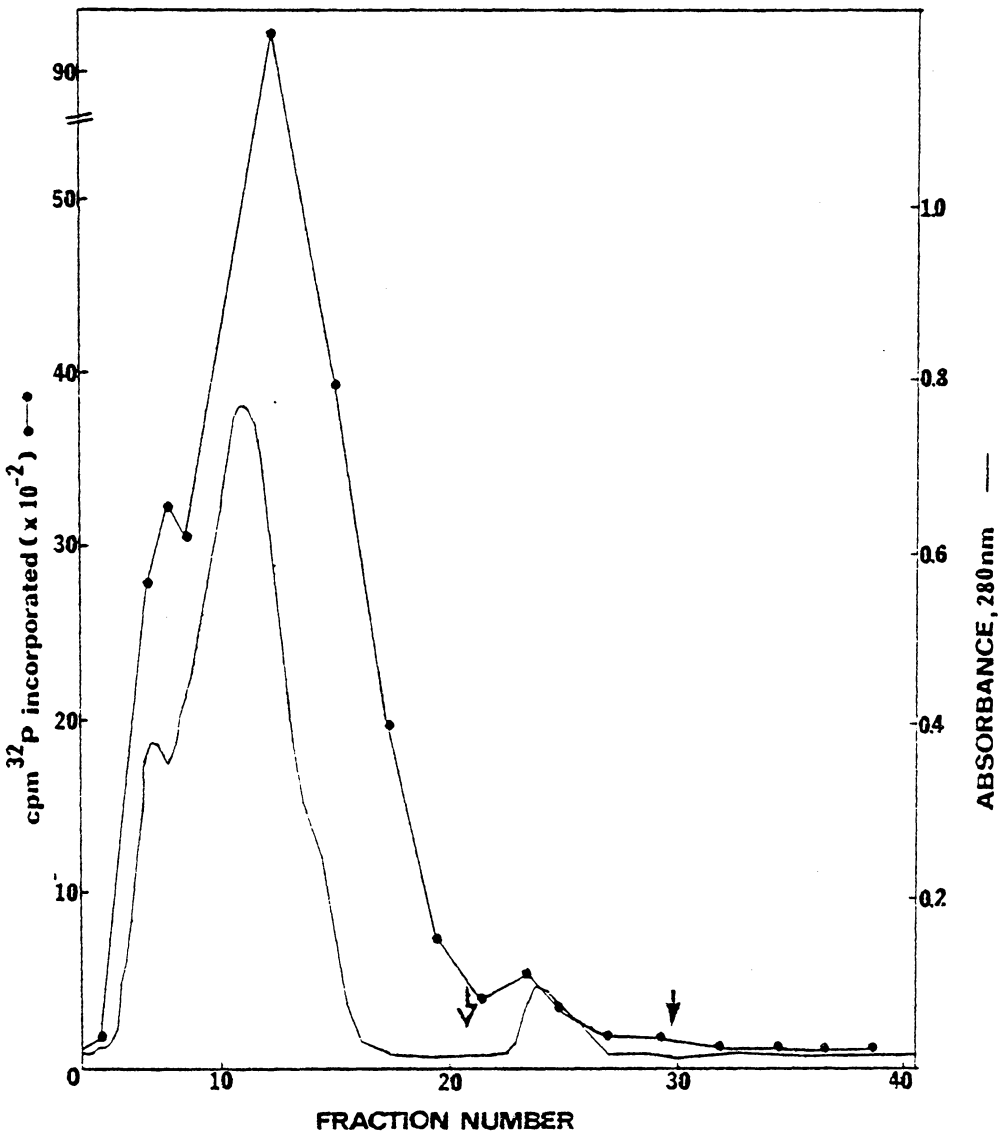


FIGURE 38

Separation of catalytic subunit of protein kinase type II. Cyclic AMP-agarose affinity chromatography of a type II protein kinase preparation from malignant tumor IDC-375 resolved an DEAE-Sephacel demonstrating the elution profile during the initial wash with 5 mM MES, pH 6.5, containing 0.1 mM EDTA, 15 mM mercaptoethanol and 100 mM NaCl (open arrow), followed by the same buffer containing 750 mM NaCl (closed arrow).

eluted during the initial wash with buffer A.

A final wash with 5 mM MES buffer, pH 6.5, containing 1.0 μM cAMP or 30 mM cAMP (Buffer B) was used to remove the regulatory subunit for cAMP-dPK types II and I, respectively. Elution with cAMP was performed in a batch manner. Approximately 50 ml of eluate were concentrated and dialyzed against MES-EDTA-ME for 48 hours at 4°C prior to evaluation of cAMP binding capacity by the filter binding assay.

3.5.3. Reconstitution of the Protein Kinase Holoenzyme: The reconstitution protocol was developed using partially purified catalytic and regulatory subunits from bovine heart (Sigma Chemical Co.) in order to validate the methods to be used with isolated mammary tumor-protein kinase subunits. Catalytic subunit (CAT) in 50 mg/ml DTT as adjusted to give a final concentration of 1 unit/μl, and regulatory subunit (REG) in 6 mg/ml DTT was adjusted to give 0.5 units/μl. Reconstitution was performed by recombining 5 units of CAT with 0.5 or 2.5 units REG in the presence and absence of 5.0 μM cAMP. Reconstitution of the bovine heart holoenzyme by adding 0.5 and 2.5 units of REG to 5 units CAT gave 32% and 74% inhibition of the catalytic activity, respectively in the absence of cAMP (Figure 39). Addition of 5.0 μM cAMP to the reaction inhibited reassociation and, in fact, a slight enhancement of phosphotransfer into histone VS was observed. The regulatory subunit preparation was tested for catalytic activity, however, none was detected.

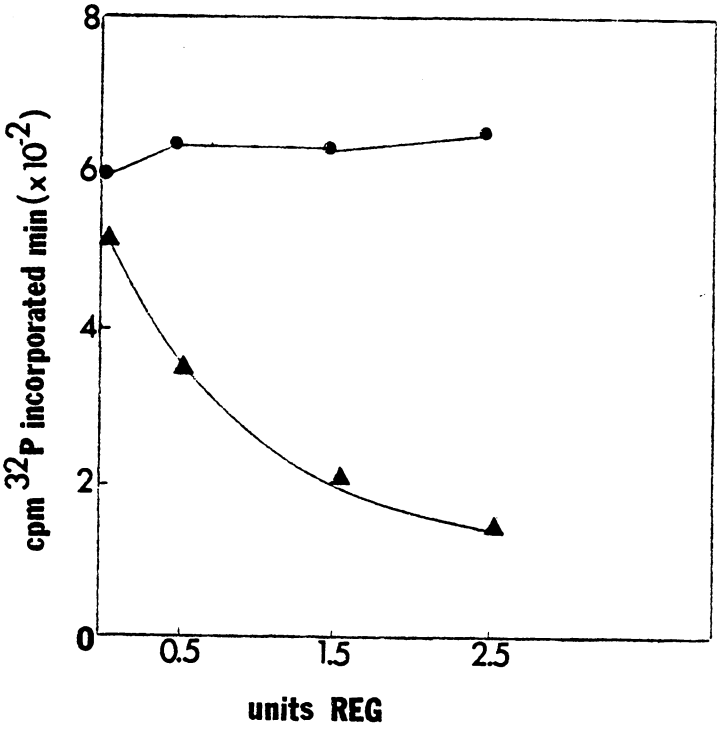


FIGURE 39

Reassociation of catalytic and regulatory subunits of cAMP-dependent protein kinase. Fifteen units of catalytic subunit (one unit will transfer 1.0 pmol phosphate from ^{32}P-ATP to hydrolyzed and partially dephosphorylated casein per minute at pH 6.5 at 30°C) were incubated in the presence of 0.5, 1.5, and 2.5 units regulatory subunit, in the absence (▲) or presence (•) of 5 µM cAMP for 10 minutes at 30°C. Phosphotransferase activity was measured as previously described. Activity values represent the mean of three replicates for each treatment. Specific activity (^{32}P)-ATP = 35.2 cpm/pmol. Substrate concentration was 2.0 mg/ml histone VS.

Similar experiments were done recombining the isolated catalytic subunit of PKII from mammary tumor IDC-375 with bovine heart REG. As indicated in Table 35, total separation of tumor CAT and PKII holoenzyme was not obtained since a 1.8-fold activation by 5.0 μM cAMP was observed. However, the preparation appeared to be enriched with CAT, as indicated by the effects of addition of the regulatory subunit, allowing 40% and 54% inhibition, respectively, with 0.5 units and 2.5 units REG. It was noted that addition of REG in the presence of 5.0 μM cAMP resulted in loss of inhibition of catalytic activity. Again, a slight enhancement of phosphorylation was observed. Protein kinase type II catalytic activity isolated from FA-1038 was also essentially free of holoenzyme contamination as indicated by activity in the presence and absence of cAMP. Inactivation of catalytic phosphotransfer was determined to be 12% and 43% in the presence of 0.5 and 2.5 units REG.

A third attempt to recombine isolated tumor catalytic subunit with bovine heart-derived regulatory subunit was done using the catalytic subunit fraction from malignant tumor IDC-3669. The catalytic subunit fraction prepared from IDC-3669-derived cAMP-dPK type II appeared to be homogeneous. Addition of 0.5 units and 2.5 units of bovine heart REG allowed a 60% and 77% inhibition of phosphorylation, respectively (not shown). These results indicated that catalytic subunit from either malignant and benign mammary tumor histone kinase showed no defect in its ability to recombine with the regulatory subunit in the absence of cAMP.

TABLE 35

REASSOCIATION OF CATALYTIC AND REGULATORY SUBUNITS FROM CYCLIC AMP-DEPENDENT PROTEIN KINASE IN MALIGNANT TUMOR IDC-375 AND BENIGN TUMOR FA-1038[1]

+/-cAMP	units REG[2]	IDC-375 cpm ^{32}P incorporated/min/mg protein ($\times 10^3$)	FA-1038
+	-	5.65	5.66
-	-	3.14	5.05
+	0.50	6.57	5.92
-	0.50	1.87	4.75
+	2.50	7.71	6.09
-	2.50	1.44	3.21

[1]Catalytic subunits of the tumor-associated protein kinase holoenzymes were isolated by cAMP-affinity chromatography as previously described. The catalytic preparations from IDC-375 and FA-1038 contained 2.65 mg/ml and 1.78 mg/ml protein, respectively (addition of 40 µg and 28 µg/reaction). Specific activity (^{32}P)-ATP = 45.6 cpm/pmol, phosphotransfer was measured using histone VS as the substrate at 2.0 mg/ml in the presence or absence of 5 µM cAMP.

[2]cAMPdPK type II regulatory subunit (Sigma Chemical Co); one unit is defined as that amount which will inhibit one unit of catalytic activity, in catalyzing the transfer of phosphate from (γ-^{32}P)-ATP to casein at a rate of one pmol per minute at 30°C, pH 6.5

In the previously described examples, reconstitution was done by preincubation of only the CAT and REG subunits in assay buffer prior to addition of the substrate, histone VS, and radiolabel. A contrasting situation was observed in the case of the cAMPd-PK type II catalytic preparation from FA-137 isolated in a similar manner (Table 36). Reconstitution was not possible unless preincubation in the presence of Mg ATP occurred for at least 10 minutes at 4°C prior to addition of the substrate and ^{32}P-ATP. This preparation was sensitive to bovine heart PK type II inhibitor. It is thought that this inhibitor is a modified regulatory subunit of protein kinase, therefore, the ability for inhibitor binding to the catalytic subunit appeared normal. Since this preparation showed behavior characteristic of a type I kinase catalytic subunit in that reassociation occurred only in the presence of Mg ATP, the holoenzyme preparation isolated by DEAE chromatography was re-examined for its dissociation patterns in the presence of high salt. The activity ratios observed in the presence and absence of 0.5 M NaCl indicated behavior representative of a type II kinase in that little, if any, dissociation occurred over a 15 minute incubation period at 30°C.

Recovery of the isolated regulatory subunits of either type I or II mammary tumor protein kinase by cAMP-agarose affinity chromatography was for the most part unsuccessful. Isolation of a regulatory subunit preparation from tumor-derived protein kinase as detected by the ^{3}H-cAMP binding assay, was possible only in the case

TABLE 36

RECONSTITUTION OF THE PROTEIN KINASE HOLOENZYME FROM BENIGN TUMOR FA-137

+/-cAMP	time preincubation w/Mg-ATP	units REG	cpm ^{32}P incorporated/ min/mg protein ($x10^{-3}$)
-	0	-	17.50
+	60	-	17.94
-	60	-	18.34
-	10	3	10.12
-	30	3	9.13
-	60	3	8.56
+	60	3	19.60

[1]Catalytic activity from FA-137 was isolated by cAMP-agarose affinity chromatography as previously described (2.3 mg/ml protein; protein addition per reaction = 34.5µg).

[2]Preincubation occurred at 30°C for the designated time intervals, in the presence of 12.0 mM $MgCl_2$ and 30 µM unlabeled ATP. The reaction was initiated by addition of the substrate, 2.0 mg/ml histone VS, and 100 nmol (^{32}P)-ATP; specific activity = 23.6 cpm/pmol.

of holoenzyme isolated from IDC-375. Even here the amount of regulatory subunit recovered was not adequate for reconstitution with tumor-associated catalytic subunit. Whether this was due to proteolytic degradation of the subunit, loss of cAMP-binding activity or failure to elute the subunit from the column was undetermined.

3.6. Human Mammary Tumor-Derived Casein Kinase

In previous studies on the substrate specificity of mammary tumor histone kinase, it was noted that relatively high levels of phosphotransfer to casein occurred. It was of interest to determine whether phosphorylation of casein was catalyzed by the histone kinase or whether a separate kinase performed this function. Casein kinase activity was detectable in several supernatant fractions prepared from malignant and benign mammary tumors. The 30,000 x g soluble fraction prepared from malignant tumor IDC-2997 was found to catalyze phosphotransfer from [^{32}P]-ATP to casein (2.0 mg/ml final concentration) at a rate of 3 to 5 pmol min^{-1}. At the termination of a 20 minute incubation at 30°C, approximately 60 pmoles ^{32}P had been incorporated into the substrate. The activity ratio in the presence and absence of 5.0 µM cAMP was determined to be 0.8 to 0.9 indicating that little, if any, response to cAMP occurred (Figure 40).

Since a high activity ratio might be indicative of cAMP degradation in the assay mixture, 500 fmoles of cAMP was added to 25 µl samples from supernatant fractions of two tumor sources, IDC-4041 and FA-1258, the reaction mixtures incubated for 0, 5, and 10 minutes

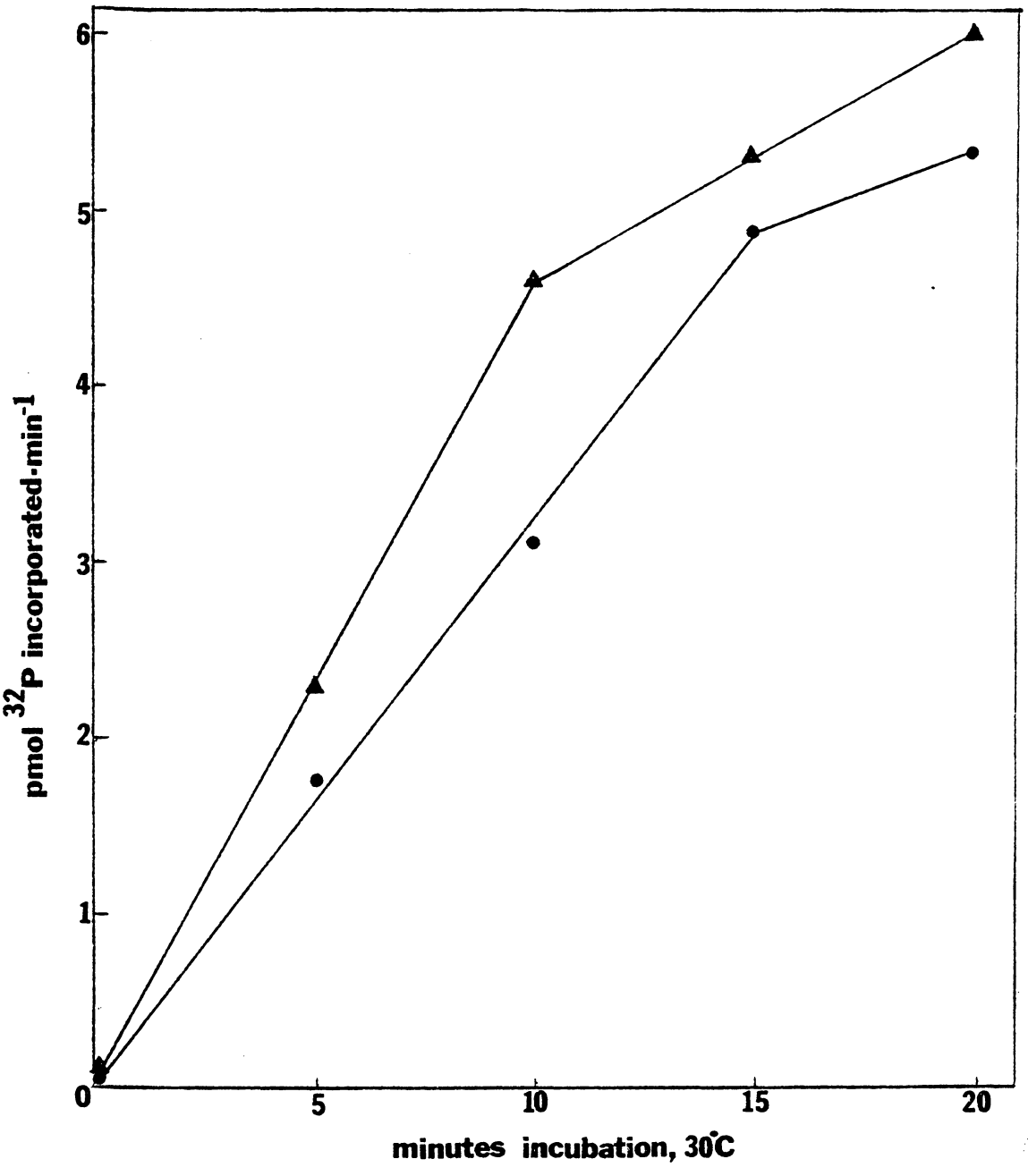


FIGURE 40

Incorporation of ^{32}P in the presence of casein (2 mg/ml) catalyzed by a 30,000 x g supernatant fraction from malignant tumor IDC-2997. Phosphotransfer was measured in the presence (▲), and absence (●) of 5.0 µM cAMP. Specific activity of (^{32}P)-ATP = 24.3 cpm/pmol.

at 30°C and the amount of cAMP remaining determined by RIA. Results indicated that cAMP was not being degraded by endogenous cAMP-phosphodiesterase activity, since greater than 95% of the added cAMP was detected after 10 minutes incubation at 30°C. Further characterization of tumor-associated casein kinase indicated that its phosphotransferase potential was linear over a 10 minute reaction period at 30°C. Measurement of casein kinase activity gave a rather broad pH profile with maximum phosphorylation occurring at pH 7.5 (Figure 41). Kinetic analysis of casein kinase indicated the apparent Km for ATP and casein to be approximately 260 μM and 1.0 mg/ml, respectively.

Addition of cAMPdPK type II inhibitor from bovine heart in amounts to inhibit 10 to 50 pmoles phosphate transfer per minute at 30°C showed no effect on casein kinase activity. Preincubation with 0.5 and 2.5 units of cAMPdPK type II regulatory subunit for 10 minutes at 4°C prior to assay had no inhibitory effect on the enzyme. These results provide additional evidence that the casein kinase did not represent a cAMP-dependent enzyme.

In addition to its presence in supernatant fractions from malignant and benign tumors, casein kinase was also demonstrated in lyophilized microsections from tumors. Activity levels ranged from 12 to 60 pmoles ^{32}P incorporated min^{-1}.mg dry wt $tissue^{-1}$, or 200 to 800 pmol ^{32}P incorporated min^{-1}.μg $protein^{-1}$. Neither tumor supernatant or tissue microsection-derived casein kinase was shown to be activated by 5 to 50 μM cAMP.

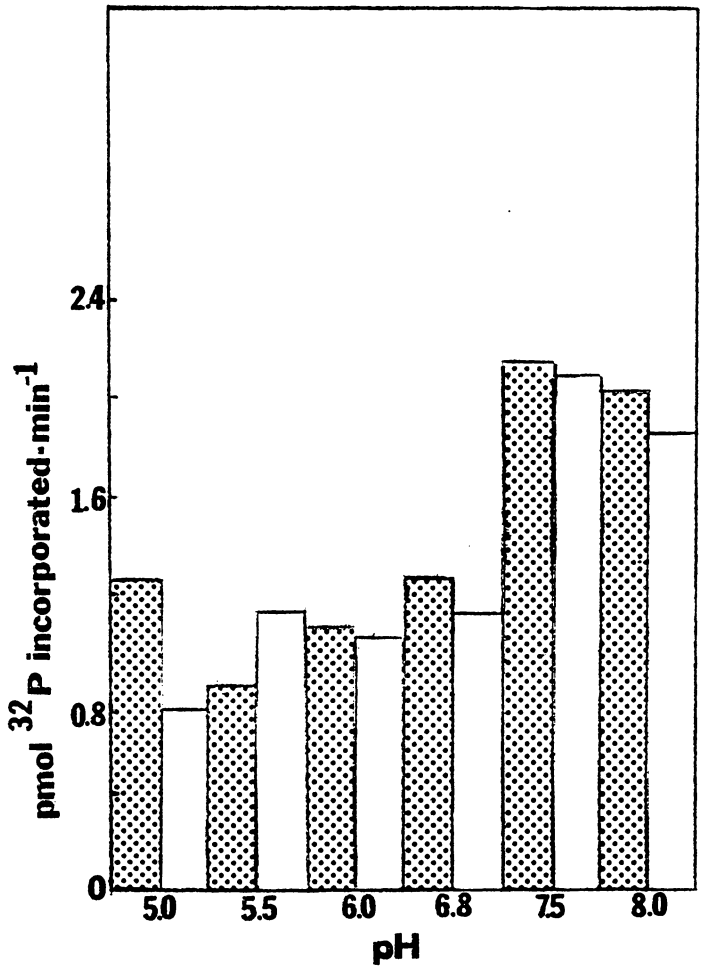


FIGURE 41

pH profile of phosphorylating activity from a cytosolic fraction isolated from malignant tumor IDC-2997 (protein addition, 55 µg) using casein at 2.0 mg/ml final concentration. Specific activity of (^{32}P)-ATP = 22.5 cpm/pmol.

Casein kinase activity was eluted from DEAE-Sephacel with 0.3 M NaCl after the resin had been cleared of cAMP-dependent types I and II protein kinase activities (Figure 42). As indicated in Table 37, tumor-associated casein kinase showed specificity for casein, and did not catalyze phosphotransfer from [^{32}P]-ATP into various histone preparations with efficiency. In addition, activation in the presence of cAMP was not possible, nor did the resolved casein kinase possess cAMP-binding activity.

3.7. Endogenous Phosphorylation in Mammary Tumor Cytosolic Proteins

An analysis of soluble polypeptides from cytosolic extracts (30,000 x g supernatant fractions) of malignant and benign human mammary tumors was made using sodium dodecyl sulfate polyacrylamide gel electrophoresis in one dimension. These analyses were made as a preliminary step to the identification of endogenous substrate proteins which might be phosphorylated by tumor-associated cAMP-dependent protein kinase. It was possible to screen several tumors for high levels of endogenous phosphorylating activity. Endogenous levels of phosphorylation in the absence of added substrate ranged from 5 to 20 pmol ^{32}P incorporated/min/mg dry weight in tumor tissue microsections and 30 to 75 pmol ^{32}P incorporated/min/mg protein in tumor cytosolic fractions.

Peptide analysis of malignant and benign tumor cytosolic fractions on denaturing polyacrylamide gels stained with Coomassie Brilliant Blue indicated that similar patterns of banding occurred, with

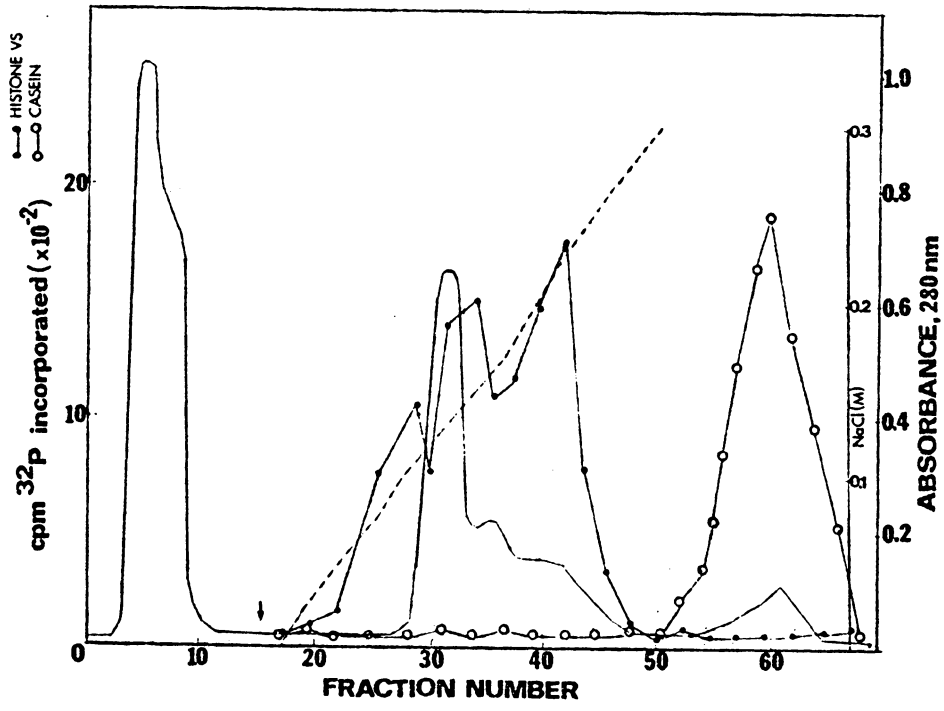


FIGURE 42

Elution profile of cAMP-dependent protein kinase (···) and casein kinase (ooo) activities from a streptomycin sulfate-treated cytosolic fraction from malignant tumor IDC-3669 partially purified using DEAE-Sephacel chromatography. The column (1.6 x 6 cm) was eluted with 20 mM Tris-HCl, pH 7.5, containing 2 mM mercaptoethanol, 1 mM EDTA, 5 mM theophylline, and 10 mM benzamidine (flow rate, 40 ml/hr), followed by a one hour linear salt gradient (arrow) using 0.0 to 0.3 M NaCl (flow rate, 80 ml/hr). Fractions (approximately 2.7 ml) were assayed for phosphotransferase activity in the presence of 2.0 mg/ml histone VS and 5.0 μM cAMP or 2.0 mg/ml casein. Specific activity of (^{32}P)-ATP = 27.9 cpm/pmol. Absorbance of column eluate was measured at 280 nM (——).

TABLE 37

SUBSTRATE SPECIFICITY OF TUMOR-ASSOCIATED CASEIN KINASE[1] FROM MALIGNANT TUMOR IDC-3669

substrate	+/-cAMP	pmol ^{32}P incorporated[2]
histone IIA	-	22
histone IIIS	-	1
histone VIS	-	31
histone VIIS	-	30
histone VIIIS	-	19
histone VS	+	14
histone VS	-	5
protamine	-	21
casein	+	206
casein	-	221

[1]The enzyme represented casein kinase activity resolved by DEAE-Sephacel chromatography of a cytosolic fraction from malignant tumor IDC-3669 (40 µg protein per reaction).

[2]Specific activity (^{32}P)-ATP = 14.2 cpm/pmol. Activity values represent the mean of three replicates for each substrate tested. Reactions were incubated for 10 minutes at 30°C prior to determination of ^{32}P incorporation into TCA-precipitable protein.

six major peptides being resolved (M_r 14,800; 38,000; 46,700; 59,800; 69,000; and 110,000) (Figure 43). Numerous additional bands were observed. Molecular weight estimations were made using the relative mobility versus molecular weight curve depicted in Figure 44.

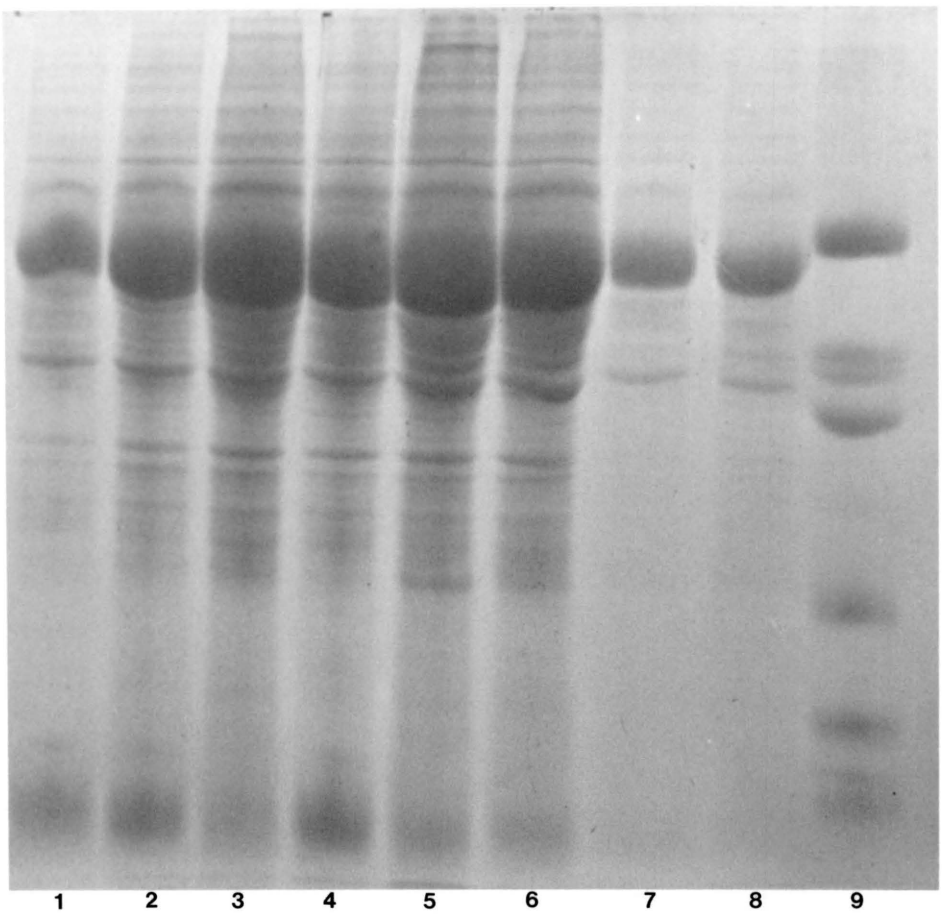
Incubation of benign and malignant tumor extracts for 2 minutes in the presence of (^{32}P)-ATP, but with no added cAMP caused several peptides with molecular weight ranging from 18,000 to 95,000 daltons to be phosphorylated (Figures 45, 46). In addition, incubation of mammary tumor extracts with 5 μM cAMP resulted in an increase in the phosphorylation in at least six bands (M_r approximately 23,000; 70,000; and four bands occurring at 80,000 to 95,000). These specific phosphorylations were seen in both malignant and benign preparations. One peptide band (M_r 59,000) was observed to demonstrate a decrease in phosphorylation in the presence of cAMP.

Evaluation of the phosphorylating potential of malignant tumor IDC-147 was examined using histone VS as the phosphate acceptor. As indicated in Figure 47, phosphorylation of H1 subfractions increased with time of incubation at 30°C. This observation verified that the majority of the phosphorylation in reactions of tumor soluble fractions with histone VS and (^{32}P)-ATP was attributable to phosphotransfer to the histone subfractions (see histone VS banding pattern in Figure 48, lanes 14, 15).

Cyclic AMP holoenzyme preparations were partially resolved from mammary tumor cytosolic extracts using anion exchange chromatography as previously described. In addition, separation of the holoenzyme

FIGURE 43

$NaDODSO_4$ polyacrylamide gel electrophoresis of malignant and benign mammary tumor cytosolic fractions. Peptide bands were visualized by Coomassie Brilliant Blue staining. Track 1, IDC-4041; 2, IDC-168; 3, IDC-3928; 4, IDC-3669; 5, FA-1258; 6, FA-3070; 7, FA-1700; 8, FA-1038; 8, molecular wt markers.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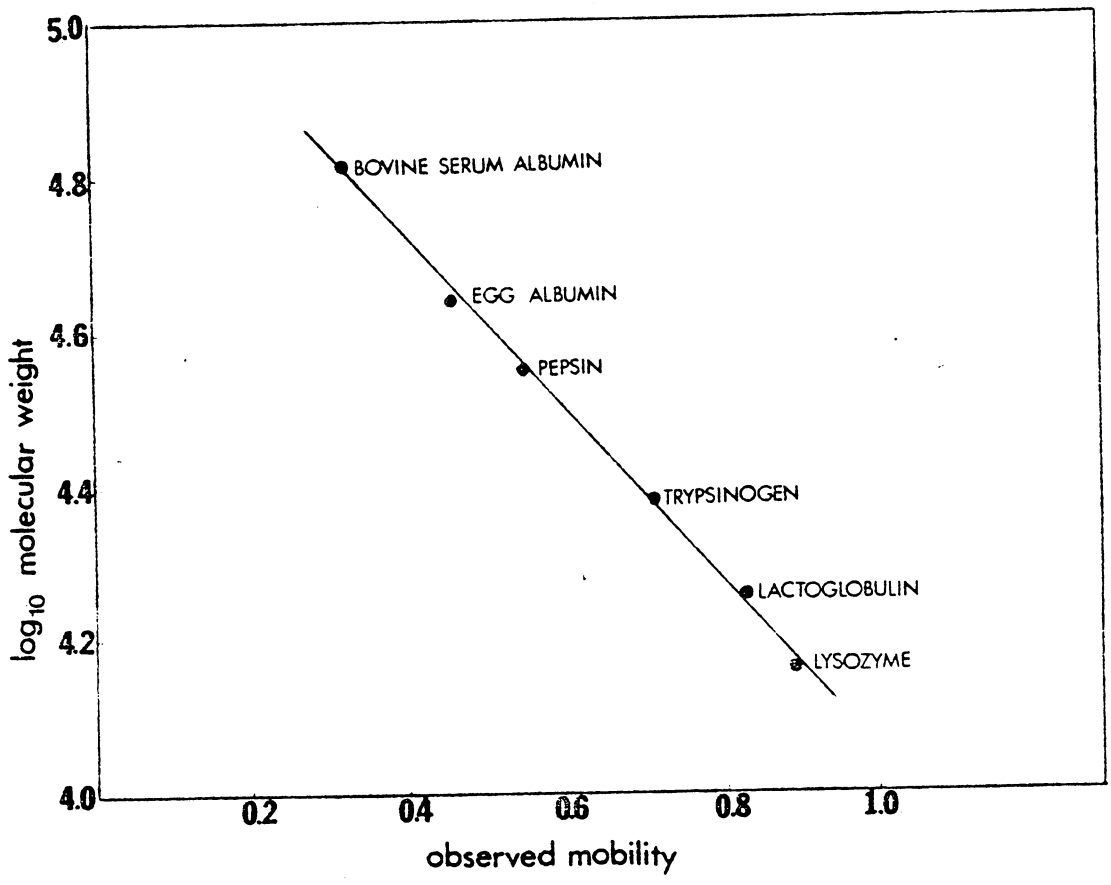

FIGURE 44

Calibration curve for $NaDODSO_4$ polyacrylamide gel electrophoresis. Details for the procedure are given in Materials and Methods. Molecular weight range; 10,000 to 70,000. Lysozyme, 14,300; β-lactoglobulin, 18,400; trypsinogen, 24,000; pepsin, 34,700; egg albumin, 45,000; bovine serum albumin; 66,000. Observed mobility represents the migration of peptides with respect to bromphenol blue as the tracking dye.

FIGURE 45

Endogenous phosphorylation in malignant and benign mammary tumor cytosolic fractions in the presence and absence of 5 μM cAMP. Phosphorylation, gel electrophoresis, and autoradiography were performed as described in Materials and Methods. Track 1, FA-1258, -cAMP; 2, FA-1258, +cAMP; 3, IDC-147, -cAMP; 4, IDC-147, +cAMP; 5, FA-3070, -cAMP; 6, FA-3070, +cAMP; 7, IDC-3928, -cAMP; 8, IDC-3928, +cAMP.

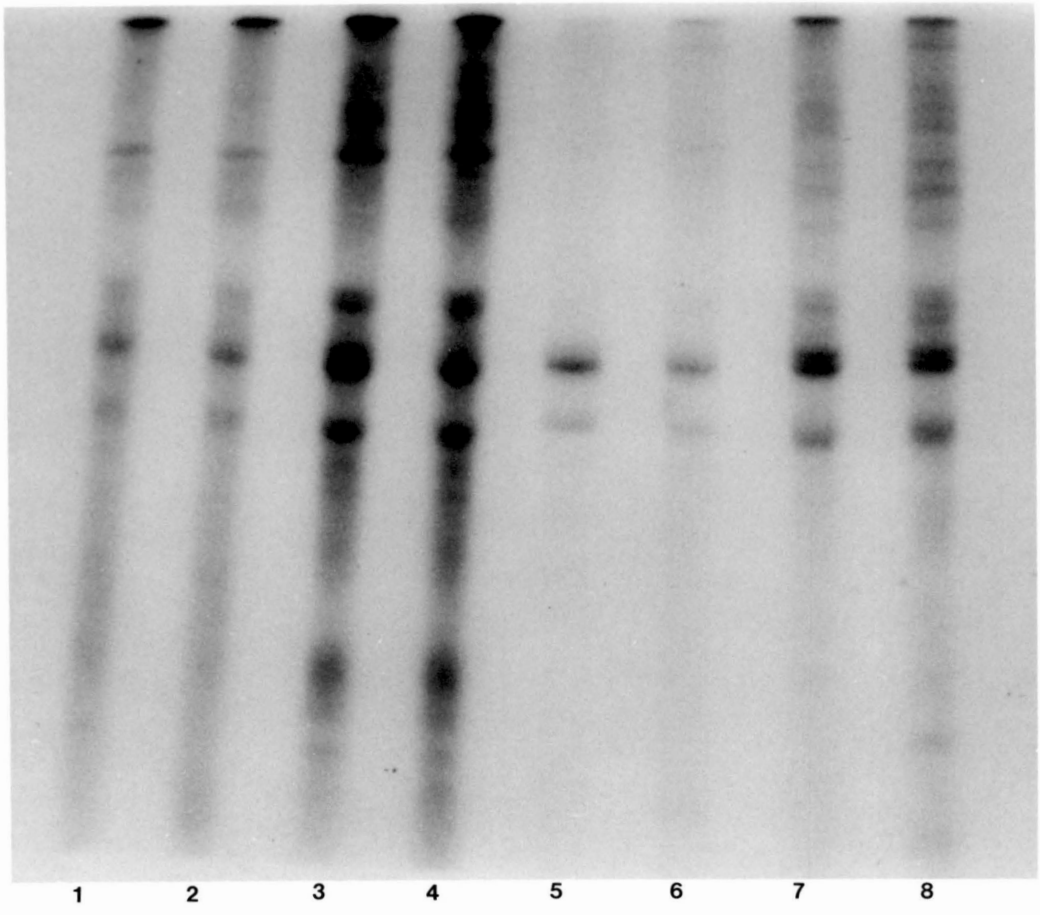
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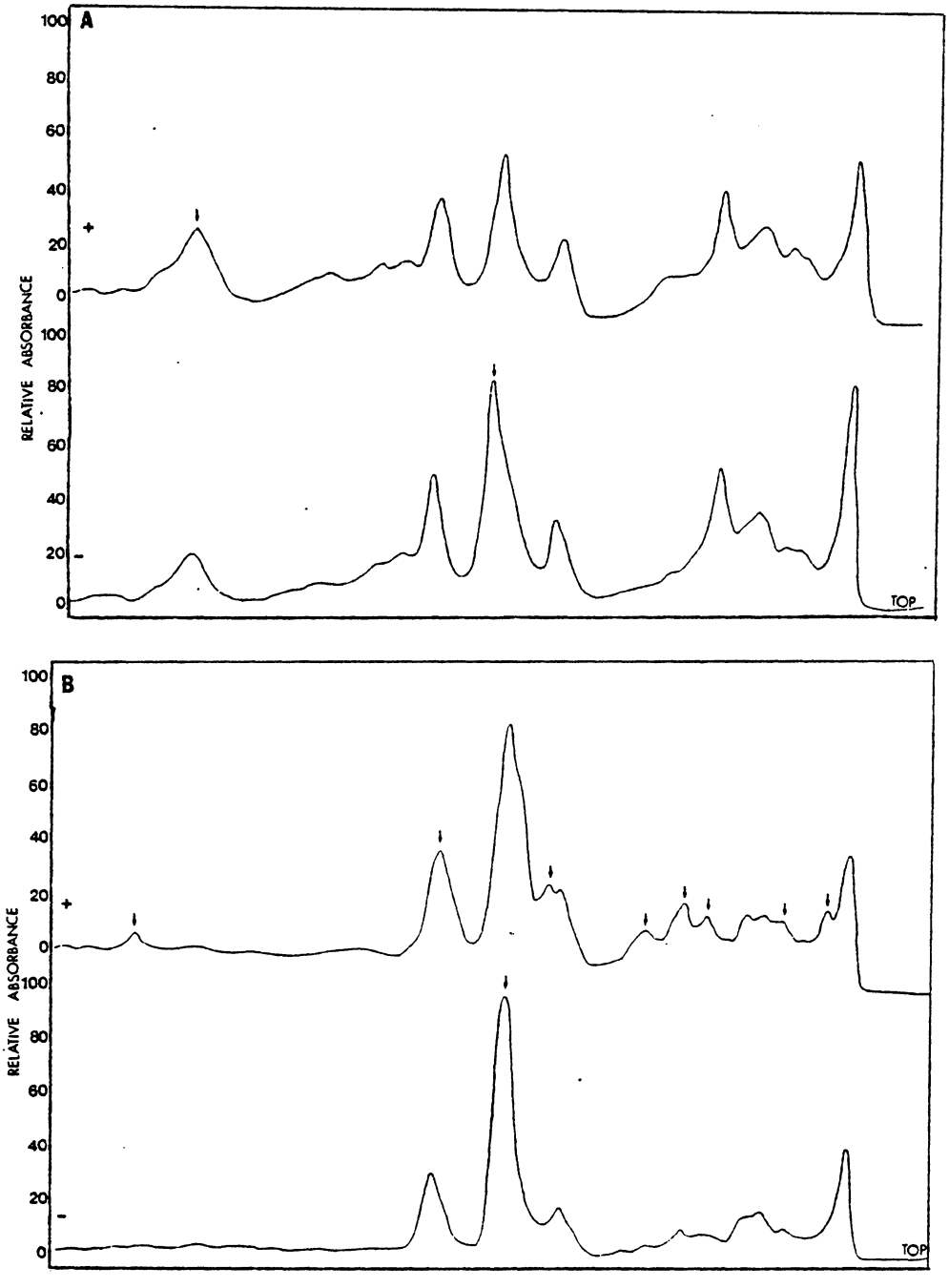
FIGURE 46

Densitometer tracings of phosphorylation patterns in cytosolic extracts from malignant and benign mammary tumors. $NaDODSO_4$ polyacrylamide gel electrophoresis and autoradiography were done as previously described. Phosphotransferase reactions were run in the absence and presence of 5 μM cAMP (indicated + or -). Arrows represent those bands showing enhanced phosphorylation by transfer from (^{32}P)-ATP. A, IDC-147; B, IDC-3928; C, FA-1258.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B
RELATIVE ABSORBANCE
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60
40
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TOP

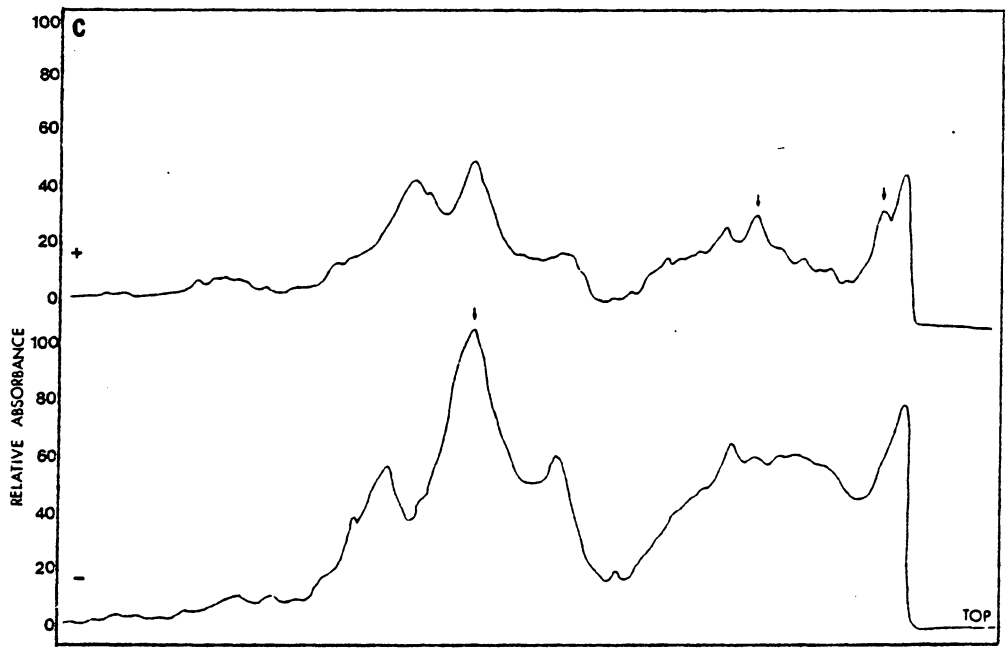
C
100
80
60
40
20
0
+
100
80
60
40
20
0
-
RELATIVE ABSORBANCE
TOP

FIGURE 47

Phosphorylation of histone VS by a 30,000 x g supernatant fraction from malignant tumor IDC-147 (2.55 mg/ml protein). Track (1) no added histone VS, 60 seconds incubation, 30°C; (2) no added histone VS, 2 minutes incubation, 30°C; (3) 100 µg histone VS added, 60 seconds incubation, 30°C; (4) 100 µg histone VS added, 60 seconds incubation, 30°C; (5) 100 µg histone VS added, 2 minutes incubation, 30°C; (6) 100 µg histone VS added, 2 minutes incubation, 30°C; (7) 100 µg histone VS added, 5 minutes incubation, 30°C.

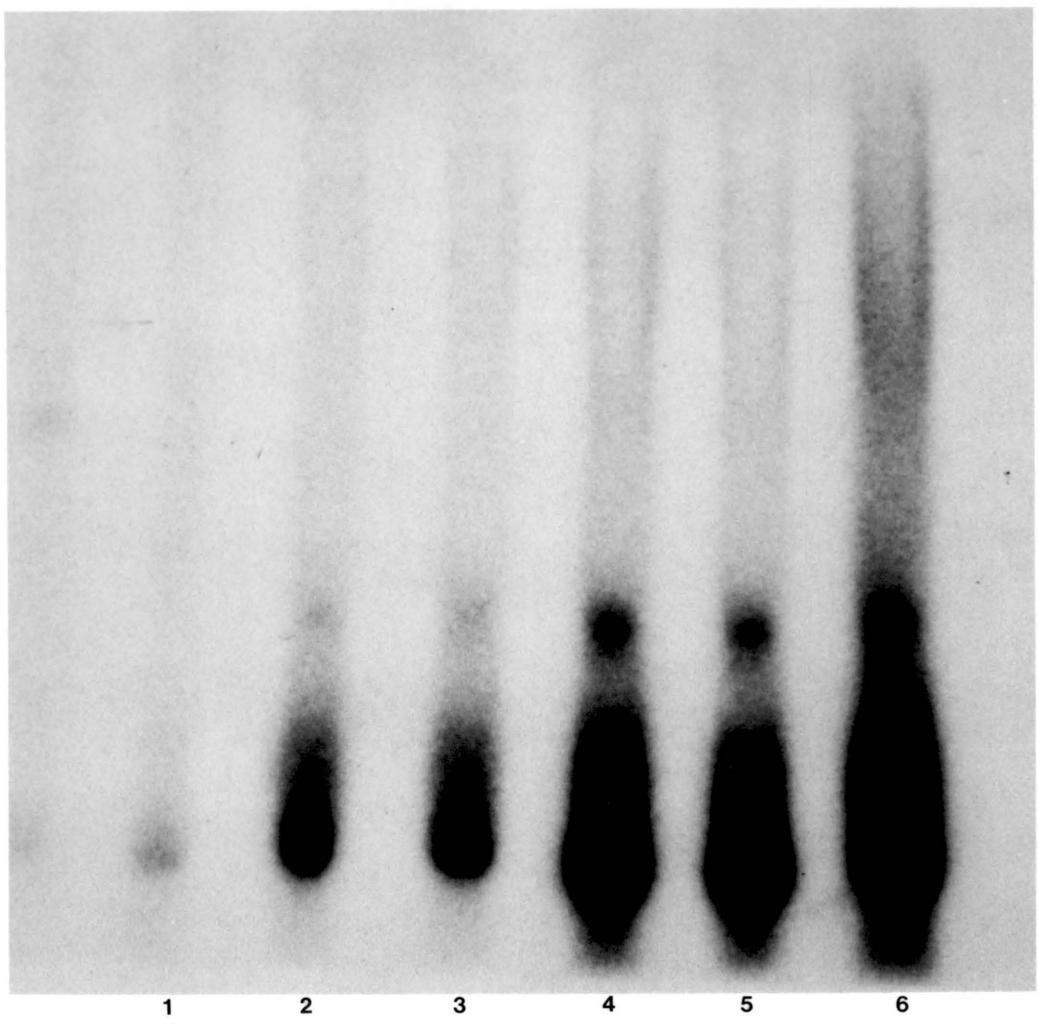
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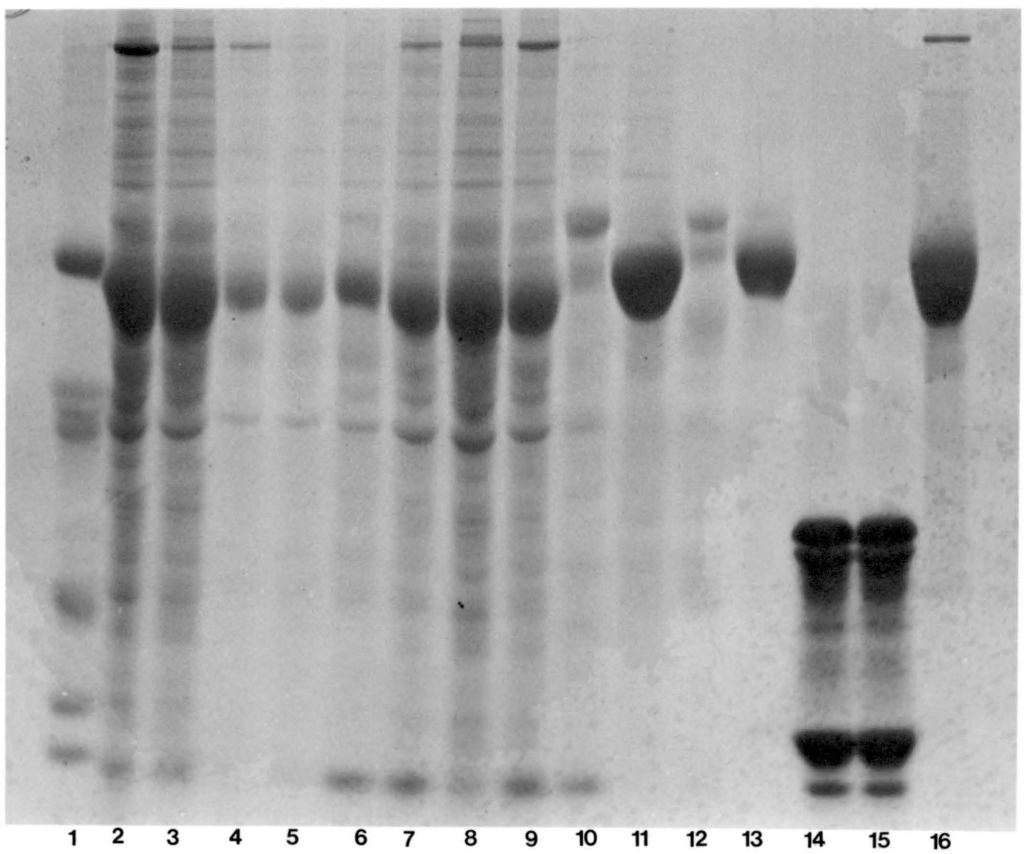
FIGURE 48

$NaDODSO_4$ polyacrylamide gel electrophoresis of malignant and benign mammary tumor extracts and holoenzyme preparations. Track (1) molecular weight markers; 30,000 x g cytosolic fractions; (2) FA-1258; (3) FA-3070; (4) FA-2752; (5) FA-1700; (6) IDC-3669; (7) IDC-147; (8) IDC-375; (9) IDC-733; holoenzyme preparations from DEAE-Sephacel, (10) FA-137 type I; (11) FA-137 type II; (12) IDC-375 type II; (13) IDC-3669 type II; (14) histone VS; (15) histone VS; (16) FA-137 type II catalytic subunit.

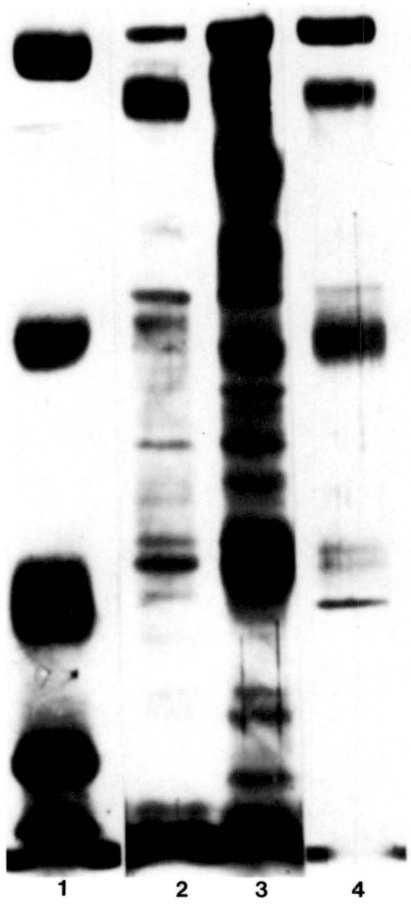

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

catalytic and regulatory subunits was done using cAMP-agarose affinity chromatography.

DEAE-Sephacel chromatography of malignant and benign human mammary cytosolic fractions resulted in the loss of several high and low molecular weight proteins (Figure 48, lanes 10-13; Figure 49, lanes 1 and 3). Partial purification of the histone kinase types I and II catalytic subunits by cAMP-agarose affinity chromatography also eliminated several peptides as seen by polyacrylamide gel electrophoresis in one dimension using a silver stain technique for visualization of peptide bands (Figure 48, lanes 2 and 4).

FIGURE 49

$NaDODSO_4$ polyacrylamide gel electrophoresis of malignant and benign human mammary tumor holoenzyme and catalytic preparations. Lane 1, molecular weight markers; 2, FA-137 type II catalytic subunit; 3, FA-137 type II holoenzyme; 4, IDC-375 type II catalytic subunit. Peptide bands were stained using the silver stain procedure described by Merril et al. (1980).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4. DISCUSSION

4.1. Statement of the Principle

That the degree of neoplastic transformation should be directly paralleled by alterations in the pattern of metabolic enzymes is the basis of the "molecular correlation concept" proposed by Weber and Lea (1967) in an attempt to interrelate the process of tumorigenesis and aberrations associated with intermediary metabolism at the time of tumor development. Applicability of this concept has been demonstrated for key enzymes and opposing pathways of carbohydrate, pyrimidine, DNA, and ornithine metabolism (Weber, 1973). The application of such a conceptual and experimental approach to the cyclic AMP system seems relevant in light of its potential role in the regulation of cell proliferation. In terms of the molecular foundation for cancer, changes in enzyme patterning would reflect adaptation of the neoplastic cell to its biochemical requirements, thus conferring to it a biological advantage as opposed to cells which constitute surrounding nontumorigenic tissues. Deviation in cellular metabolism resulting in a situation of imbalance does not necessarily represent a disordered, but rather an altered expression of the genetic potential, particularly if malignant transformation is viewed as a disease of differentiation. Consequently, it may be anticipated that various facets of gene expression such as relative cell growth rate and metabolic patterning may be linked.

In evaluating the pathological status of a tumor lesion, knowledge of the biochemical controls which favor cell proliferation and the loss of normal growth characteristics are fundamental to defining the nature of the neoplastic state. In this context, individual human mammary tumors, as well as separate loci within individual mammary tumors, have been partially characterized in terms of adenylate cyclase and cyclic AMP-dependent protein kinase activities in this study. The integrating component between these dual enzymes is cyclic AMP, whose cellular levels are balanced by synthesis by adenylate cyclase and degradation by cyclic AMP phosphodiesterase. A valid elucidation of cyclic AMP metabolism in neoplastic tissue using a system which resembles the clinical progression of cellular transformation is important. Therefore, two tumor classes were examined; infiltrating ductal carcinoma (malignant) and fibroadenoma (benign) which demonstrate differences in the degree of transformation, and consequently, biological behavior. Any deviations noted in activity levels or responsiveness to enzyme modifiers may be indicative of metabolic change either directly or indirectly associated with progression of the cancerous condition.

Human breast cancer appears to be the result of a multistage carcinogenic process (Brennan, 1975). The Berenblum hypothesis (1959); a well-known theoretical model of tumorigenesis that depicts the oncogenic process as a two-step mechanism, i.e. initiation, followed by promotion, represents a scheme in which the action of hormones important in both development and growth of mammary tumors may be

understood. Initial dependency of tumor growth on hormonal stimulation is an example of a critical component of the second stage. Removal of the hormone, or source of hormone by ablation, results in tumor regression until progression towards tumor autonomy is complete, after which hormone therapy has no influence on tumor growth or neoplastic character. Tumor regression in MTW9 rat mammary adenocarcinoma has been shown to occur not only because cell replication is arrested, but also due to increased susceptibility of cytosolic proteins to proteolysis (Rouleau and Gullino, 1977). Variation in hormonal response has been demonstrated in four human breast cancer cell lines, as well as in various human tumors, and involves differences in hormone-receptor affinity, binding patterns, and in the post-binding response (Osborne et al., 1978). Alterations in cell membrane structure observed during cell transformation events suggests that alteration in adenylate cyclase activity levels or aberrant modulation of hormone stimulation through alterations of receptor function or the transduction mechanism in neoplastic tissue, thus suppressing the intracellular transmission of information at the cell membrane through the second messenger system, or else permitting abnormal signaling effects to occur.

Secondary female mammalian reproductive hormone targets, such as the breast, are dependent upon interaction at the level of the hypothalamus, pituitary, and ovary in a complex integrated manner that reflects not only the level and duration of stimuli, but also the

responsiveness of the tissue and interactions between cell types. Under normal circumstances, the mammary gland is influenced predominantly by estrogen, progesterone, and prolactin. The complex regulation of estrogens by gonadotropin production is central to the control of ovarian function, and therefore, the secretion of progesterone. Studies focusing on the role of prolactin and estrogen receptor levels in mammary gland explants and human breast cancer cell lines such as MCF-7 demonstrated an association with N^6, O^2-dibutyryl cyclic AMP and insulin-induced stimulation of DNA synthesis (Shafie and Brooks, 1972).

Fifty percent of primary breast cancer lesions in women are positive for estrogen receptors (ER), which allows for predictions to be made concerning estrogen and androgen responsiveness, and prolactin dependency. Normal breast tissue and benign breast lesions characteristically lack the cytosolic receptor protein which appears to be induced in neoplastic cells during mammary carcinogenesis. Non-tumorigenic cells do not contain free receptor in large amounts or fail to demonstrate endrocrinologic growth stimulation (Brennan, 1975). Regression of advanced breast cancer lesions following bilateral oophorectomy or after administration of relatively high doses of estrogens during the postmenopausal state is proposed to be associated with a decrease in prolactin secretion and number of prolactin receptors (Stoll, 1969). Recent work with induction of rat mammary carcinoma by 7,12-dimethylbenz(a)anthracene (DMBA) demonstrated that B_2cAMP mimics the effects of oophorectomy, and may be coupled with

phosphorylation of a new, regression-associated nonhistone protein species which becomes the predominant substrate of cyclic AMP-dependent protein kinase in the tumor nuclei (Cho-Chung, 1977). With respect to the effect elicited by other hormones potentially involved in breast tissue development, considerable controversy exists over the role of thyroid hormones, insulin, prostaglandins, glucocorticoids, and additional pituitary hormones in the clinical course of human breast cancer.

Although relatively few studies have been made concerning hormonal regulation of cyclic nucleotide metabolism in neoplastic mammary cells, recent observations suggest that estrogen, insulin, and prolactin may be required to suppress cAMP levels in dimethylbenz(a)anthracene (DMBA)-induced mammary tumors (Matusik and Hilf, 1976; Cho-Chung et al., 1978). Ovariectomy of rats bearing DMBA-induced mammary tumors resulted in elevated cAMP levels, increased cAMP binding, and increased AC activity in tumors. This effect was reversed by estrogen administration. The relationship between estrogen and prolactin in tumor development is complex and compounded by observations that prolactin may stimulate the rate of prostaglandin synthesis (Rillema, 1975). Prostaglandins may play a role in the regulation of cell proliferation and differentiation. Furthermore, most actions of prostaglandins appear to be mediated through some aspect of cyclic nucleotide metabolism. Elevated levels of prostaglandins have been observed in DMBA-induced mammary tumors of rat and in human mammary tumors (Tan et al., 1974). In addition, inhibition of the growth rate of human

cancer cell lines by prostaglandins has been demonstrated (Jaffe and Santoro, 1977). Stimulation of AC activity by PGE_1 has been shown in murine mammary carcinoma and in Walker 256 mammary carcinoma cells (Cho-Chung et al., 1977). Little knowledge of how prostaglandins function in either normal or neoplastic mammary cells is available, consequently, the interaction between prostaglandins and cyclic nucleotides in the regulation of the mammary cell cycle remains obscure.

Intracellular redistribution of cAMP-binding proteins and protein kinase stimulated by cAMP may be a key feature to cAMP-responsive growth arrest of neoplastic cells (Cho-Chung et al., 1978). Present evidence indicates cAMP promotion of nuclear accumulation of these macromolecules occurs in much the same manner as steroid hormones promote nuclear accumulation of receptor proteins. In mammary tumors regressing due to estrogen withdrawal, the increase in cAMP-binding activity is accompanied by increased histone kinase activity, and increased levels of cAMP, as well as adenylate cyclase activity (Cho-Chung et al., 1977). An inverse relationship appears to exist between the action of cAMP and steroid hormones in the growth control of hormone-dependent mammary carcinoma in which the integrity of both cAMP-binding proteins and hormone receptors is essential (Liv and Greengard, 1976; Matusik and Hilf, 1976). The presence of integral cAMP-binding components in estrogen receptor deficient mammary tumors can be associated with autonomous growth, as can the reverse of this situation, integral estrogen-binding components in cAMP-binding

protein deficient tumors. Further clarification of this interaction is required in order to understand the hormonal control of mammary tumor growth.

Another consideration to be made when attempting to resolve the differences between malignant and benign tumors at the biochemical level are those enzymes which occupy key positions in intermediary metabolism which respond to alterations in the biochemical state of the cell, as well as to physiological stimuli. A close interrelationship exists between the control of enzyme activity by covalent (i.e. phosphorylation-dephosphorylation) and noncovalent (allosteric effector) modification. As a generality, enzymes involved in biodegradative pathways are activated by phosphorylation, whereas enzymes involved in biosynthetic pathways are inactivated by the same process. In addition, regulation of gene expression by phosphorylation of histone and nonhistone chromosomal proteins is essential for integration of cellular metabolism. Whether phosphorylation of an enzyme or structural component of the cellular framework is ultimately expressed as a change in function is dependent upon intracellular effector and substrate concentrations which may amplify or suppress such phosphorylating effects. Furthermore, the existence of several protein kinases and protein phosphatases with multiple actions allows for synchronization of metabolic pathways as well as the independent control of various cell functions. The examination of cAMP-dependent protein kinase activity in human mammary tumors could provide an index for the phosphorylation potential of a tissue, as well as

indicate whether abnormal cellular behavior is associated with an alteration in the activity level, cAMP-dependency, or isoenzyme population of tumor-specific protein kinase.

Several studies suggest that most mammalian cytosolic protein kinases are stimulated by cAMP, and preferentially phosphorylate basic proteins such as histones *in vitro* (Walsh et al., 1968; Chen and Walsh, 1971; Majumber and Turkington, 1972). In contrast, nuclear protein kinases are generally unresponsive to cAMP and prefer acidic proteins such as casein or phosvitin as substrates *in vitro* (Takeda et al., 1971; Desjardins et al., 1975). Redistribution of protein kinase from the cytoplasm to nucleus has been observed to occur in rat liver following stimulation by B_2cAMP and glucagon (Castagna et al., 1975), in rat uterus with hormonal stimulation of adenylate cyclase (Bhalla et al., 1973), and in calf ovary in response to chorionic gonadotropins (Jungmann et al., 1974). This translocation process has also been demonstrated during mammary tumor regression following B_2cAMP treatment, so that a correlation exists between growth arrest *in vivo* and nuclear accumulation of protein kinase (Cho-Chung and Clair, 1977). This evidence, in combination with cell cycle specific changes in cAMP-dependent protein kinase activity previously discussed, suggests a definite role of cAMP-mediated phosphorylation in cellular transformation and tumorigenesis. This is strongly supported by a recent report that the viral *src* gene product, a tyrosine-specific protein kinase involved in cell transformation, shows definite homology with the catalytic chain of mammalian cAMP-

dependent protein kinase (Barke and Dayhoff, 1982).

Of central concern in this thesis was a comparison between enzyme profiles obtained from preparations of whole mammary tumor extracts and those derived from individual tumor tissue micro-sections where discrete, histologically-confirmed areas of neoplastic cells could be located and analyzed using microgram amounts of tissue. Such a coupled approach for analysis of enzyme activity was taken to provide an estimation of the contribution made by surrounding non-tumorigenic tissue. However, even in mammary tumor micro-sections, the exclusion of connective tissue elements was not entirely possible. Within benign tumors, cellular regions were more evenly distributed in stroma than observed in malignant lesions, thus tissue samples derived from benign tumors contained a higher percentage of connective tissue than did similar sections from malignant tumors. Due to a lower proportion of neoplastic cells in benign tumor sections, plus the possibility of stromal material contributing to tissue dry weight measurements, the actual differences noted in enzyme activity between malignant and benign tumors may have been greater than reported during some stages of this study.

The vast majority of cancerous lesions contain numerous sub-populations of cells. According to Fisher (1974), at least four different cancer cell subpopulations may exist within any one breast lesion. Such clones vary in metastatic potential, drug susceptibility, hormone responsiveness, production of biological markers, morphology and chromosome number. In addition, tumor cells are

genetically more unstable than normal cells, so that the potential for further subpopulation development is tremendous. These factors give two degrees of heterogeneity which need to be taken into account; first, at the level of the breast tissue itself, and secondly, at the level of tumor development. Such considerations make biochemical analysis of human breast tumors a difficult, albeit challenging task, and require a novel approach to assess differences associated with increasing degree of neoplastic transformation. If transformation does reflect the abnormal expression of genomic function, then the potential difference between individual breast lesions is enormous, and necessitates that each breast tumor as a discrete unit be evaluated. The microtechniques developed and applied in this study would allow for biochemical analyses to be made using breast tumor samples in a tissue dry weight range obtainable through needle biopsy of cancerous breast tissue.

4.2. Interpretation of the Data

4.2.1. Adenylate Cyclase in Neoplastic Mammary Tissue: The physiological properties of tumor particle, as well as tumor micro-section adenylate cyclase from neoplastic human mammary tissue in the presence of Mn-ATP or Mg-ATP as the substrate were similar to enzyme characteristics previously described from other sources of mammalian tissue in terms of pH optimum, divalent cation requirement and kinetic properties. Such tumor preparations also possessed low levels of cyclic nucleotide phosphodiesterase and adenosine triphosphatase activity.

Cyclic AMP production was expressed in terms of tumor tissue dry weight, rather than on the basis of extractable protein or cellular DNA content. This allowed for direct comparison of adenylate cyclase activity levels to be made between tumor particle preparations and specific loci within individual breast tumors. It was felt that estimation of activity levels in particle fractions on a protein basis might be biased by contribution of surrounding and infiltrating connective, vascular, and adipose tissue elements. Contamination by non-tumorigenic components was largely avoidable using the tissue microdissection procedure. Likewise, estimation of enzyme activity on the basis of cellular DNA would reflect differences in tissue cellularity between malignant and benign lesions, as well as emphasize the potential for neoplastic cell heteroploidy.

In the series of malignant and benign mammary tumors examined, the potential for cAMP synthesis was 5 to 10-fold higher in benign than malignant particle fractions when measured in the presence of Mn-ATP to allow maximum activity in vitro. That the two tumor classes had distinct levels of adenylate cyclase activity is supported by the fact that the students "t" test showed the differences between malignant and benign enzyme activities to have a $t_{(20)}$ value of 5.36 ($p < .001$).

The reduction in the cAMP synthetic potential in malignant tumor tissue would be consistent with the apparent inverse relationship proposed to exist between cAMP levels and cell proliferation (see review by Ryan and Heidrick, 1974; Matusik and Hilf, 1976). It is

thought that tissues with a high metastatic potential and rate of division demonstrate lower cAMP levels and adenylate cyclase activity than those tissue considered normal or having a low metastatic potential and growth rate. As previously stated, this relationship is not clear, for conflicting reports indicate elevated cAMP levels in metastatic rat mammary carcinoma (Chatterjee and Kim, 1975) and in human malignant breast tumors (Minton et al., 1976).

In this study, analysis of microgram quantities of human breast tissue indicated that similar levels of cAMP occurred in both malignant and benign tumors using a sensitive radioimmunoassay. It must be considered that the levels of endogenous cAMP may not reflect the actual in vivo concentrations present in mammary tumor tissue due to such intrinsic factors as rapid turnover of cAMP in the tissue or extrinsic factors such as loss during the extraction procedure. It seems to be questionable whether cAMP levels might reflect the similarity in tumor growth patterns, and not necessarily be a function of the degree of transformation. It is also possible that other enzymes which regulate cellular cAMP levels act in a different manner in malignant and benign breast tumor tissue. Malignant breast tumors have been observed to show an overall decrease in activity of cAMP-phosphodiesterase (Chatterjee and Kim, 1975; Cohen et al., 1976), although levels of the low Km isoenzyme have been reported to be elevated (Singer et al., 1976; Gutmann and Rutherford, 1982). In this study, the validity of the microassay procedures for cAMP measurement and evaluation of AC activity has been demonstrated. The partial characterization of enzyme activity emphasizes the usefulness of this microtechnique in studying

neoplastic tissue.

Calcium at micromolar concentrations was observed to suppress tumor-associated adenylate cyclase in both particle preparations and tumor micro-sections from malignant and benign tumors. This inhibitory effect may have reflected competition of Ca^{2+} for divalent cation binding sites normally occupied by Mg^{2+} or Mn^{2+} on the catalytic subunit. It was of interest that no calcium effects associated with calmodulin were detected using a concentration range of added calmodulin which readily induced a response with rat brain adenylate cyclase or human breast tumor-derived cyclic nucleotide phosphodiesterase (Cheung, 1971).

Activation of adenylate cyclase from benign tumor extracts by the non-hydrolyzable GTP analog, Gpp(NH)p, in the presence of Mg-ATP or Mn-ATP was approximately two-fold higher than that observed using extracts from malignant tumors although the activation ratios determined for both tumor classes were well within the range found for activation of other mammalian kinases. This might suggest that the activation potential was greater in benign than malignant tumor tissue. Total cAMP production levels were also increased in benign tumor particle fractions compared to malignant tumor particle fractions in the presence of physiologically equivalent concentrations of Gpp(NH)p. If activation levels in benign mammary tissue resemble those obtainable in normal breast tissue, it is possible that the regulatory subunit of the malignant enzyme may be altered so as to partially suppress the stimulus response due to modified binding or transduction

of the stimulus to the catalytic subunit. Whether one or the other of these possibilities is valid remains to be discerned.

That GTPase activity for termination of the cyclase reaction which is proposed to be associated with the G/F regulatory subunit of the enzyme complex was functional in tumor derived adenylate cyclase was indirectly shown by the fact that preincubation of at least one malignant enzyme with activated cholera toxin enhanced GTP-activated AC. While differences were detected involving guanine nucleotide induction of tumor particle AC, fluoride activation was similar in both classes of mammary tumors, and gave 40 to 50% higher cAMP synthesis in vitro than observed with Gpp(NH)p.

The hormonal responsiveness of breast tumor particle AC was very complex in that each individual lesion was distinct in its response to the various hormones tested. Both tumor classes demonstrated significant variation. The lack of responsiveness in some cases could have been due to previous saturation of hormone receptors. Since only a single concentration of guanine nucleotide:hormone combination was used in this study, it is very possible that higher levels or different combinations may have allowed more response on behalf of the tumor-derived adenylate cyclase activities examined. The most noted positive effectors included PGE_1, TSH, and glucagon, although the actual implications of such stimulation is as yet unknown. Of particular interest was the recognition and positive response of malignant tumor AC to PGE_1 since interaction of this substance with cyclic nucleotides has been implicated in cell growth regulation. The actual increase in cAMP synthesis following hormone treatment varied, not

being consistent between or within tumor classes. It was interesting to note that a definite negative response of adenylate cyclase to luteotropic and lactogenic hormones occurred. Hormone receptors for hCG and prolactin have been shown to occur in human breast tissue, however, recent evidence indicates that prolactin binding to the cell membrane induces the release of an additional protein species, so that the intracellular transmission of the stimulus may not directly involve cAMP (Teyssot et al., 1982).

Both malignant and benign tumor-associated AC appear to be rather thermal labile over relatively short periods of incubation at 37°C. In all cases studied, stabilization of enzyme function was attainable by preincubation with Gpp(NH)p or fluoride. The guanine nucleotide was demonstrated to increase the thermal stability of the benign preparation significantly, and the malignant preparation to a lesser extent. Another difference observed was that the capacity for guanine nucleotide, but not fluoride activation was permanently lost in the malignant enzyme examined. The capacity for benign tumor-associated AC induction by Gpp(NH)p could be restored. This difference might suggest that the regulatory subunit in the benign AC was more thermal stable than that from malignant tissue.

The variation in AC activity across individual tumor micro-sections from both malignant and benign tumors supported the idea that different cell subpopulations existed within the cancer lesions examined. It became necessary to average values obtained from several neoplastic loci within a tumor micro-section in order to accurately estimate the

potential for cAMP synthesis within that specimen. Specific activity levels in lyophilized tumor sections were found to be approximately 100-fold higher than measured in tumor particle preparations. Several reasons may account for this discrepancy, including (1) partial inactivation of AC, release of AC inhibitors, or proteolytic degradation of AC due to the tissue disruption and washing steps, (2) loss of soluble AC activity, or loosely-bound AC from washed particle preparations which were measureable during tumor micro-section analysis, or (3) more precise estimation of specific activity levels based upon direct dry weight measurement.

Previously noted differences in AC activity levels between malignant and benign tumors disappeared upon evaluation of AC activity in tumor tissue micro-sections where definite masses of histologically-confirmed neoplastic cells could be isolated and analyzed. Basal, as well as Gpp(NH)p-stimulated cyclase activity was similar for both tumor classes, as were the activity ratios. In fact, the degree of AC stimulation by guanine nucleotide varied only slightly between tumor types, giving values comparable to findings reported for other human tissues and gram weight samples of material (Hunt et al., 1978; Matsukura et al., 1977; Stolc, 1977). However, even in these studies, wide differences in both basal and stimulated AC values were observed, again reflecting the variation possible when comparing individual tumors. If it is true that differences such as these partially account for the complexity of the second messenger system in neoplasia and allow for the contradictory experimental results observed, this

only further necessitates evaluation of individual tumors for generating useful enzyme profile patterns.

Using tumor micro-section analysis of cAMP production, again, variability in hormonal responsiveness was observed. The overall trend remained for positive induction by PGE_1 and TSH, however, in contrast to observations using tumor particle preparations, a positive response to prolactin was noted which lends doubt as to the stability of prolactin receptors during preparation of the tumor washed particle preparations. Variable responses were observed between tumors of a single pathological status, and between tumor classes, most probably reflecting discrete subpopulations of tumor cells within lesions.

Consistent with observations previously made, malignant and benign tumor micro-section AC also demonstrated thermal lability which could be partially prevented by preincubation with Gpp(NH)p. In an evaluation of enzyme partitioning in tumor micro-sections, it was found that soluble AC activity from benign tissue was greater on a percentage basis than that from similarly-treated malignant tissue, possible indicating a difference in membrane-association of adenylate cyclase activity.

4.2.2. Protein Kinase Activity in Neoplastic Mammary Tissue: The analysis of breast tumor protein kinase was initiated to provide a basis for further detailed studies of human protein phosphorylating systems. Protein phosphorylation regulated by cAMP-dependent protein kinases (PK) is related to the regulation of cell growth (Kletzien,

Miller and Pardee, 1977). Furthermore, the amount of cellular holoenzyme is under hormonal control in normal as well as in neoplastic tissue (Marvaldi et al., 1979). It is postulated that increased endogenous phosphorylation and enhanced PK activity may relate to the maintenance of the proliferative state during carcinogenesis (Raul et al., 1981; Eppenberger et al., 1976). Bechtel et al. (1978) demonstrated that specific PK activity (pmol ^{32}P incorporated/min/mg protein) was 2.5-fold higher in primary carcinoma than normal or dysplastic breast tissue, but 3 to 4-fold lower when expressed in terms of cellular density. A cytosolic cAMP-dependent PK which plays a central role in proliferation and differentiation in mouse mammary tissue and was induced in response to prolactin, mediated the action of the hormone by phosphorylation of nuclear histones as well as specific membrane proteins (Majumder, 1977). In this example, depressed levels of PK may have reflected the precancerous condition. Specific activities of the holoenzyme, catalytic and regulatory subunits, expressed in terms of cellular DNA, were markedly lower in breast carcinoma than normal mammary tissue, in addition to increased holoenzyme dissociation and decreased cAMP responsiveness. It becomes apparent that additional data on mammary tumor protein kinase is imperative for valid biochemical assessment of the phosphorylating potential of neoplastic tissue.

The physicochemical properties of cAMP-dependent protein kinase isolated from several human breast cancer lesions are similar to those characteristics described for other mammalian protein kinases.

Subcellular fractionation of breast tumor homogenates indicated a predominance of phosphotransferase activity in the cytosolic fraction, with approximately 10 to 20% of the activity being membrane-bound. Catalytic activity was thermal-stable over at least a 30 minute period, as was the capacity for enzyme activation by micromolar concentrations of cAMP. Protein kinase activity exhibited a rather broad pH optimum around 8.

Previous characterization of human breast tumor protein kinase by Bechtel et al. (1978) was done using protamine, a sperm-specific, basic protein, as the phosphate acceptor, whereas the presently described studies indicated the capacity for protamine phosphorylation was quite limited. Since cell-cycle-dependent change leading to characteristics of neoplasia in tumor cells reflects cellular reprogramming at the genomic level, it was of interest to examine the phosphorylating potential using nucleosomal and extranucleosomal histones. Regulation of gene transcription by controlling potentially active genome regions through repression/derepression (blocking/deblocking) mechanisms is a function designated to histone proteins and may be the basis for irreversible committment to particular growth characteristics demonstrated by malignantly-transformed cells. Since malignant transformation is considered to be a disease of differentiation, data on histone phosphorylation in tumor tissue is relevant in terms of understanding gene regulation in neoplastic tissue.

In this study, mammary tumor-associated cAMP-dependent protein kinase demonstrated a definite preference for histone VS. Kinetics

of histone VS phosphorylation were similar to those reported for histone kinase in other mammalian systems. According to the nomenclature of Bradbury (1975) histone VS represents the histone subgroup f1, and is analogous to histone H1. This histone is lysine-rich, extranucleosomal, with a molecular weight of approximately 21,000 daltons. It shows DNA-independent synthesis, is continuously replaced in the cell, occurs in the spacer region between nucleosomes, and is proposed to function in the packing of nucleosomes into the second order supercoil.

In addition to phosphorylation of histone VS, casein phosphorylation was also relatively high, but was determined to be cAMP-independent and will be discussed shortly. Phosphorylation of histone preparations IIA, IIIS, VIS, and VIIIS was 10 to 50% that observed with histone VS. These histone proteins represent the four major nucleosomal core histones H2a, H2b, H3, and H4; with H2a and H2b being slightly lysine-rich and H3/H4 being arginie-rich.

The most extreme variation among histone proteins occurs in the lysine-rich H1 group which may show up to five subfractions (isohistones) possessing different electrophoretic mobility and amino acid composition. In contrast to the nucleosomal histones, expressed heterogeneity in H1 is both species and tissue-specific, with differences occurring in the relative content of the different subfractions. Although no major differences in patterns of nucleosomal histones have been reported in normal and malignant tissues, quantitative differences have been observed to occur in H1 subfractions in

tumors (Hohmann et al., 1971; Hnilica et al., 1963; Desai et al., 1969). Differences in multiplicity and microheterogeneity of H1 subfractions in mouse mammary tumors is due to phosphorylation and shows a strong correlation with the rate of tumor growth (Sluyser, 1977; Balhorn et al., 1972). The disappearance of one H1 subfraction has been shown to be negatively correlated with the growth rate of rat hepatomas and may be related to malignant transformation (Lea et al., 1975).

Studies indicate the highest levels of phosphorylation to occur in groups of lysine-rich histones, specifically H1 and H2a. The arginine-rich histones show only transient cell-cycle-specific phosphorylation. The most complex pattern of phosphorylation is found in group H1 which is subjected to three types, (1) phosphorylation of one serine residue in the C terminus following synthesis, (2) phosphorylation of at least four sites in both terminal halves during mitosis, and (3) phosphorylation of one serine residue in the N terminal fragment related to hormonal stimulation. The first two types represent growth-related functions and are cAMP-independent, whereas the third type is hormone-related and cAMP-dependent function. It seems that tissue-specific patterns of H1 group expression due to phosphorylation is based upon replacement of serine residues by alanine at the major site of hormone-related phosphorylation at the N-terminus of the molecule. Probable functions of nucleoprotein phosphorylation are related to three processes: (a) correct deposition of histones on replicating chromatin

(H1, H4), (b) condensation and stabilization of chromatin during mitosis (H1), and (c) regulation of transcription (SER-37, H1; H2a, H2b, H3, H4).

Data on histone phosphorylation are not sufficient to draw final conclusions on the interrelationship between post-translational modification and the rate of RNA transcription. However, evidence supporting a correlation between phosphorylation of H1, change in conformation of the histone core, derepression of specific chromatin regions to allow active RNA transcription following hormonal stimulation has been presented (Lamy, Lecocq and Dumont, 1977).

Analysis of the histone VS preparation by SDS polyacrylamide slab gel electrophoresis indicated the presence of three major peptide bands, each of which was phosphorylated by human breast tumor cytosolic fractions in the presence of (^{32}P)-ATP. In addition to these major subfractions, at least eight other peptide bands were resolved which appeared to be phosphorylated to varying degrees. Unfortunately due to the large net positive charge on histones, these proteins often run abnormally on SDS gels and fail to exhibit the usual relationship between mobility and molecular weight (Panyimet et al., 1971). Estimated molecular weight values for the histone VS subfractions ranged from 15,600 to 28,000 daltons.

Specific criteria for classification of protein kinase activity has been described (Traugh, Ashby, and Walsh, 1979). In experimental situations, three categories of PK are recognized; type I, holoenzyme (RC), which is equivalent to cAMP-dependent PK; type II (C), which

includes free catalytic subunit; and type III which includes additional protein kinases which are not regulated by cAMP. It should be noted that free catalytic subunit is not stimulated by cAMP, nor does it bind cyclic nucleotides, but does show inhibition by regulatory subunit in the absence of cAMP and PK inhibitors in the presence or absence of cAMP. Type III kinase, exemplified by casein kinase, demonstrates a negative response to all four criteria.

Four characteristics used to classify a protein kinase as being cAMP-dependent include (1) stimulation of the enzyme by cAMP, (2) the ability to bind cAMP, (3) inhibition by free regulatory subunit, and (4) inhibition by heat-stable inhibitor proteins. Each of these characteristics has been met by the breast tumor derived histone kinase activity described.

The degree of activation of PK depends upon the cAMP concentration, amounts of regulatory and catalytic subunits, the nature and concentration of substrate, and ionic strength. In this study, a relatively low ionic strength buffer was used so as to prevent error in determination of type I and type II holoenzyme activity ratios. Activation of mammary tumor histone kinase occurred using micromolar concentrations of cAMP in a dose-dependent manner. The activity ratio increased as kinase activity was resolved by DEAE-Sephacel chromatography. The K_a value for cAMP was estimated to range from 600 to 800 nM in tumor cytosolic fractions, but decreased ten-fold following anion exchange chromatography indicating that holoenzyme preparations did possess a high affinity for cAMP. Activation

constants for cAMP in partially-purified mammalian cAMP-dependent protein kinase generally range from 15 to 350 nM (Hofman, 1978). Other cyclic nucleotides were shown to partially activate tumor histone kinase, although at much higher concentrations.

The range of histone kinase activity was similar in cytosolic preparations from benign and malignant tumors examined and were close to activity levels reported by Bechtel et al. (1977) using protamine as the phosphate acceptor. Examination of cAMP-dependent histone kinase in a limited number of tumor micro-sections revealed a difference in the levels of phosphotransferase activity, with that resolved from benign tumors being approximately two-fold higher than activity from malignant tumors, on a dry weight basis [$t(18) = 2.51$; ($p < 0.0250$].

Activity ratios determined with cAMP were higher in malignant than benign tumor cytosolic fractions. This observation could be explained in several ways. Free catalytic subunit is generated in tissues in response to hormonal stimulation, or may be dissociated from the holoenzyme during manipulations of tissue extractions and partial purification. On the other hand, malignant histone kinase may demonstrate a decreased affinity for cAMP binding, or decrease in its activation capacity following cAMP binding to the regulatory subunit. It did appear that the cAMP binding capacity of partially-purified histone kinase from malignant and benign tumors was nearly equivalent, therefore an attempt was later made to probe the catalytic subunit for functional defects.

Inhibition of tumor-derived histone kinase activity by heat-stable inhibitors of protein kinase extracted from bovine heart and rabbit skeletal muslce was observed, with inhibition curves being similar for both types of inhibitors. Inhibition of kinase activity by adenosine, ADP, and 5'AMP was the same in the presence or absence of cAMP indicating that this effect was accountable by the binding of the inhibitor to the catalytic site.

Relative to normal tissue, most tumor systems studied have shown decreased cAMP-dependent protein kinase activity which also shows a correlation with decreased cAMP-binding activity (Granner, 1972; Riou et al., 1977; Eppenberger et al., 1976). Neoplastic tissue, both of murine and human mammary origin, and particularly cAMP-unresponsive tumors, may be defective in catalytic activity, with a decreased affinity for ATP (Cho-Chung et al., 1977) or in association with regulatory subunit activity. Observations supporting the latter categorize the enzyme system defect in terms of (1) decreased affinity of binding proteins for cAMP, (2) decreased responsiveness to cAMP, or (3) decreased intracellular amounts of cAMP binding protein (Majumder, 1977; Cho-Chung et al., 1977; Steinberg et al., 1977; Lasser and Daniel, 1978). It is interesting that tissue culture lines of Walker carcinoma cells having different degrees of resistance to the cytotoxic effects of alkylating agents and dibutyryl cAMP show both quantitative and qualitative differences in cAMP-binding protein (Tisdale and Phillips, 1976). Such alterations seen included affinity for cAMP, pH optimum and stability for cAMP binding, and temperature stability.

Cyclic AMP-binding activity from human mammary tumors was determined to be in the range found for other partially purified binding proteins studied in mammalian tissues (Granner, 1974; MacKenzie and Stellwagen, 1974). No difference in affinity was observed between the two tumor classes. No evidence of two different affinity cAMP binding proteins was found using different pH values in the assay, as has been reported in neoplastic tissue (Sharma et al., 1975). The apparent K_b values for cytosolic cAMP-binding proteins determined for human mammary tumors in this study indicated a lower affinity for cAMP in comparison to binding values reported for high affinity cAMP binding sites in other tumor systems (Nambu and Terayama, 1976; Cho-Chung et al., 1977). Loss of the high affinity cAMP binding sites from tumor cytosolic extracts has been observed in neoplastic tissue (Granner, 1974). The relatively low affinity of binding protein for cAMP observed in the present study was probably not due to the presence of a specific binding inhibitor since no such activity was detectable in the various malignant and benign tumors examined.

Cyclic AMP binding activity in breast tumor cytosolic fractions was shown to be specific for cAMP, and was slightly higher in malignant than benign tumors examined. However, whether such activity levels reflected cAMP binding to the regulatory subunit of cAMP-dependent PK, and also binding to free cytosolic cAMP binding proteins was unknown. Binding activity was demonstrated to co-elute with cAMP-dependent histone kinase activity using DEAE-Sephacel chromatography. Upon

this observation, it was assumed that cAMP binding activity to the resolved enzyme did actually represent cAMP association with the regulatory subunit.

Soluble fractions from benign and malignant mammary tumors were shown to contain both cAMP-dependent histone kinase type I and II as evidenced by their behavior on DEAE-Sephacel, dissociation patterns in the presence of 0.5 M NaCl, and inhibition by specific heat-stable inhibitors in a dose-related manner. The occurrence and ratios of protein kinases classified indicated that the type II holoenzyme was the predominant isoenzyme in the human breast tumor system. Activity ratios measured for PK type I were less than for PKII, but may have resulted from partial dissociation of the type I holoenzyme during elution with the salt gradient. These results are similar to those reported by Majumder (1977) that two peaks of protein kinase activity were resolvable by DEAE-cellulose chromatography of cytosolic preparations from rat mammary carcinoma. The type I and II kinases were activated 4-fold and 8-fold, respectively, by micromolar concentrations of cAMP. According to Cho-Chung et al. (1977), DE-52 cellulose chromatography of cytosolic extracts from Walker 256 mammary carcinoma gave a single major peak of kinase activity, representing 80% of the total kinase activity resolved, which showed characteristics of protein kinase type II as described by Hofman et al. (1975).

An attempt was made to isolate the regulatory subunit from tumor-derived types I and II histone kinase by cAMP agarose affinity

chromatography. Only in one case was cAMP-binding activity recoverable following elution with cAMP. Proteins eluted by 7 M urea failed to demonstrate binding activity or to inhibit protein kinase activity when added to isolated catalytic subunit. Elution with cGMP was not found to selectively displace the regulatory subunit either at room temperature or after incubation at 30°C.

Reconstitution of isolated regulatory and catalytic subunits is a complex function of the concentrations of both proteins, ionic strength, and the presence of substrate, Mg-ATP and cAMP (Hofmann, 1978). In addition, dialysis of concentrated subunits preparations prior to reconstitution attempts is necessary since the presence of NaCl may limit reassociation. Isolated mammary tumor-derived type II catalytic subunit successfully reassociated with regulatory subunit extracted from bovine heart tissue. Catalytic activity was readily inhibited following addition of this heterologous subunit in the absence of cAMP and without preincubation with Mg-ATP in all but one tumor derived enzyme examined. One species of type II catalytic subunit possessed reassociation characteristics typical of a type I enzyme. In this case, reconstitution of the holoenzyme was possible only by preincubation of the catalytic and regulatory subunits in the presence of Mg-ATP prior to addition of the substrate and (^{32}P)-ATP. Even despite this discrepancy, the DEAE-Sephacel elution profile, dissociation, and inhibition properties indicated the enzyme to be a type II kinase.

One important consideration in this study was stabilization of the holoenzyme regulatory and catalytic fractions during homogenization

and tissue preparation for assay. It was necessary to incorporate 5 mM benzamidine in the homogenization and column buffers to inhibit proteolytic degradation of the kinase. Despite this, and the fact that all steps were carried out at temperatures from 4 to 10°C, (with exception of elution of the regulatory subunit from cAMP-agarose) in the presence of reducing agents such as dithiothreitol or 2-mercaptoethanol, lability of both holoenzyme and subunit preparations was a difficult problem to overcome. It was also not possible to recover a heat-stable inhibitor of protein kinase from either malignant or benign mammary tumors such as those described for numerous other mammalian tissue, even though there were indications of inhibitory activity in tumor homogenates.

Chromatographic analysis of casein kinase activity was made from soluble extracts of mammary breast tumors. The elution profile of casein phosphotransferase activity was resolved as a single peak eluting at 0.3 M NaCl following elution of histone kinase type II from DEAE-Sephacel. This enzyme demonstrated a pH optimum at 7.5 and showed a distinct preference for casein as the phosphate acceptor. In contrast to histone kinase activity which showed linearity with incubation time for 5 to 10 minutes at 30°C, time-dependency of casein kinase activity was linear up to 15 minutes at 30°C. Catalytic activity was not suppressed in the presence of heat-stable PK inhibitors, nor did it show inhibition by cAMP-dependent protein kinase regulatory subunit. Tumor-derived casein kinase was not activated by cAMP and did not possess cAMP binding activity. Based upon these characters, this enzyme was classified as a type III kinase

according to the system of Traugh et al. (1979). The function of this enzyme is as yet unknown, although casein represents a major protein synthesized by mammary epithelial cells during lactation and would certainly be available as a substrate for phosphorylation.

Examination of the endogenous phosphorylation of malignant and benign mammary tumor cytosolic extract indicated that both cAMP-dependent and cAMP-independent mechanisms for phosphotransfer occurred. The phosphorylation of at least six peptide bands was enhanced in the presence of cAMP, whereas, it was noted that one band showed decreased phosphorylation in the presence of cAMP. No significant differences were detected between phosphorylation in the two tumor clases, however, further resolution of the peptide profiles by two-dimensional gel electrophoresis may reveal differences in protein phosphorylation.

4.3. Implications for Future Study

With divergent views regarding the presence of ordered and specific patterns of biochemical alterations in cancer cells, it becomes necessary to relate progression of the disease to some indicator of the gradation of malignancy. If aberrant biological behavior is based upon the establishment of malignant characters through alterations in enzymatic patterns expressed, then enzyme associated with cAMP metabolism may be important indicators of development of the cancerous state. Using a coupled approach such as the one described in this treatise by adapting microtechniques to analyze microgram samples of neoplastic tissue allowed comparisons to be made with whole tumor extracts. This technique may be most helpful in discriminating specific

tumor-associated biochemical events occurring with respect to the evaluation of tumor lesions. The potential for determining specific activity levels, as well as isoenzyme patterns using this coupled approach allowed for a sensitive resolution to problems often encountered in during enzyme analysis such as profile analysis, replicability, sample quantity, and selectivity within heterogeneous human tissues. Within the kinetic, biochemical and physical parameters defined in this study, a groundwork was laid for further study of hormone-responsiveness of breast tumor-associated adenylate cyclase, as well as the potential for cAMP synthesis, and mediation of its function by cAMP-dependent protein kinase. The results obtained in this study indicate that the second messenger system involving cAMP was intact in both malignant and benign human breast tissue.

Further analyses of human mammary tumor samples need to be done in order to validate the findings presented here for tumor populations large enough to be amenable to strict statistical analysis. The trends and confidence levels for similarities and differences noted between malignant and benign breast tumors are of interest and need further examination. Indeed, the idea that each breast cancer lesion is distinct in its biochemical characters need also be considered.

Treatment of neoplastic disease requires a knowledge of the molecular biology and biochemical pharmacology of differences between normal and cancerous cells. It is a tremendous step between data generated during _in vitro_ studies, and validation of this data _in vivo_. Only based upon such information will the control mechanisms

which regulate gene expression be understood in neoplastic tissue so that a rational design of clinical prophylactic or chemotherapeutic control of cancer be feasible.

5. SUMMARY

The application of microanalytical techniques was used to elucidate the enzyme activity profiles of adenylate cyclase and cyclic AMP-dependent protein kinase within a series of malignant and benign human mammary tumors. The described methods and their application allowed for utilization of visually confirmed areas of tumor involvement and eliminated the contribution from noninvolved contaminating cell types which is a problem often encountered due to breast tissue heterogeneity. Data generated from these procedures was compared to analyses using whole breast tumor particle and cytosolic fractions.

Various physical, kinetic, and biochemical properties were examined to test the hypothesis that the growth characteristics of neoplastic tissue may be due to the lack of a functional cAMP signal to suppress cellular proliferation. The potential for cAMP formation and the effector function of cAMP was examined in malignant and benign tissue to probe the cells for defects in the levels of enzyme activity, and to examine the extent and types of regulations occurring as demonstrated in a cell free system. If the appearance of tumor-specific isoenzymes is noted, or abnormal modulation of existing enzymes does in fact occur in neoplastic tissue, this may be of importance as a signal for early stages of cell transformation and tumor differentiation.

The results obtained indicate that AC associated with particulate preparations from malignant and benign human breast tumors have characteristics similar to AC isolated from other tissues. Although

there is only indirect evidence for alterations in the catalytic subunit of AC in tumor tissue, the loss or defect of hormone receptor activity has been demonstrated in a number of malignant cultured cell lines of animal and human origin, in human renal cortical carcinoma and adrenal-cortical tumors, and murine hepatomas. In the present study, it has been shown that AC associated with membrane fragments from human breast tumor tissue are at least partially sensitive to hormones in vitro. It has yet to be determined whether Mg·ATP is the true physiological substrate for AC, and if Mg^{2+} is required for the maximum hormone response. Preliminary experiments indicate that the percent activation of AC from benign tumor tissue obtained with PGE_1 was twice as high in the presence of Mg·ATP than Mn·ATP.

The thermal inactivation studies done indicate that AC from malignant and benign tumor tissue were similarly labile after pretreatment at 37°C prior to assay, although activation by NaF was still possible and nearly complete. The accumulation of cAMP in whole cell preparations is not induced by NaF, and nonspecific stimulation of AC by NaF is proposed to be due to some alteration(s) in the enzyme system during the membrane fragmentation process. It has been shown that a partial loss in the response to Gpp(NH)p occurred in AC associated with the particulate preparation from malignant tissue after preincubation at 37°C prior to assay. This may indicate a decreased stability of the GTP-sensitive site in malignant AC. The attempts made to partially solubilize AC from malignant and benign human breast tumor tissue indicated that preparation of an active soluble AC fraction

was indeed possible and might permit the purification of breast tumor associated AC to be used in further studies on regulation.

In summary, we detect no major defect in the catalytic site of AC in either malignant or benign tumors. The enzyme appears to show response to hormone stimulation similar to other mammalian enzymes demonstrating no apparent loss of hormone control of the enzyme. The inhibitory effects of Ca^{2+}, and the lack of calmodulin response coupled with Ca^{2+} suggests that this type of regulation of adenylate cyclase does not occur in human mammary tumors.

In this evaluation, human mammary tumors have been demonstrated to contain both histone kinase and casein kinase activities. Histone kinase activity demonstrated a distinct preference for histone H1 subfractions as phosphate acceptors. Two types of cAMP-dependent histone kinase activities were resolved, based upon elution profiles from DEAE-Sephacel, dissociation in the presence of 0.5 M NaCl, and response to heat-stable inhibitors of protein kinase. Of these two isoenzymes characterized, the type II histone kinase was observed to predominate in malignant and benign breast tumor cytosolic fractions. Both histone kinases demonstrated a high affinity for cAMP and a relatively high affinity for ATP and histone VS. Activity ratios were demonstrated to be higher in type I than type II histone kinase. Inhibition by protein kinase inhibitors, adenosine, 5'AMP and ADP indicated that the holoenzymes were similar to other mammalian kinases described. The majority of histone kinase activity was soluble and relatively heat-stable at 30°C.

Levels of histone kinase activity were similar in malignant and benign mammary tumor extracts, although it was observed that activity levels were two-fold higher in benign than malignant tumors using the tissue micro-dissection technique described. In addition, activity ratios for histone kinase were higher in malignant than benign tumors, which might indicate that association or activation with cAMP may be defective in malignant tumors. However, it was also shown that cAMP binding was higher in malignant than benign cytosolic fraction, although cAMP association constants were nearly identical. Therefore, the ability for cAMP-induced dissociation of malignant kinase, or activation of catalytic subunit may be abnormal.

Attempts to isolate and characterize histone kinase regulatory subunits was largely unsuccessful. Isolated and partially-purified type II catalytic subunit was found to readily reassociate with heterologous regulatory subunit, with the exception of one catalytic species which required preincubation with regulatory subunit in the presence of Mg-ATP.

Tumor-specific casein kinase was demonstrated to be cAMP-independent and was separable from histone kinase activity by DEAE-Sephacel chromatography. This enzyme showed a distinct preference for casein in contrast to histone, and was not inhibited by protein kinase inhibitors or cAMP-dependent PK regulatory subunit.

Evaluation of the potential for endogenous phosphorylation in malignant and benign tumor extracts revealed that phosphorylation of at least six protein bands was enhanced in the presence of micromolar

concentrations of cAMP, occurring to the same extent in both malignant and benign tumor preparations.

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MICRO-ANALYSIS OF ADENYLATE CYCLASE AND CYCLIC AMP-DEPENDENT PROTEIN KINASE ACTIVITIES IN HUMAN MAMMARY TUMORS

by

Terry Ann Woodford

(ABSTRACT)

The application of microanalytical techniques was used to elucidate the enzyme profiles of adenylate cyclase and cAMP-dependent protein kinase within a series of malignant and benign human mammary tumors. The methods allow for determination of enzyme activity in histologically-confirmed neoplastic areas within individual tumors which permits comparisons to be made with particle and cytosolic fractions prepared from mammary tumors. The potential for cAMP formation by adenylate cyclase and mediation of cAMP effects by cAMP-dependent protein kinase were studied to provide information on the levels of enzyme activity and the extent and type(s) of regulation of these enzymes in a cell-free system.

Adenylate cyclase (AC) activity levels were higher in benign than malignant tumor tissue on a dry wt basis. A 2 to 3-fold activation by Gpp(NH)p and a 4 to 5-fold stimulation by fluoride was demonstrated. Calmodulin activation of tumor-associated AC was not observed. Membrane-associated AC could be stimulated by PGE_1, prolactin and TSH. Thermal

stability studies suggested that AC from malignant tissue was more labile than that from benign tissue.

Cyclic AMP-dependent protein kinase associated with human mammary tumors was primarily type II kinase based upon DEAE-Sephacel chromatography and demonstrated a preference for histone VS as a substrate. Cyclic AMP-dependency was supported by enzyme activation by cAMP, by cAMP binding, and inhibition by protein kinase inhibitors and regulatory subunit. The activity ratio was slightly higher in benign than malignant tissue. Histone kinase activity was higher in benign than malignant tumor microsections. Cyclic AMP-binding activity was higher in malignant than benign tumor cytosolic fractions, although cAMP affinity was similar. Catalytic subunit was prepared from tumor histone kinase by cAMP affinity chromatography. Reassociation of tumor catalytic subunit and heterologous regulatory subunit was shown. A cAMP-independent casein kinase was also partially characterized in human mammary tumors. Endogenous phosphorylation in tumor cytosolic fractions was examined and polypeptides identified which showed phosphorylation differences in the presence of cAMP.