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INTERRELATIONSHIPS BETWEEN PROSTAGLANDINS, CYCLIC ADENOSINE 3',5'-MONOPHOSPHATE AND NUCLEAR PROTEIN PHOSPHORYLATION DURING INDUCED REGRESSION IN RAT MAMMARY TUMORS

The Ohio State University PH.D. 1982

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INTERRELATIONSHIPS BETWEEN PROSTAGLANDINS, CYCLIC ADENOSINE 3',5'-MONOPHOSPHATE AND NUCLEAR PROTEIN PHOSPHORYLATION DURING INDUCED REGRESSION IN RAT MAMMARY TUMORS

DISSERTATION

Presented in Partial Fulfillment of the Requirement for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

Mary Katherine Foecking, B.S., M.S.

* * * * *

The Ohio State University

1982

Reading Committee:

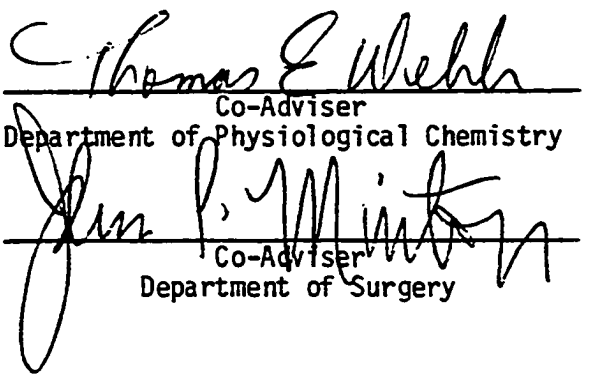
Dr. Rao V. Panganamala

Dr. Ronald W. Trewyn

Dr. Thomas E. Webb

Dr. John P. Minton

Approved By



Co-Adviser
Department of Physiological Chemistry

Co-Adviser
Department of Surgery

DEDICATED

TO MY

PARENTS

ACKNOWLEDGEMENTS

I wish to express my sincerest gratitude to Dr. Thomas Webb for constant guidance and encouragement, for helpful discussions and for his willingness to take me on as a student under unusual circumstances. I am very grateful to Dr. John P. Minton for many years of guidance and support and especially for the opportunities for experience and independence not usually afforded to graduate students.

I wish to thank Dr. Hussein Abou-Issa, whose input into this project was substantial, for many helpful discussions, and for his essential guidance on the prostaglandin binding experiments, as well as for guidance and assistance on the experiments involving the MTW9 tumors.

I thank Dr. Panganamala for many helpful discussions, both in general and regarding method development.

I am grateful to Dr. Dorothy Schumm for making laboratory space available to me, and especially for her encouragement and support.

Special thanks go to Aline Davis for her patience and expert skills which made the preparation of this dissertation many times easier than it might have been.

I also want to thank Loretta DeVecchia for estrogen receptor and progesterone receptor determinations, and especially for her friendship.

I thank Dennis Jung for his help in preparing the illustrations, and even more, for friendship and support, and for his enthusiasm for science and every undertaking.

VITA

March 30, 1954. . . . . Born - Cleveland, Ohio

1976. . . . . B.S., Chemistry, University of Dayton, Dayton, Ohio

1978. . . . . M.S., Physiological Chemistry, The Ohio State University, Columbus, Ohio

1978-1981 . . . . . Research Associate, Department of Physiological Chemistry, The Ohio State University, Columbus, Ohio

1981-1982 . . . . . Presidential Fellow, The Ohio State University, Columbus, Ohio

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1. J.P. Minton, M.K. Foecking, D.J.T. Webster and R.H. Matthews, 1979. Response of Fibrocystic Disease to Caffeine Withdrawal and Correlation of Cyclic Nucleotides with Breast Disease. Am. J. Ob. Gyn. 135:157-158.

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FIELDS OF STUDY

Major Field: Physiological Chemistry

Studies in Cyclic Nucleotides and Mammary Disease. Dr. Richard H. Matthews and Dr. John P. Minton.

Studies in Prostaglandins, Cyclic Nucleotides and Protein Phosphorylation. Dr. John P. Minton, Dr. Thomas E. Webb, and Dr. Hussein Abou-Issa.

Studies in Effects of Diet on Tumorigenesis. Dr. John P. Minton.

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LIST OF ABBREVIATIONS AND SYMBOLS

APS	Ammonium persulfate
B	Benzene
BIS	N',N'-methylenebis-acrylamide
BCG	Bacillus Calmette-Guerin
BP	Benzo(a)pyrene
$CaCl_2$	Calcium chloride
cAMP	Adenosine 3',5'-monophosphate
cm	Centimeters
cpm	Counts per minute
C. parvum	Corynebacterium parvum
DMBA	7,12-dimethylbenz(a)anthracene
DMH	Dimethylhydrazine
EA	Ethyl acetate
EDTA	Ethylenediaminetetraacetic acid
FANFT	N-[-4-(5-nitro-2-furyl)-2-thiazolyl]-formamide
gm	Grams
Gpp(NH)p	Guanylylimidodiphosphate
GTP	Guanosine triphosphate
^3H-6-Keto-$PGF_{1\alpha}$	6-[5,8,9,11,12,14,15-^3H(N)]-keto prostaglandin $F_{1\alpha}$
^3H-PGE_2	[5,6,8,11,12,14,15-^3H(N)]-Prostaglandin E_2
15-HETE	15-Hydroxyeicosatetraenoic acid
15-HPETE	15-Hydroperoxyeicosatetraenoic acid

^{125}I-ScAMP-TME	^{125}I-Succinyl cyclic AMP-Tyrosine methyl ester
IBMX	Isobutylmethylxanthine
KCl	Potassium chloride
6-Keto-PGF$_{1\alpha}$	6-Keto-Prostaglandin F$_{1\alpha}$
Lt	Leukotriene
$MgCl_2$	Magnesium chloride
MeOH	Methanol
mg	Milligrams
ml	Milliliters
mm	Millimeters
mmol	Millimoles
NaCl	Sodium chloride
ng	Nanograms
ODC	Ornithine Decarboxylase
pg	Picograms
PG	Prostaglandin(s)
PGA_2	Prostaglandin A_2
PGD_2	Prostaglandin D_2
PGE_2	Prostaglandin E_2
PGF$_{2\alpha}$	Prostaglandin F$_{2\alpha}$
PGI_2	Prostacyclin
PIF	Prolactin inhibitory factor
pmole	Picomoles
py3T3	Polyoma virus transformed 3T3 cells
RAP	Regression associated protein
RIA	Radioimmunoassay
SDS	Sodium dodecyl sulfate

SE	Standard error
TCA	Trichloracetic acid
TEMED	Tetramethylenediamine
TRIS	Tris(hydroxymethyl)aminomethane
TxA_2	Thromboxane A_2
TxB_2	Thromboxane B_2
µl	Microliters

INTRODUCTION

I. Overview

Many factors, both endogenous and exogenous, contribute to the genesis, promotion and maintenance of cancer. Identification of these factors and an understanding of their mechanisms of action in malignant cells may lead to knowledge contributing to the treatment and prevention of cancer.

This dissertation reports the results of a study investigating the possibility that prostaglandins, and in particular PGE_2, may play a role in the control of growth of hormonally dependent rat mammary cancer. Before discussing the experimental designs and the results which were obtained, the biochemistry of the prostaglandins will be briefly reviewed. In addition, descriptions of early observations which suggested a role for prostaglandins in cancer, as well as the current directions which research in prostaglandins and cancer are taking, will be summarized. A possible mechanism for the regulation of mammary tumor growth by prostaglandin via activation of adenylate cyclase will be proposed. The central tumor model in this series of experiments is the hormonally dependent DMBA-induced mammary tumor; therefore a discussion of the hormonal characteristics of this tumor will be included, along with current knowledge concerning the growth and regression of this tumor.

There is an accumulating mass of evidence in the literature that prostaglandins play some role in the control of growth of existing tumors. In conjunction with this, is the large body of evidence that cAMP plays an important role in the control of cell replication and tumor proliferation (Ryan and Heidrick, 1974). This evidence along with the identification by Cho-Chung et al (1977) of a regression associated protein, whose phosphorylation is regulated by cAMP, led us to study the regression process in a well defined tumor system, specifically the DMBA-induced rat mammary tumor.

II. Prostaglandin Biochemistry

A summary of the pathways involved in prostaglandin synthesis and metabolism is shown in Figure 1. The literature dealing with the biosynthesis, metabolism, occurrence and biological actions of prostaglandins is extensive. Numerous reviews have been written, including those by Hinman (1972), Samuelsson et al (1975, 1978), Weeks (1972) and Moncada and Vane, (1979). Only brief overviews of prostaglandin biosynthesis will be presented here.

The unsaturated fatty acid arachidonic acid [5,8,11,14-eicosatetraenoic acid (20:4)], is the precursor to prostaglandins of the 2 series (i.e. PGE_2) and to the biologically significant thromboxanes, TxA_2 and TxB_2, as well as prostacyclin, PGI_2. A requisite for prostaglandin synthesis is the release of arachidonic acid, or the appropriate polyunsaturated fatty acid, from the phospholipids in the cellular membrane by a phospholipase (Flower and Blackwell, 1976). Phospholipases with differing substrate specificities have been proposed (Bills et al, 1977; Rittenhouse-Simmons, 1979; Bell et al, 1979).

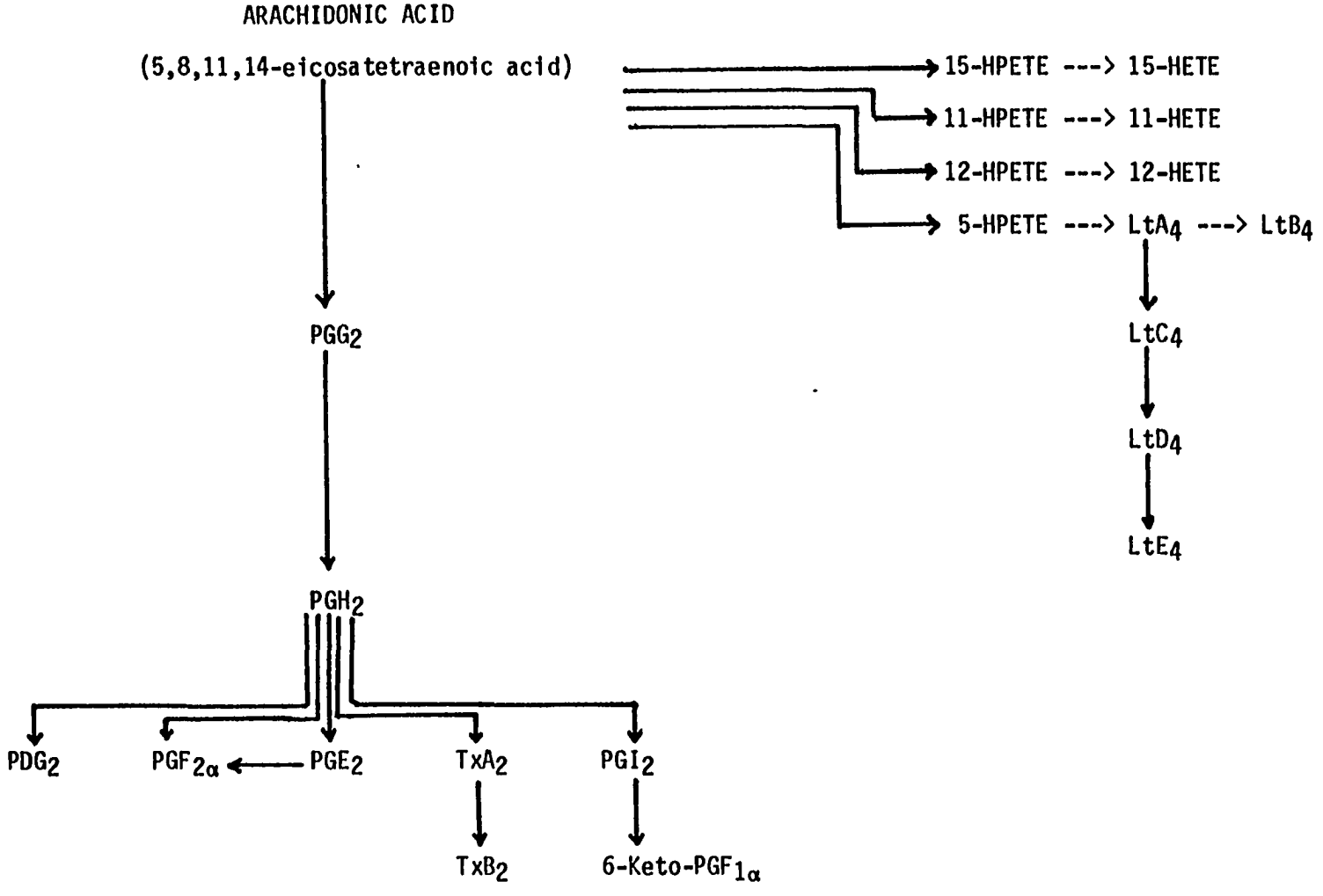


Figure 1. Arachidonic Acid Metabolism

The enzyme cyclooxygenase which converts the unsaturated 20 carbon fatty acid to the endoperoxides PGG_2 and PGH_2 has both cyclase activity and peroxidase activity. The endoperoxide PGH_2 is the substrate for a number of enzymes which catalyze the formation of the classical PGs, for example PGE_2, PGD_2, and $PGF_{2\alpha}$, along with the formation of thromboxane A_2 (TxA_2) and prostacyclin (PGI_2).

Prostaglandins have been identified in many tissues. Investigations into the biological roles of prostaglandins in smooth muscle, the reproductive system, kidney, the circulatory system, inhibition of gastric secretion (Weeks, 1972), functional hyperemia, the inflammatory response, central nervous system (Hinman, 1972), blood and bone marrow cells, fat cells, fibroblasts (Samuelsson et al, 1975) and platelet function (Samuelsson, et al, 1978) have been reviewed. The literature concerning the role of prostaglandins in cancer will be discussed in detail below.

TxA_2 and PGI_2, as products of platelets and arterial endothelium, respectively, have been considered primarily in the regulation of platelet aggregation and vascular responses (Moncada and Vane, 1979). However, the presence of TxA_2 in spleen, bone marrow, and lung from various species, and prostacyclin in corpus luteum, uterus, stomach, small intestine and lung suggest that these cyclooxygenase products may have biological activities beyond their roles in the cardiovascular system (Sun et al, 1977).

Prostaglandins are not stored, rather they are synthesized on demand, then proceed to act at the site of synthesis (Bito, 1975). Much of the catabolism of prostaglandins occurs in the lung and the liver (Ferreira and Vane, 1967; Piper et al, 1970).

Specific inhibitors of prostaglandin synthesis at the site of cyclooxygenase include non-steroidal anti-inflammatory agents, such as indomethacin and aspirin (Vane, 1971; Ferreira et al, 1971; Smith and Willis, 1971). Some other compounds which inhibit cyclooxygenase include meclofenamic acid, flufenamic acid, naproxen, and ibuprofen (Flower, 1974). In addition, 5,8,11,14-eicosatetraynoic acid has been shown to inhibit both cyclooxygenase and lipoxygenase from soybean and human platelets (Ahern et al, 1970; McDonald-Gibson et al, 1973; Downing et al, 1970).

Steroids which are anti-inflammatory, such as glucocorticoids, inhibit the release of fatty acids from phospholipid stores by inhibiting phospholipase activity (Hong and Levine, 1976). This inhibition is mediated by macrocortin, a protein induced by glucocorticoid which is inhibitory to phospholipase activity (Hirata et al, 1980; Blackwell et al, 1980).

Arachidonic acid is also a substrate for the lipoxygenase enzyme. This enzyme converts 20-carbon unsaturated fatty acids to hydroperoxy and mono-or di-hydroxy derivatives, which also have biological activity. Further products of the lipoxygenase pathway include the leukotrienes. Lipoxygenase products have been studied in regard to platelet function (Nugteren, 1975; Samuellson et al, 1975), leukocyte function in immediate-type hypersensitivity reactions (Goetzl et al, 1981) and the release of Slow Reacting Substance of anaphylaxis (SRS-A) from lung upon immunological challenge (Piper et al, 1981). Lipoxygenase activity has not been observed in mammaliam cell types other than lung, platelets and leukocytes (Moncada and Vane, 1979).

III. Prostaglandin Modulation of cAMP Synthesis

As stated earlier, the biological roles of prostaglandins are many and varied. The mechanism of prostaglandin action has been a subject for investigation. It is unlikely that all prostaglandin actions can be explained by a single mechanism, but there may be a common mechanism for prostaglandin actions in similar and analagous functions.

The possibility that prostaglandins act through the cAMP system in the regulation of intracellular actions has been investigated extensively. The volume of literature on this topic alone is overwhelming, and several reviews have been written to attempt to organize all the available information. More extensive reviews are to be found in the volume edited by Kahn (1973), the reviews by Kuehl (1974, 1976), as well as in the sections in the Annual Reviews of Biochemistry from 1972, 1975 and 1978 (Hinman, 1972; Samuelsson et al, 1975; Samuelsson et al, 1978).

In most cases, prostaglandins, particularly of the E series, have been shown to stimulate an increase in cAMP. In 1972, PGE_1, PGE_2 or both, were reported to stimulate cAMP levels in lung, corpus luteum, anterior pituitary, spleen, fetal bone, diaphragm, guinea pig heart, rat aorta, rat erythrocyte ghosts, thrombocytes, fibroblasts, leukocytes, cultured neuroblastoma cells and lymphocytes (Himan, 1972). By 1975, more direct evidence for the activation of adenylate cyclase by PGE_1 and PGE_2 in many of the same tissues was reviewed (Samuelsson et al, 1975). In the latter 1970's, specific binding of PGEs to many cells and tissues had been reported and correlated with accumulation of cAMP and/or with activation of adenylate cyclase (Samuelsson et al, 1978).

Although stimulation of cAMP levels by PGE_1 and PGE_2 has been observed in the majority of systems studied, there are exceptions. An inhibition of cAMP accumulation in the presence of PGE_1 or PGE_2 was reported in intact fat cells, in toad bladder, Purkinje cells and spleen (Kuehl, 1974; Samuelsson et al, 1975). PGE_1 inhibits hormone-induced lipolysis by decreasing cAMP in intact fat cells (Butcher, 1970), but in broken fat cells, the effect on adenylate cyclase is stimulatory (Frank et al, 1971).

Certain prostaglandin actions may be independent of cAMP accumulation in some systems. For example, inhibition of amino acid uptake by PGE_2, $PGF_{1\alpha}$ or $PGF_{2\alpha}$ in human diploid WI38 fibroblasts occurs without an alteration of cAMP levels (Polgar and Taylor, 1977). Prostaglandin $F_{2\alpha}$ and $F_{1\alpha}$ do not activate adenylate cyclase or increase cAMP in the cells and tissues which have been studied.

The regulation of platelet function has become the classical example of the action of arachidonic acid products through modulation of cAMP. PGI_2 is the major arachidonic acid metabolite in vascular tissue and the most potent inhibitor of platelet aggregation studied. PGI_2 inhibits platelet aggregation by stimulating platelet adenylate cyclase, leading to an increase of cAMP in the platelets (Gorman et al, 1977). PGI_2 has been shown to be the most potent activator of platelet cAMP production (Tateson et al, 1977). The increase in cAMP secondary to the interaction of PGI_2 with a specific receptor on the platelet membrane inhibits further prostaglandin production within the platelets, thereby preventing synthesis of the pro-aggregatory TxA_2. The effects of TxA_2 are antagonistic to PGI_2, even with respect to cAMP accumulation

since this compound can abolish the PGI_2-induced increase in cAMP under the proper *in vitro* conditions.

The fact that the well-studied increase in platelet cAMP subsequent to PGI_2 stimulation acts to decrease platelet arachidonate metabolism serves as a reminder that the interactions between prostaglandins and cAMP can be cyclic or function in a biofeedback-mechanism. There are examples of stimulation and inhibition of PG synthesis by cAMP. Dibutyryl cAMP stimulates prostaglandin synthesis in mouse ovary and rat testes (Kuehl, 1974) and in Graafian follicles, thyroid cells, adrenal cortex and adipocytes (Samuelsson et al, 1978). As mentioned above, cAMP inhibits PG formation in platelets. Thus, there is evidence for the existence of both positive feedback mechanisms and negative feedback mechanisms. Thus, it appears that, during differentiation of certain cell types, specific inhibitors or different feedback loops are formed which taylor the responses of that particular cell or tissue to the prostaglandins.

IV. Prostaglandin Production and Release by Human and Animal Tumors

Malignant tumors and transformed cells in culture synthesize and release prostaglandins.

Prostaglandins have been identified in extracts of many human cancers. Prostaglandin E_2 and lower quantities of $PGF_{2\alpha}$ were detected in medullary carcinoma of the thyroid using a bioassay quantitation method (Williams et al, 1968), in an attempt to find a causative factor for the diarrhea usually associated with this cancer. Neural crest tumors were found to have high PGE_2 (Sandler et al, 1968) and $PGF_{2\alpha}$ levels (Papanicolaou, 1975). Elevated PGE_2 and $PGF_{2\alpha}$

were also reported in ganglioneuromas, neuroblastomas and pheochromocytomas. The ability to produce prostaglandins was identified in Kaposi's sarcoma (Bhona et al, 1971). Kaplan et al reported that carcinoid tumors, as well as medullary carcinoma of the thyroid (as reported earlier) contained and secreted PGE (Kaplan et al, 1973). PGE was found to be the major prostaglandin in a renal cell carcinoma (Zusman et al, 1974), in an investigation into the antihypertensive activity of the lesion. Further studies of the causative factor in the diarrhea accompanying neural crest tumors were reported by Jaffe and Condon (1976). PGE production by colon cancers was measured by bioassay of the rat gastric fundus strip (Bennett et al, 1977a). The PGE_2 levels in the serum of patients with bronchial carcinoma, as measured by RIA, was found to decrease significantly following surgical removal of the tumors (Fiedler et al, 1980), whereas the serum PGE_2 levels in patients with non-malignant disorders did not differ from normal. The elevation of PGE_2 levels in human breast cancer has been reported. Bennett and co-workers measured PG-like material using a bioassay in normal, benign and malignant human breast tissue and found the highest levels in the cancers (Bennett et al, 1977b; Bennett et al, 1980b). The prostaglandin-like materials were also released into the blood of breast cancer patients (Stamford et al, 1980). The synthesis of PGE_2 and $PGF_{2\alpha}$ from microsomal fractions of breast tumors was measured, by Rolland et al (1980a,b), who reported that PGE_2 is the primary product of cyclooxygenase activity. An activity in the cytosol which converts PGE_2 to $PGF_{2\alpha}$ accounts for the $PGF_{2\alpha}$ content in the tumors.

The ability of animal tumors to produce prostaglandins has been reported in many systems. The report of the ability of $BP8/P_1$ tumors, $BP8/P_1$ ascites tumor cells and mouse sarcoma 180 tumors to synthesize PGE_2 by Sykes and Maddox (1972) was one of the earliest reports concerning prostaglandins in experimental tumors. There was no significant effect on tumor growth with treatment of the animal with aspirin or indomethacin. Since then, reports on the effects of anti-inflammatory drugs have appeared; these will be discussed later. The PGE_2 content in $HSDM_1$ mouse fibrosarcomas was measured by radioimmunoassay in an effort to implicate this PG in the bone resorbing activity accompanying this malignancy (Tashjian et al, 1972). The role of PGE_2 in the hypercalcemia associated with cancer was also studied in the VX_2 carcinoma in the rabbit, (Voelkel et al, 1975). Moloney-sarcoma virus-induced tumors synthesize greater than 50-times as much PGE_2 as normal mouse muscle from the rear leg (Humes and Strausser, 1974; Humes et al, 1974).

It must be noted in the interpretation of these results that comparison of cancer to normal tissue is often difficult because the normal tissue is a heterogeneous mixture of different cell types with different functions, whereas a tumor usually arises from a single cell type and is more homogeneous in nature. Thus, the apparent elevation of prostaglandins in some tissues is difficult to interpret. Nevertheless, the observation that tumors possess the ability to synthesize prostaglandins is important.

V. Possible Role of Prostaglandins in Carcinogenesis

The production of prostaglandins by transformed cells and malignant tumors, as noted above raises the possibility that prostaglandins may play a role in the tumorigenic process. Since PGE_2 was the metabolite found in the highest quantities in the malignant tissues which were studied, it seems possible that PGE_2 may be of the greatest importance.

There are several steps in the carcinogenic process at which prostaglandin research is being pursued. Some of the different stages in the carcinogenic process in which prostaglandins have been implicated included initiation, promotion, cell proliferation and differentiation, and metastasis of the tumor to distant sites, (Honn et al, 1981).

a. Initiation

The observation that an increase in prostaglandin production accompanies chemical carcinogen exposure in MDCK dog kidney cells (Levine, 1977a) suggests that prostaglandin production may be important in initiation of carcinogenesis. By definition, initiation of carcinogenesis is thought to occur when a cell endures an irreversible genetic mutation. The damage to the genome may be a result of radiation, chemical or viral insult. Often the active, or ultimate, carcinogen, in the case of chemical carcinogenesis, is not the chemical compound as it is found in the environment, but a derivative which is formed *in vivo* in the process of the cell's metabolism of toxic compounds. Oxygenation of toxic compounds by mixed function oxidases is a common pathway of metabolism for these toxic chemicals; and the oxygenated metabolites are often the ultimate carcinogens.

Cooxygenation of benzo(a)pyrene (BP) by the peroxidase activity of the prostaglandin synthetase (cyclooxygenase) enzyme is important in the

metabolism of BP by ram seminal vesicle microsomes (Marnett et al, 1975: Sivarajah et al, 1978). The prostaglandin synthetase inhibitors indomethacin and aspirin inhibit the arachidonic acid-dependent cooxygenation. BP itself is not converted to carcinogenic metabolites by cooxygenation in ram seminal vesicle microsomes, but when the starting materials is the 7,8-dihydrodiol derivative of BP, arachidonic-acid-dependent cooxygenation results in a metabolite, probably a diol-epoxide, which is strongly mutagenic (Marnett et al, 1978, Guthrie et al, 1982).

The demonstration of arachidonic acid-dependent cooxgenation of BP by ram seminal vesicles is interesting, but of questionable physiological relevance, since skin and lung are the most frequent targets of BP action. Eling and co-workers have demonstrated this activity in microsomal preparations from other tissues, including rat lung and intestine, mouse skin and human and guinea pig lung (Sivarajah et al, 1981). The same workers have reported similar results with two other carcinogens, benzanthracene and dimethylbenzanthracene (Guthrie et al, 1982).

One tissue which may be particularly susceptible to chemical carcinogenesis is the kidney, where, by virtue of its role in excretion, concentration of toxic metabolites may occur (Honn et al, 1981). The prostaglandin synthetase-dependent cooxygenation in kidney inner medulla of urinary bladder carcinogens was reported by Zenser et al (1979a, also Zenser, et al, 1980). Addition of arachidonic acid to kidney medulla microsomes, where mixed function oxidase activity is low, initiated the oxidation of benzidine and N-[-4-(5-nitro-2-furyl)-2-thiazolyl]-formamide (FANFT), and indomethacin was inhibitory. <u>In vivo</u>

confirmation of the arachidonic acid-dependent cooxygenation of benzidine was obtained in dogs (Zenser et al, 1979b).

Cooxygenation of chemical carcinogens by prostaglandin synthetase may be considered to have physiological relevance, especially in tissue where mixed function oxidase activity is low.

b. Promotion

A tumor promoter is a compound which decreases the latent period between initiation and the appearance of a tumor, and also decreases the dose of initiator required for carcinogenesis. The most common compound used in studies of promotion is the phorbol ester 12-O-tetrodecanoyl phorbol-13- acetate (TPA), and a common model for promotion is the carcinogenesis of mouse skin. It must be noted that promoters alone do not cause cancer.

Biochemical changes which follow treatment with TPA include deacylation of cellular lipids, induction of ornithine decarboxylase (ODC), and stimulation of DNA synthesis. On a macroscopic scale, inflammation is apparent. Arachidonic acid, PGE_2 and $PGF_{2\alpha}$ are released from MDCK cells after treatment with TPA, but not after treatment with 4-α-phorbol didecanoate, which does not promote tumor activity (Levine and Hassid, 1977b). Furthermore, there is a correlation between tumor promoting activity in mouse skin and the ability of some phorbol diesters to stimulate PGE_2 biosynthesis by macrophages (Brune et al, 1978a). Also, the induction of ODC by phorbol esters correlates with the ability to elevate PGE levels in Friend erythroleukemia cells (Yamasaki et al, 1979). Indomethacin prevents the TPA induction of

ornithine decarboxylase in mouse skin and treatment with PGE_1 or PGE_2 restores the ODC induction (Verma et al, 1977).

Administration of prostaglandins in the absence of TPA does not increase ODC activity in CD-1 mouse skin, suggesting that prostaglandins may play a role in the induction of ODC by TPA and subsequently tumor promotion; but are not in fact tumor promoters (Verma et al, 1977). A similar conclusion results from the observation that TPA-stimulation of DNA synthesis can be inhibited by indomethacin and PGE_2 overcomes this inhibition, but not in the absence of TPA (Furstenberger and Marks, 1978). Inhibition of tumor promotion by indomethacin in the CD-1 mouse (Verma et al, 1980) provides further evidence that prostaglandins play a role in tumor promotion.

The effect of indomethacin on the promotion of cancer in mouse skin in the SENCAR mouse can be inhibitory or enhancing, depending on the dose; thus 25-100 μg of indomethacin enhances TPA promotion in the SENCAR mouse, and greater than 100 μg is inhibitory, probably due to toxicity (Fischer et al, 1980; Fischer and Slaga, 1982). The authors suggest that lipoxygenase products may be important in promotion and that indomethacin, by inhibiting cyclooxygenase activity, increases the arachidonic acid metabolism through the lipoxygenase pathway.

The importance of inflammation and more directly prostaglandin production in tumor promotion is suggested by studies which showed that the anti-inflammatory glucocorticoid analog dexamethasone suppressed tumor promotion in mouse skin (Scribner and Slaga, 1973). Further evidence for a role of prostaglandins in tumor promotion is found in the stimulation of prostaglandin synthesis by growth factors (which are

essentially natural promoters), such as epidermal growth factor (Levine and Hassid, 1977c).

The stimulation of prostaglandin synthesis by TPA in tissues other than mouse skin has been reported. MDCK cells are sensitive to concentrations of TPA as low as 10^{-9}-10^{-10}M. TPA also stimulates PGE_2 synthesis in macrophages from the peritoneal cavity of mice (Brune et al, 1978b), in chick embryo fibroblasts (Mufson et al, 1979), in HeLa cells (Crutchley et al, 1980), in a murine epidermal cell line HEL/30 (Furstenberger et al, 1980), in Friend erythroleukemia cells (Yamasaki et al, 1979), and bone (Voelkel et al, 1980).

All the available evidence suggests that PGE_2, and possibly other prostaglandins, may play a role in tumor promotion but are not themselves tumor promoters.

c. Metastases

One of the main factors which, in the course of the neoplastic process, often becomes life-threatening, is the ability of cancerous cells to break away from a primary tumor and establish growth, or metastases, at a distant site in the body. Prostaglandins have been implicated in the process of tumor metastasis.

Initial studies on the role of prostaglandins in metastasis were based on the theory that bone metastases were promoted by the hypercalcemia which accompanies some cancers. It was thought that prostaglandins acted locally to cause osteoporesis, creating a likely spot for metastases to "take root." An alternate theory was that osteoporesis was secondary to prostaglandin-producing metastases which had already "settled in."

The action of prostaglandins in metastasis to tissues other tha n bone has also been studied. In general, treatment of tumor-bearing animals with prostaglandin synthesis inhibitors either has no effect or acts to inhibit metastasis, as reviewed by Bennett (1982). A review of the studies involving non-steroidal anti-inflammatory drugs and survival came to the same conclusion: the effect was either benefical or nil. Bennett has reported that survival of breast cancer patients was inversely proportional to the synthesis of prostaglandin-like material by the primary tumors (Bennett, et al, 1979).

Current thought favors the hypothesis that platelet aggregation plays an important part in tumor metastasis. Honn (1982) proposes that circulating tumor cells disrupt the balance between PGI_2 (which is anti-aggregatory) and TxA_2 (which is pro-aggregatory) in favor of platelet aggregation. Tumor cells have the ability to induce platelet aggregation in platelet rich plasma (Gasic et al, 1973, 1978). Therefore, it follows that modulation of PGI_2 synthesis and TxA_2 synthesis may be useful in the prevention of metastases.

PGI_2 produced a dose-related increase in experimental metastasis of B16a melanoma to the lung, liver and spleen (Honn et al, 1981a). PGE_2 did not prevent lung colony formation from B16a melanoma cells, suggesting a specific platelet effect. Inhibition of PGI_2 synthesis by 15-HPETE enhanced lung colony formation and the effect of 15-HPETE was reversed by addition of PGI_2. Inhibition of TxA_2 synthesis by endoperoxide analogs significantly reduced lung metastases of B16a melanoma cells injected into the tail vein. Furthermore, inhibition of TxA_2 synthesis with an imidazole derivative resulted in reduced metastases in two <u>in vivo</u> tumor models (Honn, 1982).

The evidence suggests that modulation of the synthesis of specific prostaglandins, in particular inhibition of TxA_2 synthesis and enhancement of PGI_2 synthesis, may be important in the prevention of metastasis.

VI. Prostaglandins and Cell Proliferation

The stage of cancer with which this dissertation is concerned is that of cell proliferation (and differentiation). The discussion of the possible roles of prostaglandins in initiation, promotion, and metastasis were given as background and general information. The ability of prostaglandins to modulate the growth of existing tumors is of primary interest here and may be significant in the design of therapies for the control of cancers after diagnosis.

Prostaglandins have been reported to affect normal and neoplastic cell proliferation. Thomas et al (1974) compared prostaglandin synthesis to growth rate in HEp-2, L, and HeLa cell lines maintained in culture, and reported that prostaglandin synthesis was inversely related to growth rate. A neuroblastoma, a glioma, and fibroblast cell line all produced PGE as the major prostaglandin product, and in these three cell lines a retardation of cell proliferation was associated with increased prostaglandin production (Hamprecht et al, 1973).

Very few other studies have been reported which correlate endogenous prostaglandin production with growth rate. However, there are many reports of the effects of exogenous prostaglandins on the rate of cell replication and proliferation of tumors.

Addition of 12-50 μg/ml PGE_1, PGE_2 or PGA_2 slowed the growth rate and increased adhesion to culture flasks of L-929 cells and mouse embryo fibroblasts in vitro (Johnson and Pastan, 1971). Cell division of mouse neuroblastoma cells in vitro was inhibited by the presence of PGE_1 or PGE_2 (1-50 μg/ml) in the medium (Prasad, 1972). The suppression of growth of the murine plasma cell tumor, MPC-11 by PGE_1 (0.1-10 μg/ml) was concentration dependent (Naseem and Hollander, 1973). PGE_2 (1-100 μg/ml) inhibited EL-14 lymphoma proliferation as measured by thymidine uptake (Sonis et al, 1977).

Santoro and co-workers have accumulated a good deal of evidence to support a suppressive role for PGE_2 on cell proliferation. The growth rate of B-16 melanoma cells in vitro was stimulated by hydrocortisone, which inhibited PG synthesis by the cells by 50%. PGE_1, when added along with hydrocortisone, normalized the growth rate. In addition, subcutaneous or intratumor injections of 16,16-dimethyl-PGE_2, which is a stable analog of PGE_2, significantly suppressed tumor growth in vivo. Inhibition of endogenous prostaglandin synthesis by administration of indomethacin caused a small but significant stimulation of tumor growth (Santoro et al, 1976). Systemic administration of 16,16-dimethyl-PGE_2 (5 μg/day) resulted in a slight but significant inhibition of B-16 melanoma growth in vivo; tumors were 32% smaller by weight (Santoro et al, 1977). Furthermore, 16,16-dimethyl-PGE_2 augmented the effects of chemotherapy on B-16 melanomas in mice (Hofer et al, 1980).

PGA_1 has also been shown to be inhibitory to cell replication. For example, PGA_1 inhibited the replication of DMSO-treated and undifferentiated Friend erythroleukemia cells (Santoro et al, 1979), this

effect may be of pharmocological importance, but is not physiological since PGA_1 synthesis has not been demonstrated in these cells.

Further evidence that PGE_2 may inhibit proliferation comes from the report that 16,16-dimethyl-PGE_2 (0.025-250 µg/kg) transiently inhibited xenograft growth, and strongly inhibited the proliferation of rat colon tumors. In addition, flurbiprofen, an inhibitor of prostaglandin synthesis, accelerated cell division in the rat colon tumors (Tutton and Barkla, 1980).

Prostaglandins E_2, E_1 and $F_{2\alpha}$ inhibited the growth of mouse leukemia lymphoblasts in culture. At 100 µg/ml, PGE_1 and E_2 inhibited incorporation of ^3H-thymidine, uridine and leucine (Yang et al, 1976). Furthermore, Honn et al (1981c) reported that the 16,16-dimethyl analogs of PGE_1, PGE_2, PGA_1 and PGA_2 inhibited DNA synthesis at doses of 1,10 and 25 µg/ml in Lewis lung carcinoma and B-16 amelanotic melanoma in vitro.

If prostaglandins, specifically PGE_2, act to inhibit cell proliferation, then inhibition of prostaglandin synthesis in tumors should stimulate tumor growth. A few of the examples listed above support this conclusion. For example, indomethacin in B-16 melanoma (Santoro et al, 1976) and flurbiprofen in dimethylhydrazine-induced rat colon tumors (Tutton and Barkla, 1980) acted to stimulate tumor growth. However, not all reports of the effects of drugs that inhibit PG synthesis have been in agreement. There are reports of treatments with non-steroidal anti-inflammatory agents having no significant effect on tumor growth; also in some cases, treatment with drugs that inhibit prostaglandin synthesis acted to inhibit tumor growth (Bennett, 1982). An interpretation of these results will be dealt with in the Discussion.

Thus, to summarize, many investigators feel that prostaglandins play an important role in the control of cell proliferation in malignant tumors and transformed cells. What this role is, remains to be clarified.

VII. Hormonal Dependence of DMBA-Induced Mammary Tumors

The DMBA-induced mammary tumors are hormone dependent, since most tumors regress in response to endocrine ablation of the host. Numerous investigators have attempted to define the specific hormonal dependence of these tumors. Whether estrogen or prolactin (or both) are of primary importance in the control of growth of these rat mammary tumors is still a matter of debate. It is generally accepted that prolactin stimulates growth of these tumors, but whether prolactin is more important than estrogen, or whether estrogen is growth promoting at all has not been settled.

Ovariectomy of rats bearing DMBA-induced mammary tumors results in regression of the tumors in 80-90% of the rats (Huggins, 1979). Replacement of estrogen in ovariectomized rats provides the hormonal requirements for growth of the tumors (Talwalker et al, 1964), suggesting that estrogen is of primary importance in the maintenance of growth of DMBA-induced mammary tumors. However, removal of prolactin by specific drugs, such as ergocornine or ergocryptine, inhibited growth of existing tumors and caused regression of the tumors (Cassell et al, 1971). Since estrogen stimulates serum and pituitary prolactin levels (Reece and Turner, 1936; Grosvenor and Tunner, 1960; Chen and Meites, 1970), it is not clear whether the depletion of estrogen due to ovariectomy, or the

subsequent decrease in prolactin, is more important in causing tumor regression.

Roughly 80% of existing mammary tumors regressed in rats which were hypophysectomized in order to remove the source of prolactin. Estrogen administration to these rats did not cause a resumption of tumor growth, whereas administration of prolactin did; furthermore, there was no synergism of estrogen with prolactin (Arafah et al, 1980). Pearson and co-workers conclude that prolactin is the major hormone which supports growth of DMBA-induced mammary tumors, based on these and the following results.

Tumors which regressed following ovariectomy resumed growth when the host prolactin level was increased by perphenazine (a stimulator of prolactin release) treatment, even in the presence of the estrogen antagonist tamoxifen. When regression was induced by an estrogen antagonist (tamoxifen) plus a prolactin release inhibitor (lergotrile mesylate), reactivation of tumor growth was obtained with perphenazine treatment. The authors sited the fact that the estrogen receptor binding was undetectable in tumors maximally stimulated by perphenazine, and the observation that estrogen could not stimulate tumor growth under conditions of undetectable prolactin levels due to lergotrile administration as strong evidence that prolactin alone supports DMBA-induced mammary tumor growth (Manni et al, 1977).

Other investigators also report evidence that prolactin is of primary and sole importance in the control of the growth of these tumors. Jabara and co-workers reported that estrogen administration following adrenalectomy and ovariectomy of tumor-bearing hosts increased tumor growth by stimulation of prolactin secretion, since estrogen did not

stimulate growth when serum prolactin levels were decreased by ergocornine. In addition, perphenazine, a stimulator of prolactin release, was able to stimulate tumor growth in the absence of estrogen (Minasian-Batmanian and Jabara, 1981).

The relative importance of prolactin and estrogen in the control of DMBA-induced mammary tumor growth was investigated in an experiment involving endocrine ablation and subsequent hormone replacement (Bradley et al, 1976), with the conclusion that prolactin is of greater importance than estrogen in maintaining growth of DMBA-induced mammary tumors.

While few investigators dispute that prolactin plays a role in mammary tumor growth, many believe that estrogen is also essential for the maintenance of growth of existing DMBA-induced mammary tumors. Evidence in favor of an essential role for estrogen in mammary tumor growth was reported by Sinha et al (1973). Lesions of the median eminence of the hypothalamus block hypothalamic releasing factors from reaching the anterior pituitary. The consequence of median eminence lesions is a permanent elevation of serum prolactin levels, and a stimulation of tumor growth in tumor-bearing rats. These tumors regressed after ovariectomy of the host, in spite of the continued elevation of serum prolactin. The tumor regression was rapidly reversed by grafting ovaries into the host, suggesting that stimulation of mammary tumor growth by prolactin is dependent on the presence of estrogen, or some other ovarian hormone.

Welsch (1972) also proposed that an ovarian hormone is essential for sensitivity of the DMBA-induced mammary tumor to stimulation by prolactin. When rats bearing DMBA-induced tumors were ovariectomized and subjected to median eminence lesions simultaneously, tumor growth

continued for 10 days, then the tumors began to regress. Median eminence lesions performed 10 days following ovariectomy in tumor bearing rats failed to stimulate tumor growth, presumably because any residual estrogen in the rat was depleted after 10 days (Welsch, 1972).

Further support for the idea that estrogen is also an essential hormone for the growth of DMBA-induced mammary tumors was obtained from tumor-bearing rats which were subjected to double endocrine ablation, specifically ovariectomy and adrenalectomy (Leung and Sasaki, 1975; Leung et al, 1975). Following ovariectomy and adrenalectomy, not all tumors responded to daily injection of ovine prolactin by reactivation of growth. Those that did not respond to prolactin alone, did respond to injection of prolactin plus estrogen with a resumption of growth. When prolactin was administered along with the estrogen antagonist nafoxidine in the ovariectomized-adrenalectomized rats tumor growth was maintained, initially suggesting that the presence of estrogen was not essential. However, when prolactin and nafoxidine were withdrawn, tumor regression was observed, as would be expected, but readministration of prolactin alone failed to reactivate tumor growth. This contrasts with the response to readministration of prolactin following an initial administration, then withdrawal, of prolactin alone, in which renewed tumor growth was observed when prolactin treatment was resumed. This result suggests that nafoxidine, the estrogen antagonist, acted to block certain estrogen-requiring events, which had to be restored by estrogen before growth could be resumed (Leung and Sasaki, 1975).

Following tumor regression due to adrenalectomy-ovariectomy, the resumption of tumor growth by prolactin administration seems to be temporary (Nagasawa and Yanai, 1970b). Furthermore, when prolactin

administration to the rat was delayed after adrenalectomy-ovariectomy, many tumors failed to resume growth (Leung et al, 1975). Another common argument for the importance of estrogen in the growth of DMBA-induced mammary tumors is the presence of estrogen receptors in the tumors (Asselin et al, 1977).

The growth of a hormonally-dependent tissue depends on the concentration and binding activity of receptor proteins as well as endogenous hormone levels. The regulation of the hormone receptors in DMBA-induced mammary tumors by endogenous hormones has been investigated in hypophysectomized tumor-bearing rats. By day 16 post-hypophysectomy, the levels of estrogen, progesterone and prolactin receptors decreased significantly. Replacement of the various hormones indicated which hormones were important in the regulation of each receptor. Administration of either estrogen or prolactin prevented the fall in estrogen receptors. Estrogen or prolactin also acted to maintain prolactin receptors. Estrogen prevented the decrease in progesterone receptors without reactivating tumor growth and prolactin had no effect on progesterone receptors (Arafah et al, 1980).

Therefore estrogen, but not prolactin, plays a role in the maintenance of progesterone receptors levels, and estrogen and prolactin can act independently to maintain both estrogen and prolactin receptor levels. It has also been reported that progesterone can act to maintain the levels of its own receptor (Asselin et al, 1976).

Although the presence of these hormone receptors in DMBA-induced mammary tumors has been cited as evidence for the hormonal dependence of the tumors, the actual levels of the receptors, particularly of estrogen

and prolactin receptors, are not predictive of responsiveness to endocrine ablation (Arafah et al, 1980; De Sombre et al, 1976).

The role of progesterone in tumorigenesis has been investigated (Jabara, 1967), but its role in the growth of existing tumors is less clear. The apparent assumption has been that since replacement of estrogen to ovariectomized rats caused a resumption of tumor growth, then the effect of ovariectomy was due primarily to the elimination of estrogen. Investigations into the results of replacement of progesterone to ovariectomized rats have been reported. Administration of 0.5 mg per day for 5 weeks following ovariectomy had no effect on tumor growth or hormone binding (Asselin et al, 1977). However, when a higher dose of progesterone (3 mg per day) was given to adrenalectomized-ovariectomized rats, static tumor growth was observed, that is, regression was arrested, but active tumor growth was not obtained (Minasian-Batmanian and Jabara, 1981). The maintenance of tumors by progesterone is independent of serum prolactin levels, since tumor stasis was observed even when prolactin release was inhibited by ergocornine.

In addition to the ovarian and pituitary sex hormones, other hormones which may also play a role in the regulation of growth of DMBA-induced mammary tumors are insulin and glucocorticoids.

When rats bearing DMBA-induced mammary tumors are made diabetic by alloxan (Hueson and Legros, 1970) or the less toxic streptozotocin (Cohen and Hilf, 1974), tumor regression is observed in 90% and 60% of the rats, respectively, and this regression can be reversed by insulin treatment. That the tumor regression is not due to generalized weight loss was demonstrated in experiments involving food restriction (Hueson and Legros, 1972). A relationship between insulin binding and the magnitude

of the biological response was reported, adding further support for the role of insulin in the regulation of rat mammary cancer (Shafie and Hilf, 1981).

A possible role for glucocorticoids in the control of DMBA-induced mammary tumor growth is suggested by the fact that glucocorticoid receptors have been identified in these tumors (Goral and Wittliff, 1975), and that adrenalectomy resulted in stimulation of tumor growth along with elevation of serum prolactin levels (Chen et al, 1976), whereas cortisol inhibited tumor growth and reduced serum prolactin levels. Tumor growth inhibition by dexamethasone in the presence of elevated prolactin levels, due to haloperidol treatment, demonstrated that glucocorticoid inhibition of mammary tumor growth is independent of serum prolactin levels (Aylsworth et al, 1980).

VIII. Mammary Tumor Regression: An Endocellular Event

Gullino and co-workers (1972a, 1972b) have studied regression due to hormonal depletion in DMBA-induced and MTW9 mammary tumors. These authors determined that regression is an endocellular event which does not involve either changes in the host immune response or a decrease in the blood supply to the tumor. Furthermore, there did not appear to be invasion by lymphocytes and macrophages into the regressing tumors; the proportion of the mononuclear immune cells to tumor cells being the same in regressing and growing tumors (Gullino et al, 1972a). In addition, there was no deficiency in the vascular system associated with regressing tumors. The tumors are able to resume growth almost immediately when returned to the proper hormonal environment (Gullino et al, 1972a).

Furthermore, the regression of these hormonally-deprived tumors is not due to degradative enzymes of extracellular origin, as evidenced by the fact that there is no increase in lysosomal hydrolases in the pericellular spaces of regressing tumors (Gullino and Lanzerotti, 1972). Thus, the cell lysis characteristic of regression is an endocellular event which does not affect the survival of neighboring cells.

It is likely that hormone dependent mammany cancer cells are "programmed" to regress upon hormone depletion by cell lysis, just as certain cells are programmed to undergo death in the process of normal vertebrate ontogeny (Saunders et al, 1962). The synthesis of new protein which occurs at the onset of regression (Gullino et al, 1972b) is consistent with the possibility that the regression event is under genetic control, and that the signal for regression is hormone (estrogen/prolactin) withdrawal.

IX. Hormonal Modulation of Prostaglandin Synthesis

That prostaglandins in the mammary gland are sensitive to ovarian and pituitary hormones is suggested by the report that PGE_2 and $PGF_{2\alpha}$ levels in normal mouse mammary gland vary with the stage of the estrus cycle (Knazek et al, 1980).

Rilemma has proposed that prostaglandins may mediate some actions of prolactin in normal murine mammary tissue. Prostaglandin E_2 and $PGF_{2\alpha}$ stimulate RNA synthesis in mammary gland explants in mice in a prolactin-like manner (Rilemma, 1976a). PGE_1 and $PGF_{1\alpha}$ alone had no effect on RNA synthesis, but when given along with prolactin, decreased the response of the tissue to prolactin. Further studies demonstrated that prolactin activated phospholipase A in membrane preparations from

mouse mammary gland (Rilemma and Wild, 1977). Other investigations have studied the possibility that cyclic nucleotides might mediate prolactin effects. Cyclic GMP was shown to mimic prolactin in the stimulus of RNA synthesis (Rilemma, 1975). Unlike prolactin, cGMP did not affect casein synthesis. However, cGMP shortened the time lag between exposure to prolactin and commencement of casein synthesis (Rilemma, 1976b). Agents which increase cAMP appear to impair prolactin responses. Cyclic AMP levels must be decreased or maintained at basal levels for prolactin effects to occur (Rilemma, 1976b).

The possibility that prostaglandin synthesis may also be modulated by estrogen in the DMBA-induced mammary tumor is supported by the fact that estrogens can modulate PG synthesis in another estrogen target tissue, the uterus (Ham et al, 1975). In the uterus, $PGF_{2\alpha}$ plays a central role in luteolysis in response to fluctuations in estrogen in several species including the rat (Ham et al, 1975), guinea pig (Naylor and Poyser, 1975) , and sheep (Caldwell et al, 1972). An increase in $PGF_{2\alpha}$ is always accompanied by a decrease in progesterone. Thus, there is a complex, cyclic relationship between $PGF_{2\alpha}$ and progesterone in the uterus.

In summary, both estrogen and prolactin appear to have the capacity to modulate prostaglandin synthesis and metabolism in target organs.

X. Rationale

The purpose of this study was to identify the intracellular mediators of the hormonal control of growth in hormone dependent mammary cancer. Prolactin and estrogen have been implicated as growth promoters of hormone dependent mammary cancer in the human and in several animal

models. The mechanism of estrogen action, through binding to an intracellular receptor, is well established, but the intracellular mediator for prolactin has not been identified in normal or neoplastic tissue. Recent evidence implicates prostaglandins and possibly subsequent changes in cyclic AMP as the intracellular mediators (Rilemma, 1975, 1976b).

The objectives of this project were to test the independent and combined effects of estrogen and prolactin on growth response and prostaglandin synthesis in the hormone dependent DMBA-induced mammary tumor in the rat, to determine if prolactin modulates PG synthesis in these tumors by measuring prostaglandin synthesis in tumors from rats with elevated or decreased prolactin and to determine if the synthesis of the prostaglandins (specifically PGE_2) correlates with the growth response of the mammary tumors which accompany the hormonal changes. In addition, it was the purpose of this project to determine if cAMP levels correlate with the tumor growth response due to hormonal changes, to investigate cAMP as a possible mechanism of prostaglandin action.

If PGE_2 is an intracellular mediator of hormone action, then PGE_2 levels and subsequently cAMP levels should correlate with the presence or absence of physiological concentrations of estrogen and prolactin as well as with the growth response of the tumor.

A further objective of this work was to identify the nuclear site of action of cAMP in the regressing mammary tumor and to study this in relation to the sequence of events initiated by ovariectomy. A final objective was to demonstrate directly that PGE_2 does in fact enhance the synthesis of cAMP in regressing mammary tumors. An understanding of the sequence of biochemical events central to the regression process may

contribute to understanding possible deficiencies in control which occur in hormonally-dependent tumors.

MATERIALS AND METHODS

I. Tumor Induction by 7,12-Dimethylbenz(a)anthracene

The tumor model used throughout most of these studies was the dimethylbenz(a)anthracene (DMBA)-induced mammary tumor in female Sprague-Dawley rats. The rats were an outbred strain obtained from Lab Supply, Indianapolis, Indiana. The carcinogen was consistently administered at 50 days of age, since this age has been reported to be optimal in terms of tumor incidence and survival of the animal (Huggins, 1979). During the course of these studies, three different methods of exposure or protocols for dosing were used. In the first method, a 1 ml emulsion of 5 mg of DMBA, as obtained from Dr. Paul Schurr (Upjohn Co., Kalamazoo, Michigan) was injected into the tail vein of 50-day old female Sprague-Dawley rats (Schurr, 1969). A second method of exposure consisted of gastric intubation of 20 mg of DMBA in 1 ml of sesame oil (Huggins, 1961). Solution of the DMBA in sesame oil was achieved by gentle heating. This solution was intubated into the stomach of the rat using a curved 16 gauge x 3 inch animal feeding needle (Popper and Sons, Inc., New Hyde Park, New York).

In an attempt to increase the fraction of treated rats having tumors, a procedure involving multiple exposures of lower doses of DMBA was employed (Cohen and Hilf, 1974). The procedure was modified to consist of four weekly doses of DMBA. The first dose, given at 50 days of age by gastric intubation, consisted of 10 mg DMBA in 0.5 ml of sesame

oil; this was followed by three weekly doses of 5 mg DMBA in 0.25 ml of sesame oil. The third procedure resulted in earlier overall appearance and high tumor incidence in our hands. Compared to a single gastric intubation, multiple exposures decreased the average tumor latency from 4.5 months to 3 months; with the earliest tumors appearing at 45 days after DMBA exposure.

At one point in the study, it became necessary to supplement the animal colony with tumor bearing rats from Hazelton Laboratories (Vienna, Virginia).

II. MTW9 Mammary Tumors

Several distinct lines of the MTW9 mammary tumor in Wistar-Furth rats were used as another model of hormone dependent mammary cancer in some of the experiments. The MTW9 series of tumors was provided by Untae Kim of Roswell Park Memorial Institute, Buffalo, N.Y.; the tumors were maintained by serial transplantation in 60 day old female Wistar-Furth rats. Three stages of hormonal progression are represented by the MTW9 tumor lines. The MTW9-A tumors are slow-growing, estrogen-dependent and hormonally responsive, while MTW9-B tumors are hormonally autonomous with an intermediate growth rate. The MTW9-D tumors represent advanced hormonally autonomous fast-growing tumors. When endocrine ablation, such as ovariectomy, is performed, the MTW9-A tumors regress, whereas the MTW9-B and MTW9-D tumors continue to grow.

III. Vernier Caliper Measurements of Tumors

The growth of tumors *in vivo* can be assessed by monitoring their dimensions. The size of the DMBA-induced mammary tumors was measured

t specific intervals using vernier calipers. The measurements recorded were the longest diameter of the tumor and its perpendicular. Care was taken to ensure that the pressure of the calipers against the tumor was consistent. All tumor measurements within a given experiment were made by the same investigator (MKF).

To determine if a significant change in the size of a tumor had occurred, the sum of the length plus width of the tumors was compared over a selected time interval. More specifically, if an increase of 20% or greater was observed in the sum of length plus width compared to the measurement at the beginning of the treatment period, the tumor was classified as growing. Alternatively, if the sum of the diameters decreased by 20%, the tumor was classified as regressing. If the change in length plus width was less than 20%, the tumor was considered static.

IV. Modifications of Tumor Growth by Hormonal Treatments

The DMBA-induced mammary tumor in Sprague-Dawley rats is dependent on hormonal stimulation for growth. Ovariectomy of rats bearing DMBA-induced mammary tumors results in regression of the tumors in 80-90% of the rats (Huggins, 1979). Replacement of estrogen in ovariectomized rats provides the hormonal requirements for growth of these carcinogen-induced tumors (Talwalker et al, 1964). These results suggest that estrogen is of primary importance in the maintenance of growth of DMBA-induced mammary tumors.

However, drugs which decrease endogenous prolactin, such as ergocornine or ergocryptine, inhibit the growth of existing tumors and cause regression of the tumors (Cassell et al, 1971). Since estrogen stimulates serum and pituitary prolactin levels (Reece and Turner, 1936;

Grosvenor and Turner, 1960; Chen and Meites, 1970), it is not clear whether the depletion of estrogen upon ovariectomy or the subsequent decrease in prolactin levels is more important in the tumor regression which ensues.

In order to characterize hormonal dependence of the mammary tumors and to correlate hormonal stimulation of tumor growth with biochemical changes, various hormone manipulations were performed in tumor-bearing rats as described below.

Bilateral ovariectomy was performed to deplete estrogen levels. Prolactin levels were altered using injection of appropriate drugs. Various combinations of these treatments were performed in order to further characterize the hormonal dependence of the DMBA-induced mammary tumors.

a. Elevation of Endogenous Prolactin by Haloperidol

Endogenous prolactin levels were elevated in tumor bearing rats by injection of haloperidol (4'-fluoro-4[4-hydroxy-4(4-chloro-phenyl)-piperidino]-butyrophenone), which was a gift from MacNeil Laboratories, Fort Washington, Pa. Haloperidol has been shown to increase release of prolactin from the anterior pituitary by inhibiting the activity of Prolactin Inhibitory Factor (PIF) from the hypothalamus (Dickerman et al, 1972). Haloperidol and other neuroleptic drugs, such as reserpine, chlorpromazine and others, are thought to inhibit PIF due to their ability to inhibit catecholamines as neurotransmitters in the hypothalamus (Abuzzahab, 1971; Meites et al, 1972). Hypothalamic catecholamines act as neurotransmitters to increase the release of PIF,

which in turn depresses release of prolactin from the pituitary; therefore, lowering catecholamine activity increases prolactin.

In the first study, haloperidol was injected subcutaneously each of 4 consecutive days at a dose of 120 µg in 0.2 ml olive oil (Bradley et al, 1976). Control rats received 0.2 ml injections of olive oil. Tumor prostaglandin content was measured on the fifth day after beginning the treatment. The rats were decapitated and the tumors were rapidly excised and frozen in liquid nitrogen (-196°C).

On a modification of the above protocol, used in a separate experiment, an aqueous vehicle for haloperidol was used (after Dickerman et al, 1972) and the drug injections were extended to 10-14 days. The injection dose of 120 µg/0.2 ml was prepared by dissolving 6 mg haloperidol in 0.125 ml of 1N HCl, 0.25 ml ethanol and 9.625 ml of distilled water. Tumor-bearing rats were injected subcutaneously for 10-14 days, and paired control rats were injected with vehicle alone. At the end of the treatment period, the rats were decapitated and the tumors were rapidly excised and used immediately for determination of prostaglandin release *in vitro*.

b. Depletion of Endogenous Prolactin by Ergocornine

For depletion of endogenous prolactin levels, the ergot alkaloid ergocornine (as ergocornine hydrogen-maleate, a gift from Prof. E. Fluckiger, Sandoz Ltd., Basel, Switzerland) was used. Ergocornine (EC) lowers serum and pituitary prolactin levels (Nagasawa and Meites, 1972a) by increasing hypothalamic PIF activity (Wuttke et al, 1971) and also by directly inhibiting prolactin release from the pituitary (Meites et al, 1972).

The EC was administered subcutaneously at dose of 0.5 mg per 250 gm rat daily for 10-14 days. In the studies 12.5 mg EC was dissolved in 1 ml 70% ethanol, then diluted with 4 ml of 0.85% NaCl to a final concentration of 14% ethanol (Bradley et al, 1976). The drug was administered in a total volume of 0.2 ml.

V. Measurement of Tumor Prostaglandin Content

a. Reagents and Chemicals

Glass microfibre filter discs (GF/B) were purchased from Whatman, Inc., Clifton, New Jersey. Brock Mini-Columns were obtained from Anderson Glass Company, Fitzwilliam, New Hampshire. Silicic acid, SIL-R, 100-300 mesh, was obtained from Sigma Chemical Company, St. Louis, Missouri. Benzene, ethyl acetate and methanol of spectroquality, were purchased from MCB Manufacturing Chemists, Cincinnati, Ohio. Ethyl acetate was redistilled from glass before use. [5,6,8,11,12,14,15-^{3}H(N)]-Prostaglandin E_2 (150-165 Ci/mmol) was obtained from New England Nuclear, Boston, Mass. Activated charcoal was purchased from Sigma, while Dextran T-70 (MW 70,000) was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden.

b. Homogenization of Frozen Tumors

Tumor samples were prepared for radioimmunoassay in groups of 8. Tumor specimens stored in liquid nitrogen were pulverized in a stainless steel mortar and pestle, which had been previously cooled in liquid nitrogen. The powdered tissue was weighed into a tared tissue grinder with a teflon pestle (150 mm x 32 mm grinding area, 55 ml capacity), then immediately placed in ice. The powdered tissue was resuspended in

9 volumes of cold 50 mM Tris - 44 mM EDTA, pH 7.5, then homogenized at 0-4°C. One ml aliquots of each homogenate were placed into duplicate test tubes containing 100 µl of 1 M citric acid. A "trace" of ^{3}H-PGE_2 (1500 cpm in 50 µl) was added to each sample to follow recovery throughout the purification procedure. Duplicate blanks containing 1.0 ml of 50 mM Tris-44mM EDTA, pH 7.5, 100 µl citric acid and 1500 cpm ^{3}H-PGE_2 were subjected to the same extraction and purification procedure as the samples.

c. Extraction of Prostaglandins

The extraction and purification procedures were based on the methods of Auletta et al, (1974). Prostaglandins were extracted from the homogenized tumor tissue into cold redistilled ethyl acetate. Five ml of ethyl acetate was added to each sample, which was then vortexed for two 30 second intervals, followed by centrifugation for 10 minutes at 2000 rpm in a Model HN-S Centrifuge (International Equipment Co., Needham, Mass.) The ethyl acetate fraction (upper layer) was drawn off with Pasteur pipettes and placed into individual 16 X 125 mm culture tubes. The entire extraction sequence was repeated with 3 ml of cold redistilled ethyl acetate, and the upper layer of each sample was combined with the first fraction. The samples were dried under a stream of air prior to separation of the PG fractions on columns of silicic acid.

d. Silicic Acid Column Chromatography

Prostaglandins were fractionated on 0.5g silicic acid mini-columns. Glass fibre filter paper discs, cut to the inner diameter of the columns by use of a No. 4 cork borer, were seated into the bottom of Brock

mini-columns which were 9 mm inner diameter and 160 mm in length. The columns were set up in a rack which was designed to accommodate 20 columns at a time. Culture tubes (16 x 125 mm) were set up beneath each column to catch the effluent.

A slurry of silicic acid was prepared by placing 12.5 g of silicic acid (SIL-R, Sigma Chemical Co., St. Louis, Mo.) in a 50 ml Erlenmeyer flask, then bringing the volume to 50 ml by the addition of 60:40:2 (benzene:ethyl acetate:methanol, v:v:v). Two ml of the silicic acid slurry was pipetted rapidly into each mini-column with continuous stirring using a Clay-Adams 1 ml pipette gun, with a wide bore. After the silicic acid was washed down the sides of the columns with 3-5 ml of 60:40:2 (B:EA:MeOH) the effluent from the columns was collected prior to sample loading.

During the preparation of the columns, 0.2 ml of 60:40:10 (B:EA:MeOH) was added to each sample tube. In order to ensure complete reconstitution of the extracted lipids, the samples were allowed to leach for 30 minutes, with occasional vortexing. The samples were then loaded onto the columns using Pasteur pipettes. Subsequently, 0.6 ml of 60:40 (B:EA) was added to each sample tube, the tubes were briefly vortexed, and then the contents were added to the appropriate columns.

After the resulting effluent had been collected, 1 ml of benzene was added to each column to remove neutral lipids. After this solvent had run through, 4.0 ml of 60:40 (B:EA) was added to each column, to elute prostaglandins of the families A and B. The combined eluates of the columns up to this point were discarded, and 20 ml scintillation vials were set up in the rack under each column. The E prostaglandins,

eluted from the columns in 13 ml of 60:40:2 (B:EA:MeOH), were stored at -20° overnight for subsequent radioimmunoassay.

For the PGE_2 radioimmunoassay, (as described below) the samples obtained from the columns were dried under a stream of air, then taken up in 1.0 ml of ethanol. 200 µl of each sample was removed for liquid scintillation radioassay in order to estimate the recovery from the columns. In general, 60-70% recovery was achieved, and the specific recovery value for each sample was used in the calculation of PGE_2 content.

Three dilutions of each sample were included in the radioimmunoassay. Typically 1/20 (50µl), 1/10 (100µl) and 1/5 (200 µl) dilutions of the samples in ethanol were placed in 12 x 75 mm culture tubes and dried. All the samples were subsequently taken up in 100 µl of assay buffer.

VI. Radioimmunoassay for PGE_2

The assay buffer used in PGE_2 radioimmunoassay (RIA) consisted of 10 mM sodium phosphate, pH 6.7, 0.9% NaCl, 0.1% bovine gamma globulins and 0.1% sodium azide.

The ^3H-PGE_2 used in the RIA was purified by silicic acid column chromatography according to the procedure described above. The repurified ^3H-PGE_2 was taken up in assay buffer to give 10,000 cpm per 100 µl and stored at -20°C for no more than 1-2 weeks.

The antiserum to PGE_2, which was developed in rabbits to the albumin conjugate of PGE_2, was provided by Dr. Thomas Ferris, Department of Medicine, Ohio State University. The stock antiserum was stored as 100 µl aliquots at -20°C, then diluted 500-fold with assay

buffer immediately before use. The antibody titer selected is that which results in approximately 50% binding (ratio of trace to total labelled antigen) since this is the titer which results in maximum sensitivity (Skelley et al, 1973).

The specificity of the PGE_2 antibody is shown in Figure 2. Prostaglandin E_1 showed 32% cross-reactivity with the antibody to PGE_2. The cross-reactivity of $PGF_{2\alpha}$ to the PGE_2 antibody was 2.5% and $PGF_{1\alpha}$ showed 0.6% cross-reactivity. The values for cross-reactivity are calculated by the ratio of the amount of PGE_2 to the amount of competing prostaglandin which results in 50% binding.

Prostaglandin E_2 was a gift from Dr. John Pike, Upjohn Company. A stock standard solution containing 1 mg PGE_2 in 10.0 ml ethanol was prepared and stored at -20°C under nitrogen. From this solution, 100 µl was taken and diluted to 10 ml in ethanol, giving a 1 µg/ml solution. Standard solutions, ranging from 2.5 pg PGE_2/0.1 ml to 10 ng PGE_2/0.1 ml were prepared in ethanol from the second stock standard and stored at -20°C under nitrogen.

a. RIA Protocol

The RIA protocol for assay of PGE_2 was modified from Caldwell et al (1971; also Auletta et al, 1974). When the RIA was used to determine PGE_2 in samples which were in ethanolic or organic solution, the samples were placed in glass 12 x 75 mm culture tubes and dried under a stream of air. The blanks, trace and standards were set up in duplicate. The samples had been homogenized in duplicate, as stated earlier.

After all the ethanolic samples were dried, 100µl of assay buffer was added to each tube. 200 µl assay buffer was added to the tubes which

Figure 2. Specificity of Antiserum to PGE_2

Unlabelled PGE_2, PGE_1, $PGF_{2\alpha}$ and $PGF_{1\alpha}$ were incubated in the presence of ^3H-PGE_2 (10,000 cpm) and antibody to PGE_2 according to the RIA protocol. The cross-reactivity was calculated by computing the ratio of unlabelled prostaglandin which gave 50% binding to the concentration of PGE_2 which gave 50% binding. The cross-reactivities of the prostaglandins were PGE_1-32%, $PGF_{2\alpha}$-2.5% and $PGF_{1\alpha}$-0.6%.

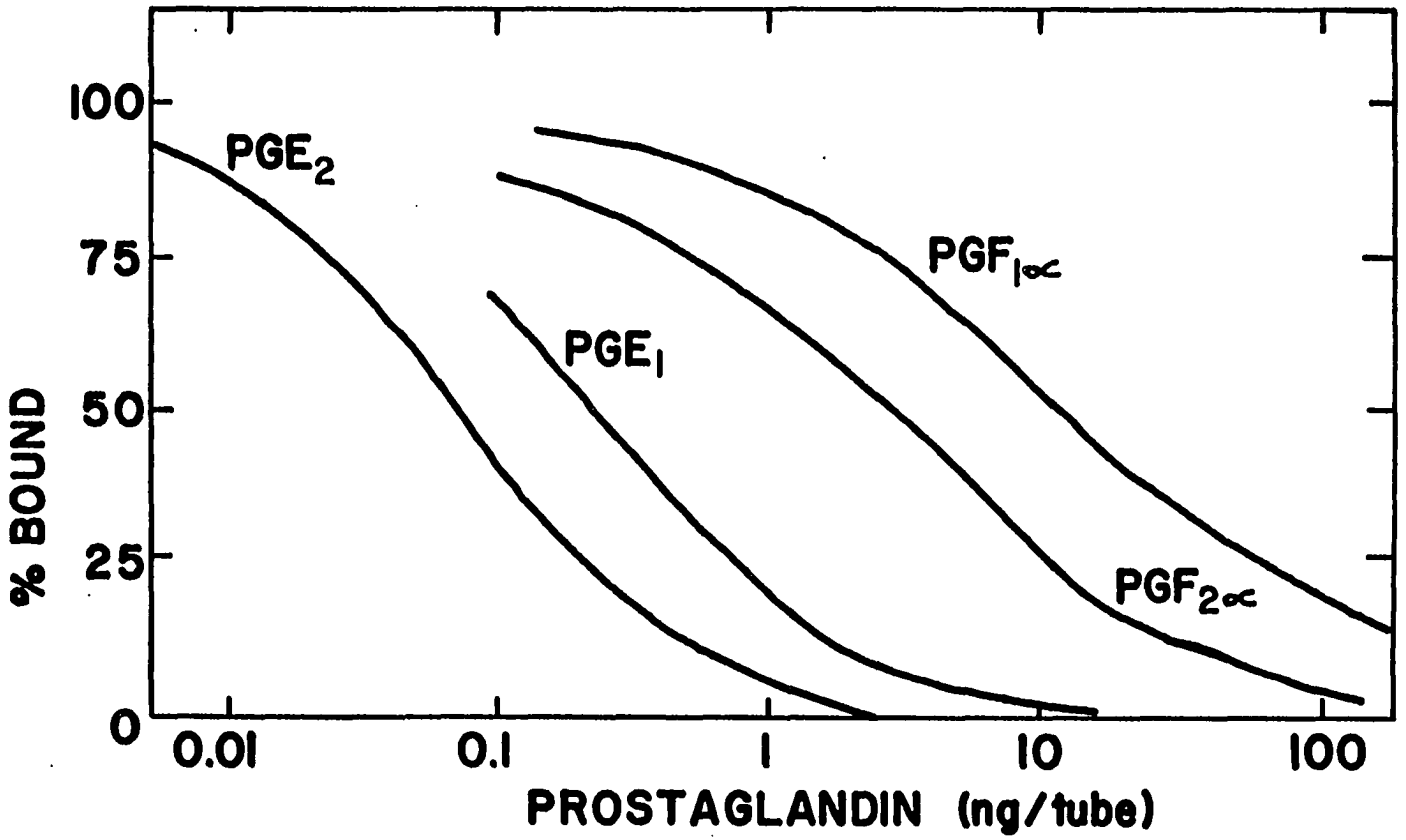


Figure 2. Specificity of Antiserum to PGE_2

served as blanks. Next, 100 µl of ^{3}H-PGE_2, which had 10,000 cpm in 100 µl of assay buffer, was added to each tube. In addition, 100µl of ^{3}H-PGE_2 was placed into each of duplicate scintillation vials for measurement of "total" PGE_2. Finally, 100 µl of a 1:500 dilution of the antibody to PGE_2 was added to all the tubes except the blank tubes. The incubation volume was 300 µl. The tubes were vortexed twice, then incubated overnight at 4°C.

Separation of the bound antigen-antibody complex from free antigen was achieved by treatment with dextran-coated charcoal. The charcoal-dextran solution was made fresh within 24 hours of use for each assay. Dextran T-70 (25 mg) was dissolved in 100 ml of cold PG assay buffer, then placed on a magnetic stirrer. To this solution was added 250 mg of activated charcoal. This solution was stirred on a magnetic stirrer for 1 hour at 4°C, whether stored at 4°C overnight or used immediately. The temperature of the charcoal dextran extraction is critical, since at room temperature, the charcoal-dextran will strip the bound antigen from the antibody.

While the charcoal-dextran solution was continuously stirred on ice, 1 ml was rapidly added to each of the assay tubes, using a 1 ml Clay-Adams pipette. The tubes were incubated at 4° for 15 minutes, then centrifuged at 3000 rpm for 15 minutes in a CRU -5000 centrifuge. One ml of the clear supernatant was transferred from each tube to a scintillation vial, 10 ml of Beckman Ready-Solv EP scintillation fluid was added, and the vials were counted in Beckman LS-7100 or LS-8100 Liquid Scintillation Counter.

TABLE 1

Radioimmunoassay for Prostaglandin E_2

	Ethanol Samples and Standards	Assay Buffer	^{3}H-PGE$_2$ (10,000 cpm)	1:500 dilution of antiserim
Blank	--	200µl	100µl	--
Trace	--	100µl	100µl	100µl
Standards	100µl	100µl	100µl	100µl
Samples	50µl, 100µl or 200µl ↓ Dry under stream of air	100µl	100µl	100µl

b. Calculations

Corrected bound = sample cpm - blank

% Bound = percentage of trace

$$= \frac{\text{corrected bound}}{\text{Trace}} \times 100$$

A standard curve was obtained by plotting % Bound vs. pg PGE_2 on 4 cycle semi-log graph paper. The resulting curve was sigmoidal. PGE_2 values of the samples were interpolated from the standard curve. Each assay included a standard curve.

VII. Radioimmunoassay of 6-Keto-$PGF_{1\alpha}$

Prostacyclin is a short-lived molecule in aqueous solution at neutral pH, with a half-life of 3.5 minutes at 25°C and 14.5 minutes at 4°C. (Cho and Allan, 1978). Therefore, 6-keto-$PGF_{1\alpha}$, the metabolite of PGI_2, was measured by radioimmunoassay as a reflection of PGI_2 production in tumor slices.

6-Keto-$PGF_{1\alpha}$ was a gift of Dr. John Pike, Upjohn Company. The labelled antigen, 6-[5,8,9,11,12,14,15-^{3}H(N)]-keto-prostaglandin $F_{1\alpha}$ (120 Ci/mmole) was purchased from New England Nuclear. The specific antibody to 6-keto-$PGF_{1\alpha}$ was obtained from Seragen Inc. (Boston, Mass.). The antiserum to 6-keto-$PGF_{1\alpha}$ cross-reacted with competing prostaglandins to the following extents: $PGF_{2\alpha}$ - 9.5%; PGE_2 - 3.0%; PGA_2 - <0.2%; PGD_2 and TxB_2 - <0.1% (as reported by Seragen). The assay buffer for the 6-keto-$PGF_{1\alpha}$ RIA was composed of 50 mM Tris, pH 7.6, 150 mM NaCl and 0.1% bovine serum albumin (Cohn fraction V). The standard curve for the assay ranged from 10-8000 pg 6-keto-$PGF_{1\alpha}$. The standards were prepared in the Tris-saline-albumin buffer and stored at 4°C.

The protocol for the 6-keto-$PGF_{1\alpha}$ RIA was a modification of the procedure recommended by Seragen. The assay tubes (12x75 mm) were numbered and set up in a rack in an ice bath (4°C). To each tube was added 200µl of the Tris-saline-albumin buffer. Then 100µl of the standards or samples in assay buffer was added to the appropriate tubes. The blank tubes received 200µl assay buffer and the trace tubes received 100µl assay buffer. ^{3}H-6-Keto-$PGF_{1\alpha}$ was diluted in Tris-saline-albumin buffer to a specific activity of 10,000 cpm/100µl, and 100µl of this solution was added to each tube and to each of 2 scintillation vials, for confirmation of total counts.

The lyophilized antibody was reconstituted in 20 ml of Tris-saline-albumin buffer to give an amount sufficient for 200 tubes. To each tube, except the blanks, was added 100 µl of reconstituted antibody. The tubes were vortexed and incubated overnight at 4°C.

The following day, the free antigen was separated from the bound antigen using the charcoal-dextran differential adsorption technique. The charcoal-dextran solution was prepared by adding 25 mg Dextran T-70 and 250 mg charcoal to 50 ml of Tris-saline-albumin buffer. Each tube received 500 µl of the 4°C charcoal dextran solution. The tubes were vortexed, then immediately centrifuged at 3000 rpm for 15 minutes in an IEC CRU-5000 centrifuge. The supernatant fluids from the tubes were decanted into scintillation vials for counting. The 6-keto-$PGF_{1\alpha}$ content per tube was calculated by determining percent of Trace for both standards and samples, as described earlier, and by interpolating the sample values from the standard curve.

TABLE 2

Radioimmunoassay of 6-Keto-$PGF_{1\alpha}$

	Tris-saline Albumin buffer	Sample or Standard	^{3}H-6-keto-$PGF_{1\alpha}$ (10,000 cpm/100µl)	Antiserum to 6-keto-$PGF_{1\alpha}$
Blank	400µl	--	100µl	--
Trace	300µl	--	100µl	100µl
Standards	200µl	100µl	100µl	100µl
Unknown Samples	200µl	100µl	100µl	100µl

VIII. Radioimmunoassay of cAMP

^{125}I-succinyl-cAMP-tyrosine methyl ester (hereafter referred to as ^{125}I-ScAMP-TME) and the antiserum to cAMP were kindly supplied by Dr. Richard Fertel of the Department of Pharmacology, Ohio State University.

The antibody to cAMP was developed in New Zealand white rabbits to ScAMP-TME coupled to keyhole limpet hemocyanin. The titer used for the assays varied with each new batch of labelled antigen. The specificity of the cAMP antiserum was very good. The cross-reactivity of the cAMP antiserum against 5'AMP, ATP, 5'GMP, GTP, ADP and GDP was less than 0.001%. The cross reactivity of the cAMP antiserum to cGMP was 0.1%. The percentages were calculated by determining the concentration of the specific antigen which results in 50% binding relative to the concentration of competing compound which results in 50% binding.

a. Tissue Processing

Tumor-bearing rats were decapitated, and the tumor tissue was rapidly excised and frozen in liquid nitrogen (-196°C) until processing and assay. Approximately 300-400 mg of tissue was pulverized in a stainless steel mortar and pestle which had been previously cooled in liquid nitrogen. Duplicates of each specimen were made. Therefore, 100-200 mg of pulverized tissue was weighed into a tared tissue grinder (A.H. Thomas Co., Philadelphia, Pa.) to which 2 ml of cold 5% trichloroacetic acid (TCA) was added. The tissue samples were kept at liquid nitrogen temperature until homogenization in TCA and subsequently on ice (4°C).

The homogenates were transferred to 16.1 x 81.4 mm polycarbonate round bottom centrifuge tubes (Oak Ridge type) using Pasteur pipettes.

For convenience in handling, 6 samples were processed at a time in this manner (giving 12 centrifuge tubes). The samples were centrifuged in a Beckman L3-40 Ultracentrifuge, using a type 40 rotor, at 34,000 rpm (100,000 xg) for 30 minutes.

The supernatant was then drawn off into large test tubes (approximately 16 x 150 mm test tubes or culture tubes). The TCA was extracted from the samples with water-saturated ether (24 ml distilled water per liter of ether). The extraction procedure, in which 5 ml of water saturated ether was added to the tube, vortexed and then drawn off, was repeated three times. The tubes were then heated at 50°C in a water bath for 5-10 minutes to evaporate the remaining ether. The samples were transferred to 12 x 75 mm disposable culture tubes. The pH of the samples was brought to 5.9 - 6.0 by addition of 50-100 µl of 1 M sodium acetate buffer, pH 6.5. The pH was tested with Fisher Scientific Short Range Alkacid Test Papers. One to ten (1:10), 1:50 and 1:100 dilutions were made in 0.05 M sodium acetate buffer pH 6.2. The samples were stored at -20°C until the assay was performed. When stored at -20°C at this stage, the samples were stable for months.

b. Radioimmunoassay Standard Curve Preparation

A vial containing an aliquot of the cyclic AMP standard containing 100 pmoles cAMP per ml, and stored at -20°C, was thawed just prior to use. Each vial was used only once, then discarded, since with repeated freezing and thawing the concentration is likely to change due to water condensation. The standard curve contained 13 points ranging in concentration from 0.00125 pmoles cAMP per tube to 5 pmoles per tube. These standards were made from serial dilutions (1:2) of the stock standard.

c. The Radioimmunoassay Protocol

The cAMP radioimmunoassay was adapted from the method of Steiner (1974). The modification used was that of Dr. Gopi Tejwani (personal communication).

The assay tubes (12 x 75 mm culture tubes) were numbered and set up, allowing tubes 1 and 2 for Blank, 3 and 4 for Trace and 5 through 30 for the standard curve of 13 points. The remaining tubes were numbered allowing for duplicates of each of the unknown samples. The procedure given in the following paragraphs is summarized in Table 3. All additions were made by Eppendorf pipette or by variable volume Pipetman pipettes. 100 µl of 0.05 M sodium acetate buffer pH 6.2 was added to tubes 1, 2, 3 and 4. To tubes 5 through 30 were added 100 µl of the corresponding standards which had been set up previously. 100 µl of each sample was added to the remaining tubes in duplicate.

An acetylation of the samples was done to raise the reactivity of the cAMP with the antiserum, since the antiserum was developed to the succinyl derivative of cAMP associated with protein. The acetylating reagent, which was made fresh for each assay, contained 0.4 ml of acetic anhydride and 1.0 ml of triethylamine (Frandsen and Krishna, 1976). The acetylation procedure was carried out in the hood. While each tube was being stirred on the Vortex, 10 µl of acetylation reagent was added, using a 10 µl Eppendorf pipette. The samples were then incubated for 10 minutes at room temperature. After this point, the tubes were kept on ice (at 4°C) for the remainder of the assay. To tubes 1 and 2 was added 0.35 ml of 0.05 M sodium acetate buffer pH 6.2, using a variable volume Pipetman pipette. 0.30 ml of 0.05 M sodium acetate was added to the remaining tubes in the assay. The vial containing ^{125}I-ScAMP-TME,

TABLE 3

RADIOIMMUNOASSAY FOR CYCLIC NUCLEOTIDES

Samples	Acetylation reagent	0.05 M Acetate buffer pH 6.2	1:100 dil. ^{125}I-cyclic (AMP) Succinyl TME derivative (diluted in 0.05 M Acetate buffer pH 6.2 containing 0.5% bovine globulins)	1:1000 dil. cyclic AMP (GMP) antisera (dil. in 0.05 M acetate buffer pH 6.2 containing 1% bovine serum albumin)
1. Blank 100 µl 0.05 M acetate buffer pH 6.2	10 µl	0.35 ml	50 µl 5000 cts/min	None
2. Trace 100 µl 0.05 M acetate buffer pH 6.2	10 µl	0.3 ml	50 µl	50 µl
3. Standards 0.001 to 5 p moles dil. in 0.05 M acetate	10 µl	0.3 ml	50 µl	50 µl
4. Unknown Samples 100 µl 0.05 M acetate buffer pH 6.2	10 µl	0.3 ml	50 µl	50 µl
5. Total T_1, T_2, T_3	None	None	50 µl	None

which was stored frozen (-20°C), was thawed. This vial contained a 1:100 dilution of ^{125}I-succinyl-cAMP, tyrosine methyl ester (TME) derivative, in 0.05 M sodium acetate buffer pH 6.2 with 0.5% bovine gamma globulin. This reagent was not thawed and refrozen more than once. Three tubes were labelled T_1, T_2, and T_3. Fifty (50)µl of ^{125}I-ScAMP-TME was added to these tubes, as well as to all the tubes in the assay. The cAMP antiserum was stored at -20°C in 100 µl aliquots as a 1:10 dilution. One aliquot (100 µl) was thawed and diluted to a final dilution of 1:1000 in 0.05 M sodium acetate buffer pH 6.2 containing 1% bovine serum albumin. At this point, tubes 1 and 2 were covered with parafilm and 50 µl of the diluted antibody were added to all the other tubes (except T_1, T_2, and T_3 which were set aside). All the tubes were vortexed. The rack was then covered with aluminum foil and incubated overnight at 4°C. (Optimum incubation time was found to be 18 hours).

The following day, 2.5 ml of 60%-saturated ammonium sulfate (195 g in 500 ml distilled water) was added to all the tubes (except total) using a Cornwall syringe. The contents of the tubes were vortexed thoroughly, then incubated for 20 minutes on ice to salt out the bound antigen-antibody complex. After incubation, the tubes were centrifuged in an IEC CRU-5000 centrifuge at 3000 rpm at 4°C for 20 minutes and the supernate from all tubes except "total" was discarded. The tubes were then thoroughly drained.

The precipitates in the tubes were counted in a Beckman gamma counter for 2 or 5 minutes. The total tubes generally had a count of roughly 10,000-20,000 cpm.

d. Calculations

Blank = averages of tubes 1 and 2

Trace = (average of tubes 3 and 4) - blank

Corrected Bound = average of duplicate tubes - blank

% Bound = percentage of trace

$$= \frac{\text{corrected bound}}{\text{Trace}} \times 100$$

The standard curve was set up by plotting % Bound vs pmoles cAMP on 3 or 4 cycle semi-log paper. The standard curve was sigmoidal. The value obtained from the standard curve was called graphical pmoles cAMP.

The cAMP of the specimen was calculated as follows:

$$\frac{\text{pmoles cAMP}}{\text{mg protein}} = \frac{\text{graphical pmoles} \times 20}{\text{mg protein}}$$

The fact that 0.1 ml of a total volume of 2 ml of sample was used for cAMP assay is the basis of the dilution factor of 20. The ratio of Trace to total is used to calculate the ratio of bound antigen to free antigen. For maximum sensitivity a ratio of Bound to Free of 50% is desirable (Skelley et al, 1973).

IX. Measurement of Prostaglandin Release from Tumor Slices

The production of prostaglandins by tumor tissue was determined by measuring the release of prostaglandins from tumor slices into an aqueous incubation medium. The method for tumor slices was modified from the method described for measuring prostaglandin release from rat aorta (Panganamala et al, 1981). Fresh tumor tissue, obtained from a decapitated rat, was divided into 4 approximately equal aliquots prior to weighing. Each aliquot (usually 100-200 mg) was sliced to a thickness

of 0.35 mm using a McIlwain Tissue Chopper. The chopping surface was cooled to 4°C prior to slicing, and the slicing operation was carried out in the cold room to avoid warming of the tumor tissue. The slicing of the tissue to the thickness of 0.35 mm ensures optimal diffusion of oxygen to all parts of the tissue and release of exported metabolities to the extracellular medium (McIlwain, 1975). The sliced tumors were placed into 16 x 125 mm culture tubes and washed in 0.5 ml of cold (4°C) incubation buffer (50 mM Tris, 150 mM NaCl, pH 8.0) for 5 minutes. The cold buffer was discarded and replaced with 1.0 ml of 20°C incubation buffer at specific intervals. Triplicate analyses were run on each tumor while the fourth tumor fragment was incubated in the presence of 300 μM indomethacin, which inhibits prostaglandin production. The samples were incubated at 20°C for 1 hour with gentle agitation, and aliquots of 40-60μl were removed at 6 time points during the incubation, as shown below.

Time (minutes)	4	8	12	20	30	60	Extract
Aliquot Volume (μl)	60	60	60	40	40	40	80

These aliquots were brought to 200 μl with 50 mM Tris, pH 8.0, 150 mM NaCl, containing 0.1% bovine serum albumin and stored in parafilm sealed tubes at 4°C until radioimmunoassay, which was usually performed within 7 days. An alternative buffer for storage was modified PGE_2 RIA buffer, which was 10 mM phosphate pH 6.7, 150 mM NaCl, 0.1% sodium azide and 0.1% bovine serume albumin (Cohn fraction V).

Dilution of the aqueous aliquots from the tumor-slice incubation to a specific volume permitted measurement of several different prostaglandin species by the corresponding specific radioimmunoassays.

When the PGE_2 RIA was used to determine PGE_2 in the aqueous samples a few minor changes were made in the standard RIA protocol. The assay buffer was modified (see above) by exchanging albumin for bovine gamma globulin. The standards were also prepared in the modified assay buffer and stored at 4°C. Further, the RIA incubation mix was modified in the following manner: An aliquot of 50 µl was removed from the total sample of 200 µl and placed into 12 x 75 mm tubes for RIA. To these samples were added 50 µl of RIA buffer, followed by 100 µl of ^3H-PGE_2 (10,000 cpm) and 100 µl of diluted antiserum. The remainder of the assay was performed as described earlier.

In Summary:

	Aqueous Samples or Standard (µl)	Assay Buffer (µl)	^3H-PGE_2 (10,000 cpm/100µl)	1:500 dilution of antiserum (µl)
Blank	---	200	100	---
Trace	---	100	100	100
Standards	100	---	100	100
Samples	50	50	100	100

Each tumor segment was extracted with ethanol at the end of the incubation period. Following removal of the 60 minute aliquot, the volume remaining in the incubation mix was 700 µl. An equal volume of ethanol was added to each tube and the contents were vortexed for 30 seconds to extract the prostaglandins. Eighty (80) µl was removed from

this extraction mix, dried under a stream of nitrogen gas, and reconstituted in 200 µl of modified assay buffer for subsequent RIA. The prostaglandin content of these tubes was compared to the 60 minute time points in order to demonstrate that the prostaglandins measured in the aqueous incubation mix were a true reflection of the prostaglandin synthesis in the tumors. This comparison showed that 90% ± 3% (mean ± S.E.) of the PGE_2 found in the ethanol extract of the sample was present and measured in the aqueous medium at the 60 minute time point. Thus, most of the synthesized prostaglandins are released to the medium and are not bound by the tissues.

Representative time courses for the release of PGE_2 and 6-keto $PGF_{1\alpha}$ from tumor slices are shown in Figures 3 and 4, respectively. The curves shown are the means of 3 determinations on a single tumor. No PGE_2 or 6-keto-$PGF_{1\alpha}$ release was detected from tissue slices incubated in the presence of 300 µM indomethacin. Furthermore, extraction of these tumors at the final (60 min) time point showed that PGE_2 and 6-keto-$PGF_{1\alpha}$ levels were undetectable, implying that the inhibition was at the level of synthesis and not release.

The comparisons of prostaglandin production by tumors was made by interpolation of the value of ng prostaglandin released per gram of tissue at 10 minutes. This value reflected an initial rate of prostaglandin synthesis from endogenous precursors. (Efforts to reduce the initial rates of prostaglandin release were unsuccessful; tumor slices incubated at 10°C showed no prostaglandin production, while at 15°C no prostaglandin production was detected before 20 minutes).

The prostaglandin release was measured in fresh tumor tissue as opposed to frozen tissue since prostaglandin production was found to be

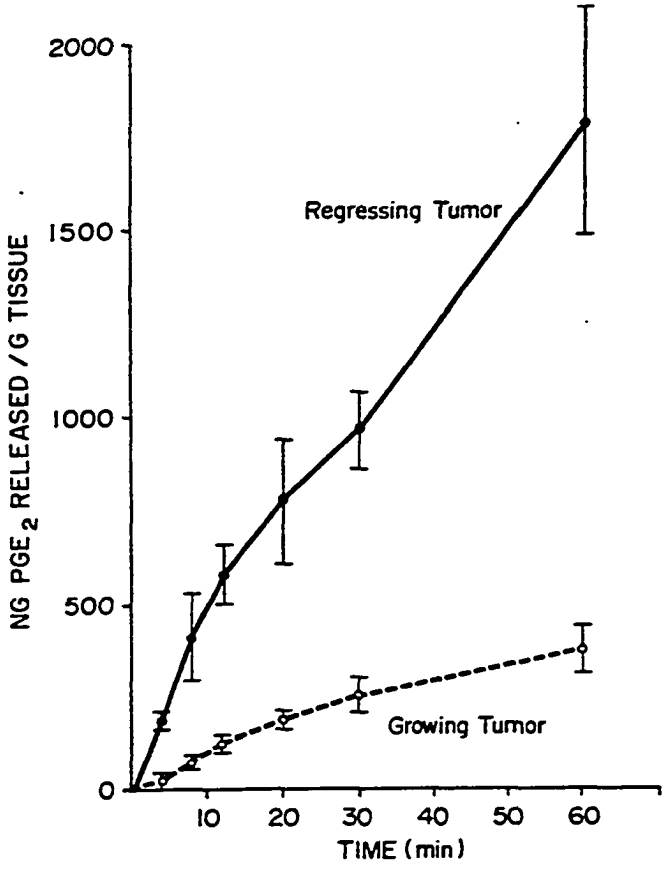


Figure 3. Prostaglandin E_2 Release from Fresh Tumor Slices as a Function of Time

Representative time courses for PGE_2 release from a growing and a regressing tumor are shown. The error bars represent standard deviation. Each curve is the mean of triplicate analyses of a single tumor. PGE_2 production was inhibited in tumor slices incubated in the presence of 300μM indomethacin.

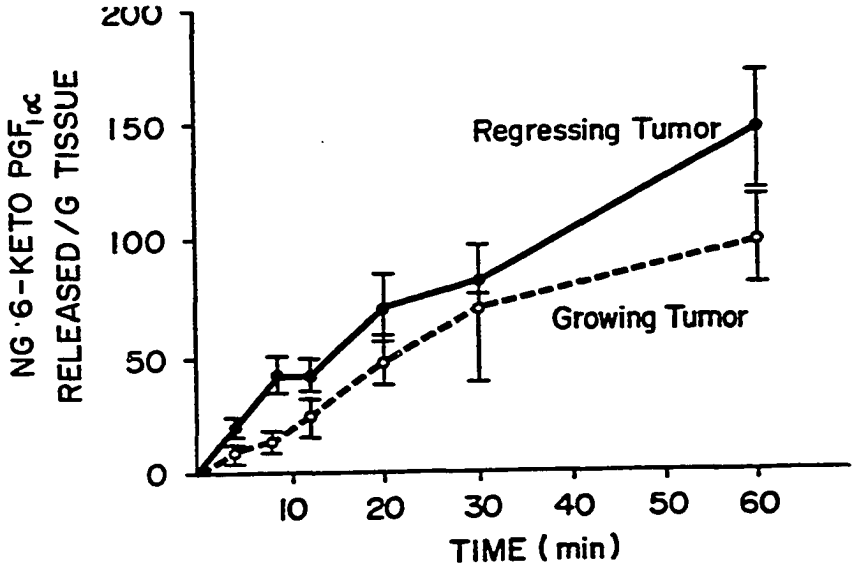


Figure 4. 6-Keto-prostaglandin $F_{1\alpha}$ Release from Fresh Tumor Slices as a Function of Time

Representative time courses for 6-keto-prostaglandin $F_{1\alpha}$ release from tumor slices are shown. Each curve is the mean of triplicate analyses of a single tumor. The error bars represent standard deviation. 6-Keto-$PGF_{1\alpha}$ production was inhibited in tumor slices incubated in the presence of 300μM indomethacin.

abnormally high in frozen tumors. The average PGE_2 released from six frozen tumors was 4 times greater at 30 minutes of incubation and 6 times greater after 90 minutes of incubation than the release seen in 4 tumors assayed as fresh tissue. An increase in prostacyclin synthesis in rat aorta upon freezing and subsequent thawing has been reported (Ts'ao et al, 1978). It is probable that the membrane disruption incident to freezing alters the activity of membrane-bound enzymes, such as phospholipase A_2 and cyclo-oxygenase.

X. Incorporation of ^{32}P-Orthophosphate into Tumor Phosphoproteins In Vitro

Tumors which were classified as growing or regressing based on the criterion described earlier, were labelled with ^{32}P-dipotassium phosphate (2.5 milliCuries) to compare phosphorylated proteins. Tumors were rapidly excised from decapitated rats, weighed, uniformly sliced to 0.35 mm at 4°C using a McIlwain Tissue Chopper. The tumor slices (approximately 300-500 mg each) were subsequently washed once with 1 ml of (4°C) 0.9% NaCl then incubated in 1 ml of phosphate-free Dulbecco's modified Eagle's medium containing 2.5 milliCuries ^{32}P-dipotassium phosphate, for one hour at 37°C with agitation. After incubation the tumor slices were washed once with ice cold Buffer A (0.25 M sucrose, 2mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM KCl, 20 mM Tris HCl, pH 7.5), then homogenized in 5 volumes of Buffer A. For isolation of the nuclei (Cho-Chung and Redler, 1977) the homogenates were centrifriged at 770 xg at 4°C for 10 minutes, and the pellets were resuspended and rehomogenized in 5 volumes of Buffer A, then filtered through three layers of gauze. These samples were again centrifuged at 770 xg for 10 minutes, and the

pellets were resuspended in 5 volumes of Buffer A before sedimenting for a third time at 770 xg for 10 minutes. The crude nuclear pellet was then suspended in 2.2 M sucrose in Buffer A, from which the nuclei were sedimented at 60,000 xg in an SW 50.1 rotor in a Beckman L3-40 ultracentrifuge for 45 minutes at 4°C. The purity of the nuclei was confirmed by phase contrast microscopy after erythrosin B staining.

For isolation of the regression associated and other nuclear phosphoproteins (Cho-Chung et al, 1977), the nuclear pellets were taken up in 0.3-0.5 ml of 10 mM sodium phosphate, pH 7.5, containing 1% sodium dodecyl sulfate (SDS) and 1% mercaptoethanol. The proteins were extracted by incubating the lysed nuclei for 4 hours at 37°C without agitation. The extract was dialyzed against 500 volumes of 10 mM sodium phosphate, pH 7.0, containing 0.1% SDS and 0.1% mercaptoethanol for 3 hours at room temperature.

The nuclear proteins obtained from this procedure were separated by electrophoresis on 0.1% SDS - 10% polyacrylamide gels, according to the procedure of Laemmli (1970), as described below.

XI. Analysis of Nuclear Phosphoproteins by SDS-Polyacrylamide Gel Electrophoresis

a. Reagents and Chemicals

Electrophoresis grade ammonium persulfate (APS), N,N,N',N'-tetramethylenediamine (TEMED) and N,N'-methylenebis-acrylamide (BIS) were obtained from Bio-Rad Laboratories (Richmond, California). Gel electrophoresis grade acrylamide was purchased from Bethesda Research Laboratories, Gaithersburg, Maryland.

b. Gel Electrophoresis

Gels were prepared using a Pharmacia GSC-8 Slab Gel Casting apparatus. The running gel consisted of 0.1% SDS - 10% polyacrylamide, and the stacking gel was 0.1% SDS - 3% polyacrylamide, with APS as the initiator of polymerization and TEMED as the catalyst. Proteins in an aliquot of the sample were quantitated by the method of Lowry (1951). A sample volume containing 40 µg protein was diluted with an equal volume of 2X sample buffer, which consisted of 0.5 ml of 0.1% bromphenyl blue, 2.5 ml of 0.5 M Tris, pH 6.8, 4 ml of 10% SDS, 1.0 ml of glycerol, 1.0 ml of β-mercaptoethanol and 1.0 ml of H_2O.

The samples to be compared were loaded onto adjacent wells in the slab gels. Duplicate gels were run; one for the determination of molecular weights by staining with Coomassie Blue (as described below), and the other for autoradiography.

Electrophoresis was run in a GE-2/4 Gel Electrophoresis Apparatus (Pharmacia Fine Chemicals, Uppsala, Sweden). The electrophoresis chamber buffer consisted of 0.05 M Tris, 0.383 M glycine and 0.1% SDS. The power source was a Heathkit regulated H.V. power supply, Model I.P. 17, set to 35 V. A minimum of 12 hours was required for the dye front to run the full length of the gel.

After the power source was disconnected, the gels were removed and immediately fixed in 50% methanol-10% acetic acid for several hours with gentle rocking.

The gels for molecular weight determination of the proteins were then stained with 0.25% Coomassie Blue in 50% methanol-10% acetic acid overnight. These gels were destained the following day in several

changes of 50% methanol - 10% acetic acid, until the protein bands were clearly visible and the background was essentially clear.

For the determination of the molecular weights of specific sample protein bands, MW standards were subjected to electrophoresis beside the sample proteins in the gels. The MW standards (SDS-PAGE Molecular Weight Standards, 10,000-100,000; Bio Rad) contained the following proteins: phosphorylase B - 94,000 MW, bovine serum albumin - 68,000, ovalbumin - 43,000, carbonic anhydrase - 30,000, soybean trypsin inhibitor - 21,000 and lysozyme - 14,300. The log of the molecular weights of these standards was plotted against relative mobility in comparison to the dye front. The molecular weights of the sample bands were interpolated based on their relative mobilities. A representative stained gel with samples and MW standards is shown in Plate I.

The gels intended for autoradiography were removed from the fixing solution then placed in water containing 1% glycerol for several hours prior to drying. The glycerol treatment helped to prevent cracking of the gels during drying. The gels were then dried on a Bio-Rad Model 224 Gel Slab Dryer (Hofer Scientific Instruments) for 4 hours prior to autoradiography. Dried gels were wrapped in Saran Wrap, then exposed to Kodak X-OMAT AR (X-ray) film in a Kodak X-matic Cassette with regular intensifying screens for a minimum of 48 hours. The films were developed using Kodak GBX Developer.

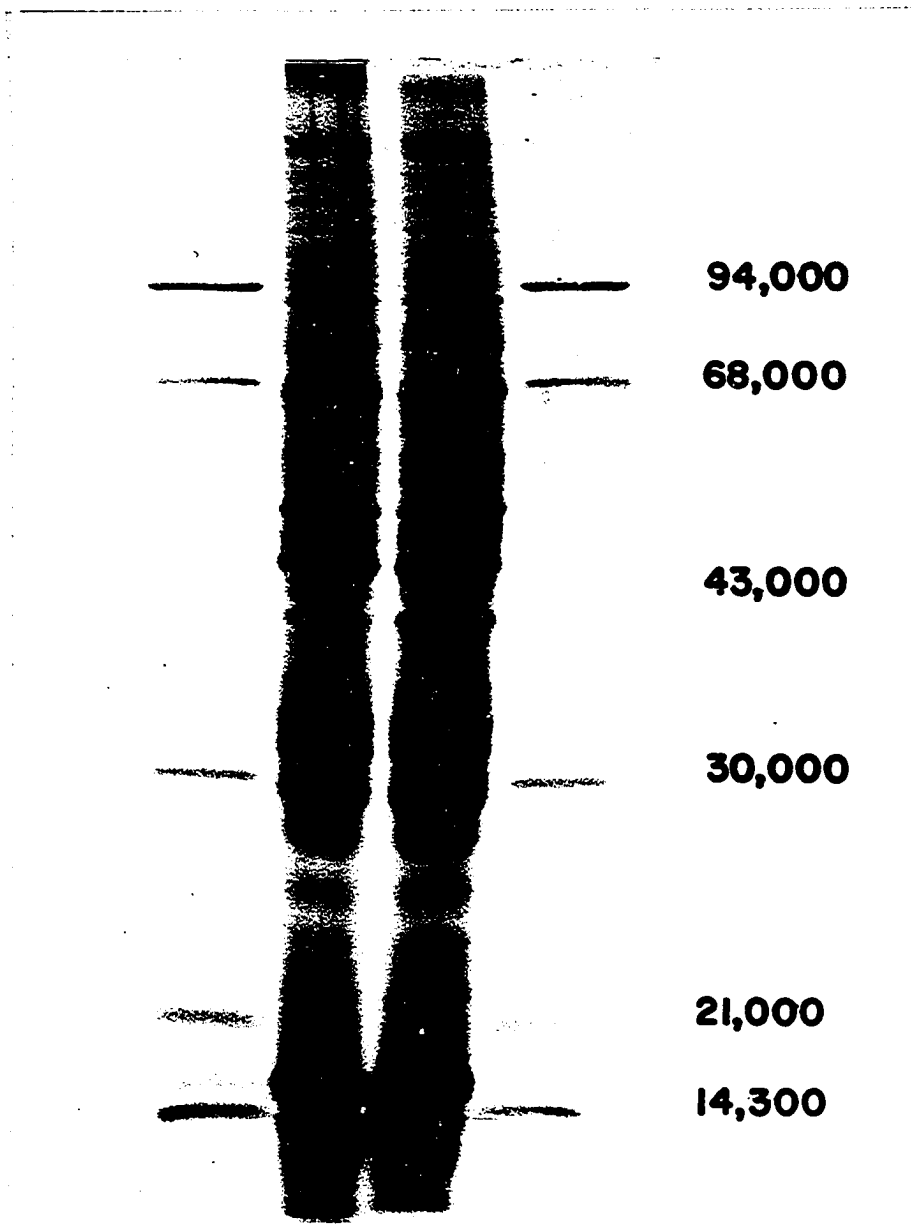
XII. Assay of Adenylate Cyclase Activity

Wistar-Furth female rats bearing the MTW9 series of mammary tumors were decapitated when the tumor sizes were in the range of 2 to 3 cm. The tumors were immediately excised and homogenized in a teflon-glass

Plate I. Molecular Weight Determination by SDS-Polyacrylamide Gel Electrophoresis: Representative Gel.

Sample proteins were separated by electrophoresis on 10% polyacrylamide gels. The standards of known molecular weights were loaded onto adjacent wells. Protein bands were made visible by a Commassie Blue stain. The molecular weights of sample proteins were interpolated from a graph of relative mobility vs log of the molecular weight.

PLATE I: Molecular Weight Determination by SDS-Polyacrylamide Gel Electrophoresis: Representative Gel



Potter-Elvehjem type homogenizer in 4 volumes of 0.25 M sucrose, 50 mM Tris buffer, pH 7.6, at 4°C. The crude homogenate was filtered through cheese cloth, then centrifuged at 10,000 xg for 15 minutes. The pellet was reconstituted in 0.25 M sucrose, 20 mM Tris, pH 7.6, and used for the assay of basal and PGE_2-stimulated adenylate cyclase activity.

The assay of adenylate cyclase activity was performed as described by Salomon et al (1974), based on the formation of ^{32}P-cAMP from α-^{32}P-ATP in a reaction mixture containing 40 mM Tris, pH 7.6, 5 mM $MgCl_2$, 10 mM creatine phosphate, 0.2 mg/ml creatine phosphokinase, 1.0 mM α-^{32}P-ATP (approximately 1µCi), 0.25 mM 3-isobutyl-1-methylxanthine and 100-200 µg of membrane protein, in a total volume of 0.4 ml. PGE_2 and Gpp(NH)p were added as indicated in individual experiments. The reaction was initiated by the addition of the homogenate and the mixture was incubated for 15 minutes at 32°C. The reaction was terminated by immersion of the tubes in a boiling water bath for 5 minutes. Approximately 50,000 cpm ^3H-cAMP was included to assess recovery of cAMP, and the samples were brought to a volume of 1 ml with water. This mixture was centrifuged at 4°C for 10 minutes at 10,000 xg. The resulting supernatants were chromatographed on Dowex AG-50 WX4, and then on neutral alumina to remove traces of unreacted α-^{32}P-ATP.

The radioactivity in the final column eluates was counted in 2 channels (optimal for ^3H and ^{32}P) a Beckman LS-7500 liquid scintillation spectrometer. Controls without homogenate or with boiled homogenate were processed in parallel with the assay as blanks, and these values were subtracted from each sample value.

The rate of ^{32}P-cAMP formation increased linearly with time for 30 minutes at 32°C and was linear with respect to the protein added to the

assay within the range of 0 to 1.0 mg. The enzyme activity is expressed as pmoles cAMP formed/mg protein/minute.

XIII. Assay of Prostaglandin Binding to Tumor Cell Membranes

Prostaglandin binding was assayed as previously described (Rao et al, 1981) using 5,6,8,11,12,14,15-^{3}H(N)-PGE_2 (130 Ci/mmol, New England Nuclear).

Tumors were excised from the tumor-bearing rats and homogenized in 5 volumes of 0.25 M sucrose, 0.01 M Tris, pH 7.4, 1.0 mM $CaCl_2$, 0.1% gelatin at 4°C. The homogenates were filtered through 2 layers of cheese cloth, then the filtrates were centrifuged at 2,000 xg for 10 minutes at 4°C. The pellets were reconstituted in the homogenizing buffer and used for the PGE_2 binding assay.

Aliquots of the membrane fraction (approximately 500 µg protein) were incubated with ^{3}H-PGE_2 in the absence and presence of a 200-fold excess of unlabelled PGE_2 to determine the specific and non-specific binding. Both labelled and unlabelled PGE_2 were prepared from stock ethanol solutions immediately before use in the binding assay. In some experiments, the ethanolic PGE_2 solutions were dried under a stream of nitrogen, then redissolved in 0.1 ml of 0.01 M Tris, pH 7.0, immediately prior to addition to the binding assay. The incubation was for 1 hour at 37°C. Following incubation, bound and free ^{3}H-PGE_2 were separated either by centrifugation or by Millipore filtration.

In the experiments where binding capacity was determined, aliquots of membrane fractions were incubated as described above with ^{3}H-PGE_2 in the presence and absence of a 200-fold excess of unlabelled PGE_2.

The background (non-specific) binding in the presence of excess unlabelled PGE_2, was subtracted from the total ^3H-PGE_2 bound and expressed as specific binding.

RESULTS

I. Correlation of the Hormonal Dependence of DMBA-Induced Mammary Tumors With Prostaglandin E_2 Content

The purpose of this series of experiments was to observe tumor growth response and PGE_2 content in DMBA-induced mammary tumors in response to *in vivo* hormonal manipulations in tumor-bearing Sprague-Dawley rats.

The tumors in this set of experiments were induced with a single 20 mg dose of DMBA at 50 days of age. The method of prostaglandin measurement, in which the prostaglandins were extracted from homogenates of tissues which had been frozen, then pulverized, resulted in a value which was defined as prostaglandin content. The hormonal manipulations which were carried out in this set of experiments included (i) ovariectomy, to deplete estrogen and induce regression; (ii) administration of haloperidol to increase pituitary prolactin release, and (iii) the combination of haloperidol treatment plus ovariectomy.

a. Effect of Tumor Latency on PGE_2 Content on DMBA-Induced Mammary Tumors

Induction of tumors using the single dose regimen (as described in Methods) resulted in the appearance of mammary tumors over a long range of time, the earliest tumors appearing at 2 1/2 months and the latest at 11 months after exposure to the carcinogen. Since the biochemical

TABLE 4

Effect of Tumor Latency on PGE_2 Content in DMBA-induced Mammary Tumors

Average tumor latency, days	No. of tumors	ng PGE_2/g tissue mean ± SE
80	13	117±19
97	10	76±16
183	7	67±35
289	8	14±4

characteristics of tumors which appear at different times may differ, the data were analyzed in order to determine whether PGE_2 content varied as a function of latency. The results in Table 4 show that prostaglandin content varied inversely with tumor latency (r=0.94), suggesting that the tumors of greater latency possess a decreased ability to synthesize prostaglandins. The decrease in prostaglandin-synthesizing capacity may be a result of the changing hormonal status of the animal due to age, or it may be a characteristic due to the nature of the tumors which appear at various times after initiation due to different selection pressures.

It has been reported that tumors which appear sooner after induction by DMBA exhibit a greater degree of hormonal responsiveness than tumors with longer latency (Bradley et al, 1976). Therefore, these variations with latency must be taken into account in the design of experiments concerning hormonal effects on prostaglandin synthesis.

Thus, in order to eliminate complicating variables, experimental comparisons were made in latency-matched tumors from age-matched rats.

b. PGE_2 Content and Growth Response in DMBA-Induced Tumors From Hormonally Manipulated Rats

The purpose of this experiment was to observe the growth response and PGE_2 content in tumors from hormone-manipulated rats. The hormonal manipulations were performed as described in Materials and Methods. Estrogen was depleted by bilateral ovariectomy. In this set of experiments, haloperidol was injected subcutaneously in olive oil daily for 4 consecutive days, and the tumors were excised on the fifth day for assay. The response of mammary tumor PGE_2 content to hormonal manipulation is shown in Table 5.

TABLE 5

PGE_2 Content and Growth Response in DMBA-induced Tumors from Hormonally Manipulated Rats

Treatment	No. of rats	ng PGE_2/g tissue, mean±SE	P-value, compared to OVX	Change in tumor size,[a] % mean±SE
Intact	10	65±15	<0.05	118±8.6
Ovariectomy	8	120±28		79±6.2
Haloperidol	11	75±23	<0.1	116±9
Ovariectomy + haloperidol	4	64±32	<0.1	110±9

[a]Tumor size at beginning of treatment=100%

The PGE_2 content was elevated approximately two-fold in tumors from ovariectomized rats compared to the PGE_2 content in intact rats ($p<0.05$), suggesting that a decrease in estrogen is accompanied by an increase in tumor prostaglandin synthesis, or a decrease in metabolism. However, since estrogen stimulates prolactin release (Grosvenor and Turner, 1960), ovariectomy also causes a decrease in circulating prolactin levels, and prolactin may be more important than estrogen in the control of growth of these tumors (see Introduction). The possibility that elevation of PGE_2 content in ovariectomized rats was more directly related to the alteration in serum prolactin levels was investigated by maintaining serum prolactin levels in ovariectomized rats with haloperidol administration. The PGE_2 content in tumors from the haloperidol-treated ovariectomized rats (therefore in the presence of prolactin but in the absence of estrogen) was not different from the levels in tumors from non-hormone-manipulated intact rats, suggesting that tumor PGE_2 content is more closely associated with endogenous prolactin levels than with endogenous estrogen. Haloperidol administration to intact rats also resulted in tumor PGE_2 content similar to intact non-hormone-manipulated rats.

The causal connection between hormonal manipulations and PGE_2 content in the malignant mammary tissue has not been firmly established. However, on the basis of these results, the relationship between prolactin and prostaglandin content appears to be an inverse relationship, in which prolactin suppresses prostaglandin synthesis. If prostaglandin synthesis is inhibited by the presence of prolactin, one might expect a greater inhibition of prostaglandin synthesis in the presence of elevated prolactin. The dose of haloperidol with which the

rats were treated was a replacement dose of ovariectomized rats (Bradley et al, 1976); therefore, in the intact rats haloperidol treatment caused a doubling of endogenous prolactin levels, at maximum. Further experiments would determine whether larger increases in serum prolactin would result in a depression of PGE_2 levels, or whether the PGE_2 levels observed in intact tumors is a basal level.

The alterations in endogenous hormone levels resulting from the various treatments induced alterations in the growth response of the DMBA-induced mammary tumors. For example, ovariectomy resulted in regression of the mammary tumors in 90% of the endocrine-ablated rats. In contrast, when the rats were treated with haloperidol after ovariectomy, tumor growth was maintained, as it was in haloperidol-treated intact rats. It was of interest to note that the changes in the tumor PGE_2 content reflected the growth response of the tumors to the hormonal manipulations. To wit, the PGE_2 content in the growing tumors from ovariectomized rats treated with haloperidol was similar to the tumor PGE_2 content from intact rats, as was the PGE_2 content in the tumors in which growth was maintained by haloperidol treatment of intact rats. In contrast, PGE_2 content was doubled in regressing tumors, which were obtained from ovariectomized rats.

These results suggest that the hormones which are depleted by ovariectomy are involved in the regulation of prostaglandin biosynthesis in the malignant mammary tissue, along with regulating tumor growth. More specifically, the experiments in haloperidol-treated rats demonstrated that prolactin overcame the effect of ovariectomy on tumor growth response as well as on PGE_2 content.

c. PGE_2 Content in Growing, Static and Regressing DMBA-Induced Mammary Tumors

Although most of the tumors in intact rats were indeed growing, there were some atypical tumors in which growth had stabilized. Thus, to permit a correlation of growth rate of all tumors with PGE_2 content, the tumors were categorized as growing, stable or regressing (as described in Materials and Methods), without regard to hormonal treatment. Using this classification, an inverse relationship between tumor growth and tumor PGE_2 content was observed, as shown in Table 6. The PGE_2 tissue content was highest in regressing tumors, intermediate in stable tumors and lowest in growing tumors. These results indicate that PGE_2 may be functionally involved in the regression of tumors which respond to endocrine ablation. These results have been published in JNCI-August, 1982.

II. Prostaglandin Release From Fresh Tumor Slices

In the course of these studies, a novel method for measurement of prostaglandin synthesis from fresh tumor tissue, based on that described for aorta by Panganamala et el, (1981) was developed and standardized. The details of this procedure were discussed extensively in the Materials and Methods section of this dissertation. Basically, the method involves measurement of prostaglandin release from the tissue into an aqueous medium. Some of the advantages of the new method are that (1) it is far less time-consuming than the procedure which required homogenization, extraction and purification of each sample (2) it allows for the measurement of more than one time point per tissue aliquot, allowing for kinetic studies since sampling is non-destructive (3) since the

TABLE 6

PGE_2 Levels in Growing, Static, and Regressing DMBA-induced Tumors[a]

Tumor Status	ng PGE_2/g tissue mean ± SE
Growing	39±15
Static	84±26
Regressing	161±42

[a]P-values: Growing vs. regressing - $P<0.005$; static vs. regressing-- $P<0.1$.

radioimmunoassay is performed on aliquots of aqueous samples, measurement of different prostaglandin species by specific RIAs is possible, and (4) the values which are obtained reflect the capacity of the tissue for prostaglandin biosynthesis from endogenous substrate.

Prostaglandin $F_{2\alpha}$ was measured in both fresh and frozen tumor slices using this method. In fresh slices, PGE_2 release was generally 2 times the release of $PGF_{2\alpha}$. However, in tumor slices which were frozen then thawed, $PGF_{2\alpha}$ release was slightly greater than (20%) PGE_2 production. This discrepancy is probably due to the membrane disruption which results from freezing and thawing.

III. Further Studies on the Growth of Hormonally Dependent Mammary Tumors and PGE_2 Synthesis

The purpose of this next series of experiments was essentially the same as the first; specifically, to determine if a correlation exists between the tumor growth response to hormonal manipulations and prostaglandin synthesis. However, some of the conditions of the experiment were altered in an attempt to improve the design somewhat. For example, the method of tumor induction was modified, in that the carcinogen was administered in 4 weekly intubations. Under these conditions, the tumors appeared much earlier, i.e. 6-12 weeks after exposure to DMBA. The benefits of this modification were two-fold; first, the lag time between dosing and the appearance of usable tumors was obviously decreased, reducing housing expenses and lag time between experiments. Secondly, the importance of using tumors of early and matched latency was demonstrated above. Within this series of

experiments, only tumors with a latency of less than 3.5-4 months were used, in order to insure that the degree of hormonal responsiveness was optimal.

Another modification of this second series of experiments was the method of measurement of prostaglandin synthesis. The release of prostaglandins from fresh tumor slices into an aqueous medium was used. The advantages of this method were listed above.

A third modification was made in the protocol for hormonal manipulations by haloperidol. The treatment period with haloperidol was extended from 4 days to 14 days, to ensure adequate time for the observation of tumor growth response. In addition, the vehicle for haloperidol was changed from olive oil, which contains a high level of the prostaglandin precursor arachidonic acid which may alter the balance in PG synthesis, to an aqueous vehicle.

As shown in Figure 5, the rate of PGE_2 release from fresh tumor slices was inversely related to tumor growth in agreement with the data on tumor PGE_2 content as measured by direct analysis of tumors (Table 6).

As shown in Figure 6, the assay of 6-keto-$PGF_{1\alpha}$ release by the tumor slices into the culture medium showed that prostacyclin (PGI_2) synthesis also varied inversely with growth.

The above results on PG content and synthesis in vitro suggest that the elevation of the PGE_2 content in tumors which we have observed during tumor regression is due to elevated synthesis of prostaglandins from endogenous precursors. This result, together with the observation that the responses of PGE_2 and PGI_2 were qualitatively similar, is

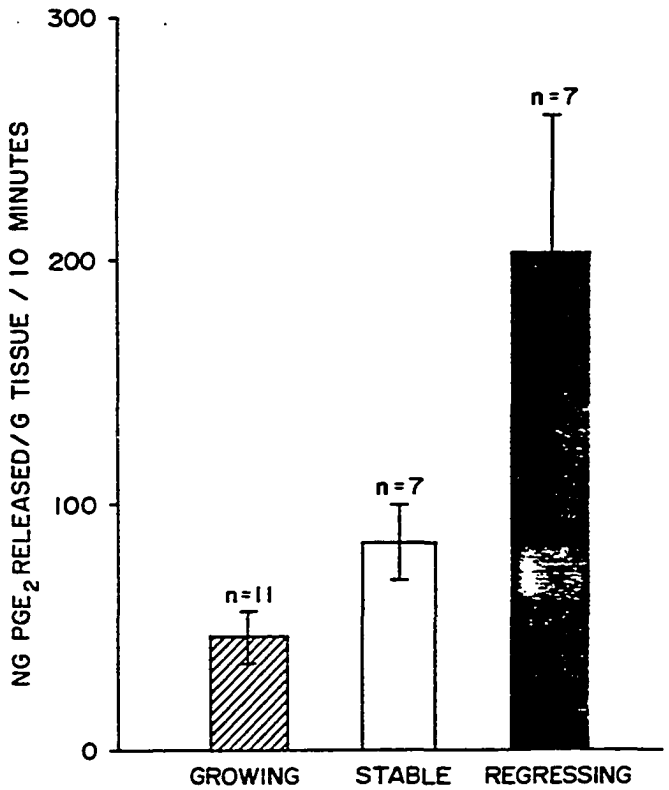


Figure 5. Inverse Relationship Between Prostaglandin E_2 Production and Growth in DMBA-induced Rat Mammary Tumors

Prostaglandin E_2 was measured as release from tumor slices, as described in Materials and Methods. The tumors were classified as growing, stable or regressing based on the criteria described in Materials and Methods. N equals the number of tumors measured. Error bars represent standard error of the mean. PGE_2 production is increased 4-fold in regressing tumors compared to growing tumors (growing vs regressing, $p<0.025$, Student's t test). Stable tumors produce 1.8 times more PGE_2 than growing tumors (growing vs stable, $p<0.05$; stable vs regressing, $p<0.025$).

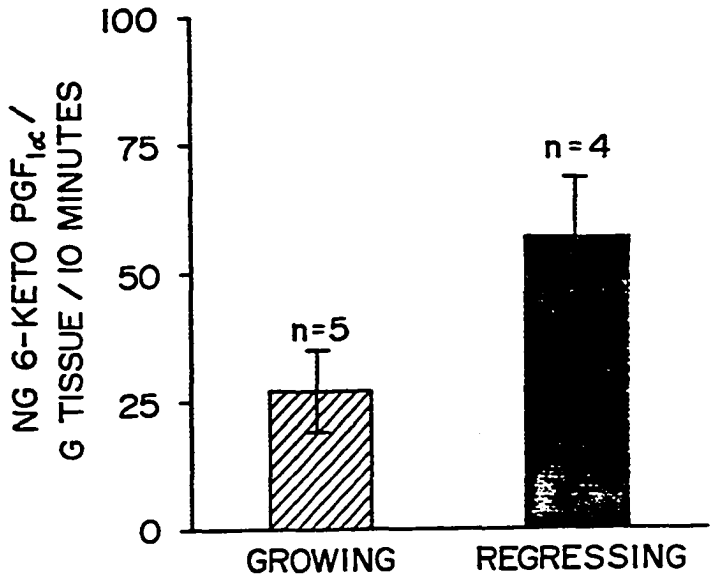


Figure 6. 6-Keto-prostaglandin $F_{1\alpha}$ Production in Growing and Regressing DMBA-induced Rat Mammary Tumors.

Prostaglandin production was measured as release from tumor slices, as described in Materials and Methods. The tumors were classified as growing or regressing based on the criteria in Materials and Methods. N equals the number of tumors measured. Error bars represent standard error of the mean. The difference between growing and regressing tumors was significant ($p<0.025$) based on Student's t-test.

consistent with the possibility that the point of regulation may be cyclo-oxygenase or the availability of substrate fatty acid.

Prostaglandin production in non-regressing tumors from ovariectomized rats was significantly different from regressing tumors from ovariectomized rats. For example, non-regressing tumors from ovariectomized rats released 102±26 ng of PGE_2 per gram of tissue per 10 minutes, which is half the level of PGE_2 production found in regressing tumors, and is similar to the value found in stable tumors (86±15 ng PGE_2 per gram of tissue per 10 minutes). Since the growth response of these non-regressing tumors fell under the classification of stable (as described in Material and Methods), PGE_2 production appeared to correlate more closely with the growth response of the tumor than with the endogenous hormonal environment.

In view of the above results, and in view of the complex interactions between estrogen and prolactin, endogenous prolactin levels were manipulated in tumor-bearing rats, independent of changes in endogenous estrogen, with the use of neuroleptic drugs. Daily subcutaneous injections of 120µg of haloperidol were given to rats to increase endogenous prolactin levels. Control rats received daily injections of the vehicle, which was 1% 1N HCl, 2.5% ethanol (Dickerman et al, 1972). Decrease of endogenous prolactin was accomplished by daily subcutaneous injections of 0.5 mg ergocornine hydrogenmaleate (per 250g rat) (Bradley et al, 1976). After a 14 day treatment period, the tumors were excised and PGE_2 release was assayed.

The response of the growing tumors to elevated endogenous prolactin induced by haloperidol was continued growth. In fact, no tumors

regressed in rats treated with haloperidol. At the dose of ergocornine administered, the mammary tumors regressed slowly; the average decrease in the sum of 2 diameters was 15%-20% over the 14 day treatment period. In comparison, control tumors increased over 20% in size.

The PGE_2 production by the tumors after the 14 day treatment period is shown in Figure 7. PGE_2 production in growing tumors from rats treated with haloperidol was similar to PGE_2 production in growing tumors in general. Tumors from rats treated with ergocornine showed PGE_2 production which was 75% greater than PGE_2 production in vehicle-injected controls ($p<0.1$). The PGE_2 production in the tumors from the ergocornine-treated rats was not significantly different from the value in stable tumors (87.3±15 ng as compared to 86.±15 ng PGE_2 released per gram of tissue per 10 minutes). Based on the criteria defined in Methods, these tumors were classified as stable in growth response. Therefore, these data are in complete agreement with those of our initial experiments, which showed that the tumor growth response correlates inversely and significantly with PGE_2 production.

a. A Note on Static (Stable) Tumors

Tumors which do not regress following ovariectomy have a defect at some point in the mechanism of hormone action. Certainly, tumors which do not possess hormone receptors do not require hormonal stimulation for growth, and are not affected by hormonal deprivation. However, there may be hormonally dependent tumors which grow in the presence of hormones but fail to regress by a hormone dependent mechanism based on one of the following flaws in the regulation mechanism responsible for regression: 1) a defect in the prostaglandin synthesizing machinery 2) defective or

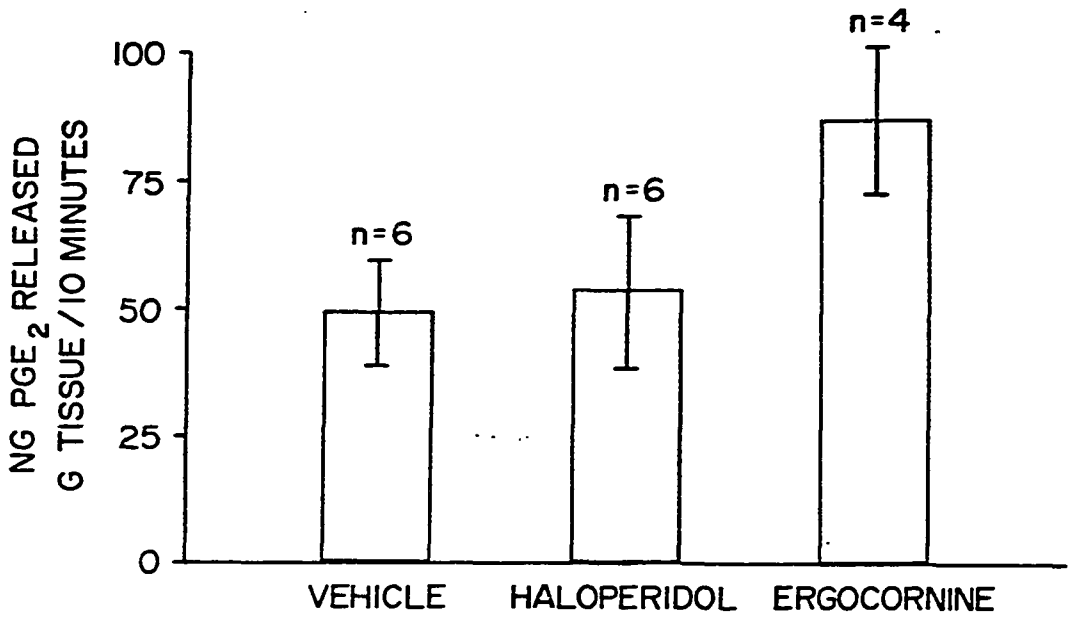


Figure 7. Tumor Prostaglandin E_2 Production after Alterations in Endogenous Prolactin.

Prolactin levels were increased in tumor-bearing rats by daily subcutaneous injections of 120µg haloperidol in 0.2 ml of 2.5% ethanol. Control rats received daily injections of the vehicle solution alone. Decrease of endogenous prolactin was accomplished by daily subcutaneous injections of 0.5 mg of ergocornine hydrogenmaleate. After a 14 day treatment period, the tumors were excised and PGE_2 release was assayed. N equals the number of tumors assayed. Error bars represent standard error of the mean. PGE_2 production is increased 75% in tumors from ergocornine-treated rat compared to vehicle-injected controls ($p<0.1$).

absent prostaglandin receptors 3) a defect in the coupling of the prostaglandin receptor to the adenylate cyclase enzyme 4) a non-stimulable adenylate cyclase 5) a cAMP binding protein with lowered or no activity or 6) a protein kinase holoenzyme with altered activity.

Other host factors also limit tumor size. In tumors which are static in growth, whether in ovariectomized or intact rats, tumor size is dependent on the degree of vascularization. Therefore, size increases are limited by the extent to which the host has supplied the tumor with blood vessels (Folkman, 1976). In stable tumors the production of new cells and the destruction of old cells by necrosis is at an equilibrium.

IV. Cyclic AMP as a Mediator of the Growth Inhibitory Effects of PGE_2

Cyclic AMP is an established regulator of cell growth (Otten et al, 1972; Ryan and Heidrick, 1974). Prostaglandins have been shown to activate adenylate cyclase in many cells (see Introduction), including mammary cells (Bar, 1973; Schorr and Russell, 1974) and thus to increase intracellular cAMP content. This stimulation of cAMP levels by prostaglandins has not been reported in malignant mammary tissue.

To investigate the possibility that the growth inhibitory effects of PGE_2 on the mammary tumors may be acting through alterations of cAMP levels, the assay of the cAMP content in growing, stable and regressing DMBA-induced mammary tumors was performed. The results are shown in Table 7. The data indicate that the cAMP content in growing, stable and regressing tumors varied inversely with tumor growth, in parallel with PGE_2 production, supporting the theory that there is a relationship between these two parameters.

TABLE 7

cAMP Content of Growing, Static and Regressing

DMBA-induced Tumors

	pmoles cAMP/mg tissue (mean ± SE)
Growing	0.69±0.1
Stable	1.0±0.2
Regressing	1.8±0.4

Growing vs. regressing $p<0.01$

Regressing vs. stable $p<0.05$

In order to demonstrate a more direct link between the elevation of PGE_2 and the elevation of cAMP in regressing tumors, experiments were designed to determine whether PGE_2 could indeed cause an increase in cAMP synthesis in hormone dependent mammary tumors.

Slices of DMBA-induced mammary tumors were incubated in the presence of PGE_2 in a Tris-saline (pH 7.4) incubation medium. The PGE_2 concentration ranged from 10^{-3}M to 10^{-8}M. As shown in Figure 8, in growing tumor slices, the cAMP content was increased at PGE_2 concentrations as low as 10^{-6}M in the presence of the phosphodiesterase inhibitor, isobutylmethylxanthine (IBMX, 0.1mM); maximal stimulation occurred at 10^{-5}M PGE_2. Generally, 10^{-5}-10^{-6}M is considered to be a higher than physiological concentration for prostaglandins in most tissues. However, PGE_2 production is higher in some tumors than in most other tissues, so that tumor tissue is probably exposed <u>in situ</u> to PGE_2 concentrations somewhat higher than normal tissues. The data suggest that DMBA-induced mammary tumors are sensitive to stimulation by PGE_2. Nevertheless, the prostaglandin concentration required for stimulation in growing tissue is so high that stimulation of cAMP levels by PGE_2 in growing tumors is probably of little physiological importance. This conclusion is supported by the observation that no increase in cAMP content in the presence of 10^{-3} to 10^{-8}M PGE_2 was detected in growing tumors (Figure 9) in the absence of IBMX.

In contrast, cAMP content increased more than two-fold in regressing tumor tissue at exogenous PGE_2 concentrations as low as 10^{-7}M, even in the absence of the phosphodiesterase inhibitor, as shown in Figure 10.

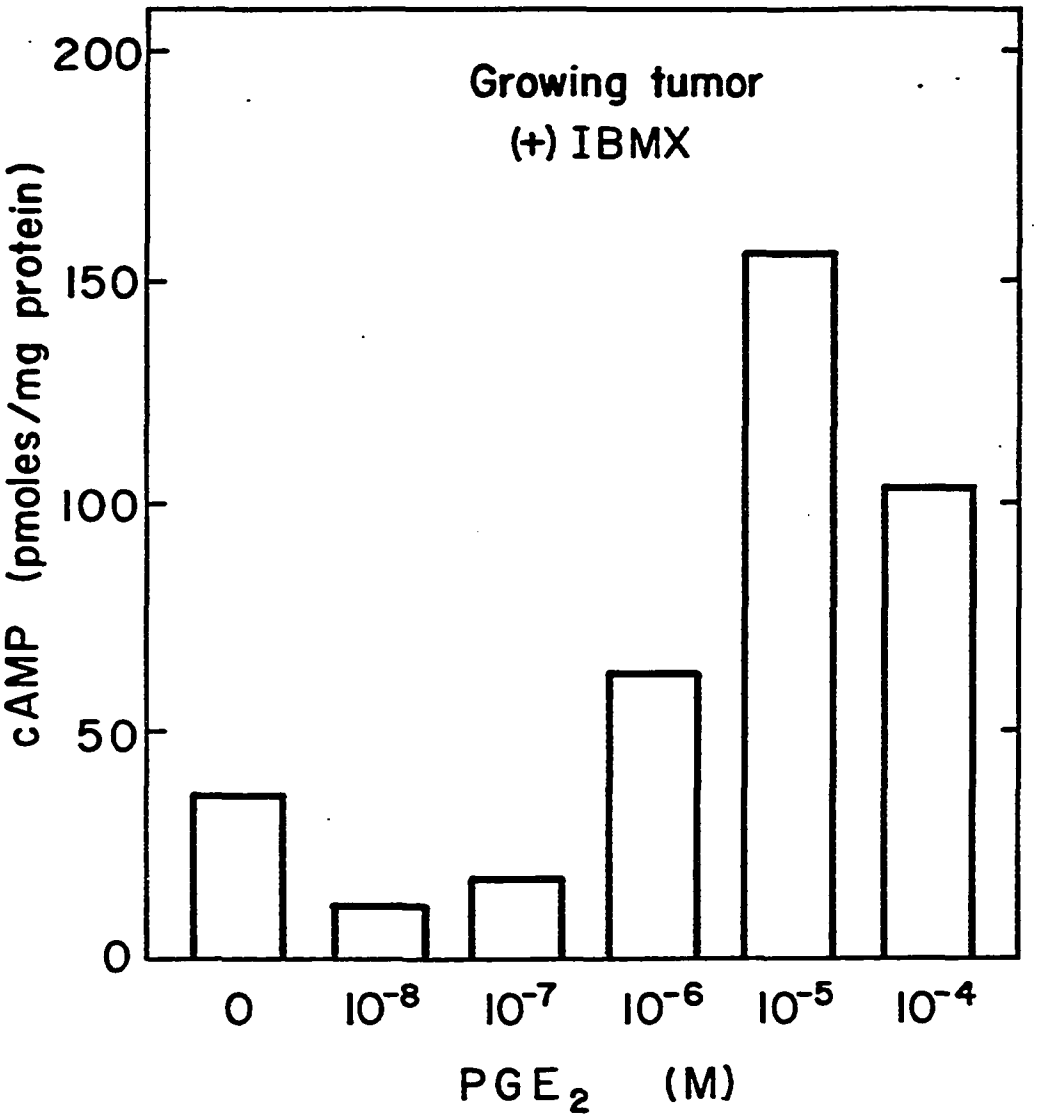


Figure 8. Effect of Exogenous PGE_2 on cAMP Content in Growing Tumor Slices in the Presence of Isobutylmethylxanthine

Slices of growing tumor tissue were incubated in the presence of exogenous PGE_2 at the concentrations shown in Tris-saline, pH 7.4 at 30°C for 10 minutes in the presence of 0.1 mM isobutylmethylxanthine. The reaction was terminated by freezing the tissue slices in liquid nitrogen. Cyclic AMP content was determined by radioimmunoassay.

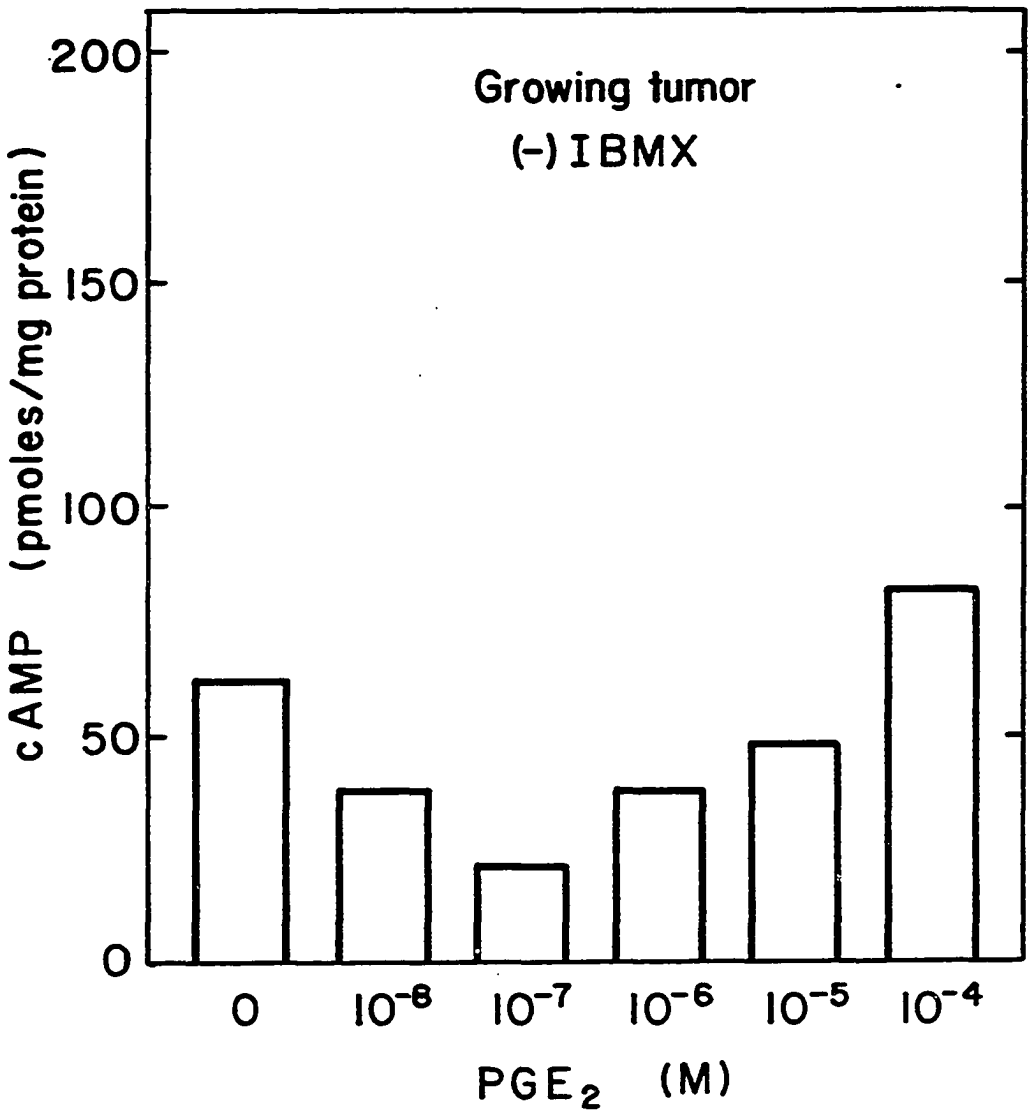


Figure 9. Effect of Exogenous PGE_2 on cAMP Content in Growing Tumor Slices in the Absence of Isobutylmethylxanthine

Slices of growing tumor tissue were incubated in the presence of exogenous PGE_2 at the concentrations shown in Tris-saline, pH 7.4 at 30°C for 10 minutes. No isobutylmethylxanthine was present. The reaction was terminated by freezing the tissue slices in liquid nitrogen. Cyclic AMP content was determined by radioimmunoassay.

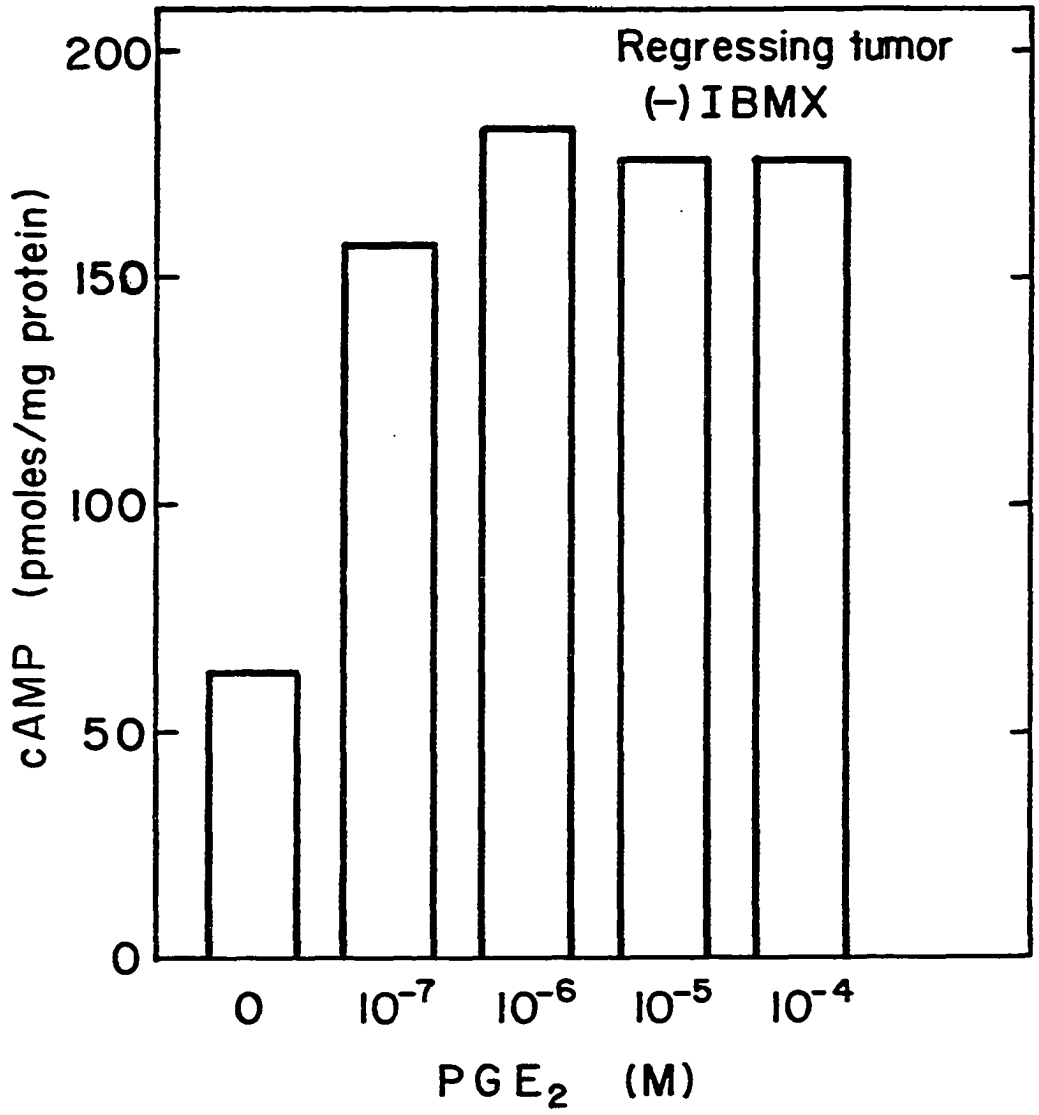


Figure 10. Effect of Exogenous PGE_2 on cAMP content in Regressing Tumor Slices.

Slices of regressing tumor tissue were incubated in the presence of exogenous PGE_2 at the concentrations shown in Tris-saline, pH 7.4 at 30°C for 10 minutes. No isobutylmethylxanthine was present. The reaction was terminated by freezing the tissue slices in liquid nitrogen. Cyclic AMP content was determined by radioimmunoassay.

These data show that regressing tumors are more sensitive to stimulation by PGE_2, since cAMP production can be stimulated by a lower, more physiological concentration of PGE_2. The fact that an increase in cAMP is apparent even in the absence of IBMX in regressing tumors, whereas the measurable stimulation of cAMP by PGE_2 in growing tumors requires the presence of the phosphodiesterase inhibitor further supports the conclusion that elevation of cAMP by PGE_2 is more marked in regressing tumors than in growing tumors. An increase in the amount or activity of prostaglandin receptors in regressing tumors could be the explanation.

V. Prostaglandin Binding to DMBA-Induced Mammary Tumors

If the elevation of the cAMP content in tumors slices is indeed a result of specific stimulation by PGE_2, then specific binding of PGE_2 to DMBA-induced mammary tumor tissue should be demonstrable.

TABLE 8

PGE_2 Binding to DMBA-induced Mammary Tumors

Exogenous ^3H-PGE_2	Specific Binding (fmoles PGE_2/mg protein)
1.8×10^{-9}M	36.9
4.5×10^{-9}M	81.6

As shown in Table 8, the plasma membrane fraction specifically bound PGE_2 at 30°C in a concentration dependent manner. This implies that DMBA-induced mammary tumors possess specific receptors for PGE_2.

VI. PGE_2 Binding in Hormonally Dependent and Independent Mammary Cancer

Further support for the possible interrelationship of PGE_2, cAMP and hormonal growth regulation in mammary tumors comes from studies on a second model system of hormonally dependent rat mammary cancer, the transplantable MTW9 tumors in Wistar-Furth rats.

The hormonal characteristics of the MTW9 series of mammary tumors are summarized in Table 9. The MTW9-A tumor is both estrogen receptor and progesterone receptor positive and is hormonally dependent, that is, it regresses in response to hormonal ablation. The MTW9-B mammary tumor is estrogen and progesterone receptor positive, but has progressed to hormonal autonomy, as evidenced by the fact that the tumors continue to grow after hormonal ablation. The MTW9-D tumor is totally hormonally autonomous, since it is both estrogen and progesterone receptor negative and totally unresponsive to hormonal ablation. The cAMP content of these tumors was higher in the hormonally dependent tumors than in the hormonally independent tumors. Cho-Chung has proposed that a functional cAMP system and a functional estrogen system are both necessary constituents for hormone dependent growth and regression in mammary tumors (Bodwin et al, 1978, Cho-Chung et al, 1978). The capacity to synthesize cAMP in hormone dependent (MTW9-A) tumors is consistent with the proposal that a functional cAMP-generating system is an essential requirement for tumor regression due to hormonal ablation. One possible explanation for the fact that the MTW9-D tumors do not regress upon endocrine ablation is that they lack the ability to synthesize cAMP, an essential step in the regression process. This reasoning is further supported by the reduced basal cAMP content in MTW9-B tumors, which

TABLE 9

Hormone Receptor and cAMP Status of MTW9 Mammary Tumors

	Estrogen Binding Sites (fmoles/mg protein)	Progesterone Binding Sites	cAMP pmoles/mg protein
MT-W9-A	84±13	502±56	31.5±4
MT-W9-B	33±5	71±16	19.5±3
MT-W9-D	8.0±2	17±3	9.7±1.4

still have some hormone receptors, but fail to regress after endocrine ablation.

Within the framework of our hypothesis, PGE_2 is elevated upon the withdrawal of hormone stimulation and acts directly to activate the cAMP system in the hormone dependent mammary tumors. Since PGE_2 action is probably contingent on the ability of a tissue to bind PGE_2, the binding activity of PGE_2 to MTW9-A and MTW9-D tumors was measured. As shown in Table 10, the binding capacity of the hormonally dependent MTW9-A tumors in the presence of 0.5 nmole/ml of PGE_2 was 6 times the capacity for binding PGE_2 in the hormonally autonomous MTW9-D tumors.

The data obtained from the MTW9 series of tumors confirmed an observation made earlier while comparing growing to regressing DMBA-induced mammary tumors. Table 10 shows that cAMP content is elevated in regressing MTW9-A tumors compared to growing tumors. Furthermore, in the tumors which do not regress following ovariectomy (both MTW9-B and MTW9-D), cAMP content was not different 5 days following ovariectomy than the cAMP content prior to ovariectomy. These results again support the proposal that a functional cAMP generating system is essential to hormonally induced regression. Furthermore, these results show that not only is basal cAMP content lower, but also that cAMP content is not stimulated in tumors which do not respond to endocrine ablation.

The results in Table 10 suggest that hormonally autonomous tumors have a reduced capacity to bind PGE_2 compared to hormonally-responsive tumors. A greater than 2-fold increase in the PGE_2 binding activity was seen following ovariectomy in the regressing MTW9-A tumors. In comparison, there was no change in the minimal PGE_2 binding of the

TABLE 10

PGE-Binding and cAMP Content in MTW9 Mammary Tumors

Before and After Ovariectomy

	Before Ovariectomy		After Ovariectomy	
	PGE_2-Binding[a]	cAMP[b] pmoles/mg protein	PGE_2-Binding[a]	cAMP[b] pmoles/mg protein
MT-W9A	44±7	31.5±4	104±20	51.4±4
MT-W9B	20±4	19.5±3	29± 8	22.3±3
MT-W9D	7±2	9.7±1.4	8± 2	9.2±2

[a]PGE_2 binding was assayed as described in Materials and Methods in the presence of 1.5×10^{-9}M ^{3}H-PGE_2 before and 5 days after ovariectomy. The binding results are expressed as femtomoles of specifically bound ^{3}H-PGE_2 per mg of particulate protein.

[a,b]Each value represents the mean ± S.E. of three experiments.

MTW9-D tumors, and only a small increase (45%) in the PGE_2 binding activity of the non-regressing MTW9-B tumors, 5 days after ovariectomy.

These results are consistent with the hypothesis that PGE_2 binding activity is increased in amount or activity in regressing tumors compared to growing tumors, which was seen earlier in connection with the increased stimulation of cAMP synthesis by PGE_2 in regressing DMBA-induced mammary tumors.

In order to establish a more direct relationship between PGE_2 binding and cAMP content in MTW9 tumors, the stimulation of adenylate cyclase activity by PGE_2 was investigated in MTW9-A and MTW9-D tumors.

GTP is an essential activator of adenylate cyclase (Rodbell, 1980). The activation of adenylate cyclase subsequent to the binding of an extracellular hormone is dependent on the interaction of the hormone receptor with proteins (associated with the cyclase) which bind GTP (Rodbell, 1980). In vitro adenylate cyclase assays contain GppNHp, which is a non-hydrolyzable analogue of GTP, to promote optimal enzyme activity.

The stimulation of adenylate cyclase activity by PGE_2 in the presence and absence of GppNHp was assayed, and the results are shown in Figure 11. In the hormonally responsive MTW9-A tumor, adenylate cyclase activity was activated by PGE_2. However, the adenylate cyclase activity of the hormonally autonomous MTW9-D tumors was insensitive to stimulation by PGE_2. Therefore, stimulation of adenylate cyclase activity by PGE_2 was a characteristic of hormonally dependent rat mammary tumors, but not of hormonally independent tumors of the same series.

Figure 11. Basal and PGE_2 Stimulated Adenylate Cyclase Activity in MTW9 Mammary Tumors.

Adenylate cyclase activity was assayed at 30°C after a 15 minutes incubation as described in Materials and Methods in the absence and presence of $4x10^{-5}M$ PGE_2 and/or $2x10^{-5}M$ 5'-guanylyl imidodiphosphate (Gpp(NH)p). Each value represents the mean of three experiments.

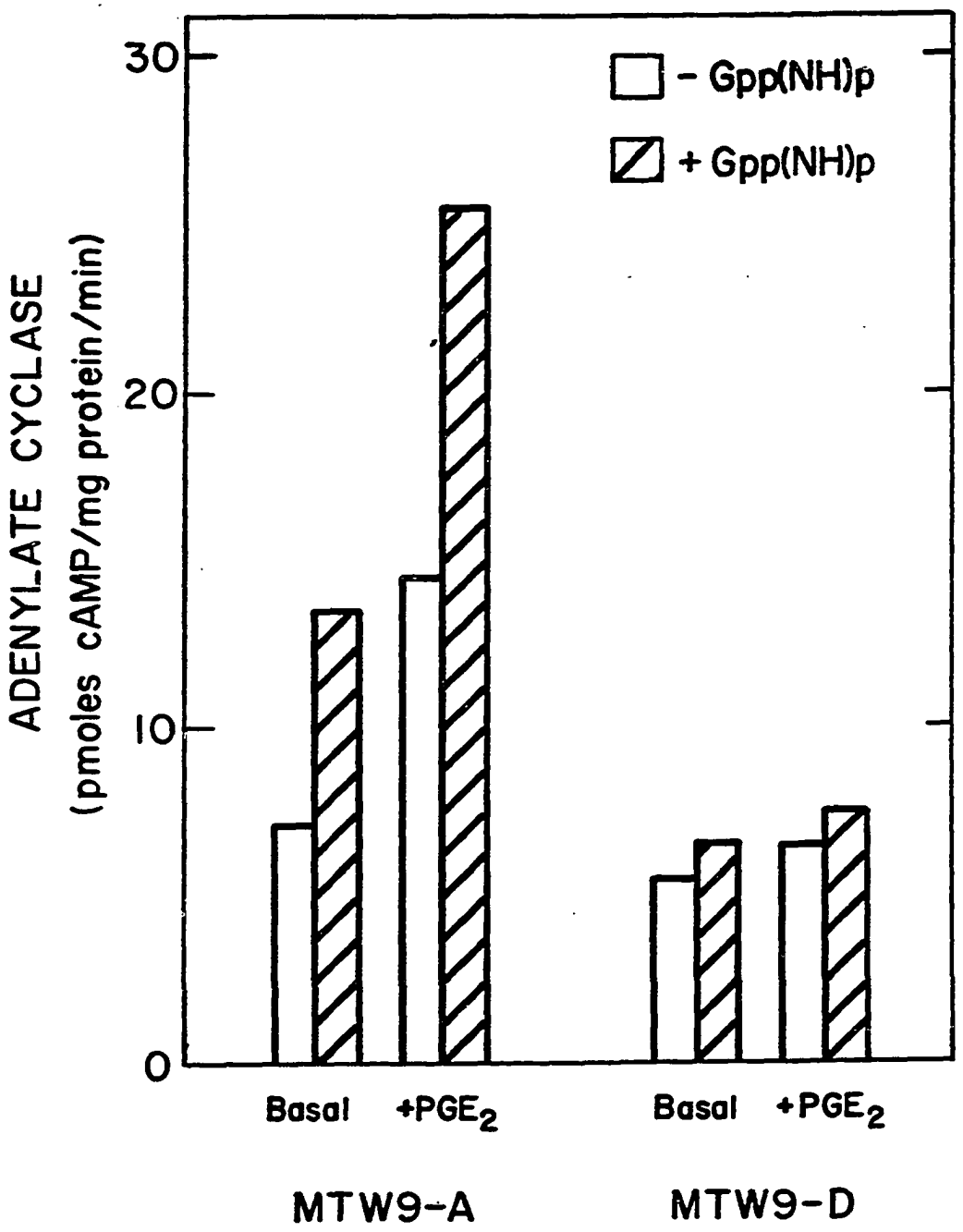


Figure 11. Basal and PGE_2-Stimulated Adenylate Cyclase Activity in MTW9 Mammary Tumors.

Thus, the hormonally autonomous MTW9-D tumors may be defective at two points in the regression process. First of all, the cAMP synthesizing system appears to be deficient even in basal activity, compared to the hormone dependent MTW9-A tumors. Secondly, even if the adenylate cyclase enzyme were responsive, PGE_2 binding by the MTW9-D tumors is so low that stimulation of cAMP by PGE_2 would be impossible. Therefore it is not clear whether the cAMP system cannot be activated or whether the total effect is attributable to the lack of PGE_2 binding.

VII. Nuclear Protein Phosphorylation in Regressing Tumor Nuclei

If prostaglandins and cAMP are directly and sequentially involved in the regression of hormone dependent mammary cancer, the outstanding question is, by what mechanism does cAMP induce tumor regression. In every eukaryotic system studied, cAMP acts to stimulate the activity of protein kinases by binding to their regulatory subunit, thereby freeing the catalytic subunit for activity (Reimann et al, 1971; Gill and Garren, 1971). The activated protein kinases phosphorylate specific intracellular proteins, thereby modifying their activities. The protein kinase substrate and its function are specific to the particular cell type in which cAMP acts. It seems reasonable that the action of cAMP in tumor regression is likely to be mediated through a protein kinase and ultimately through phosphorylation of a specific protein or proteins.

Cho-Chung has reported an increase in the rate of phosphorylation of a 76,000 MW nuclear protein in rat mammary tumors induced to regress by ovariectomy or treatment with dibutyryl cAMP (Cho-Chung and Redler, 1977). The phosphorylation of this protein was demonstrated under optimal conditions, in an *in vitro* system, in which tumor cytosols had

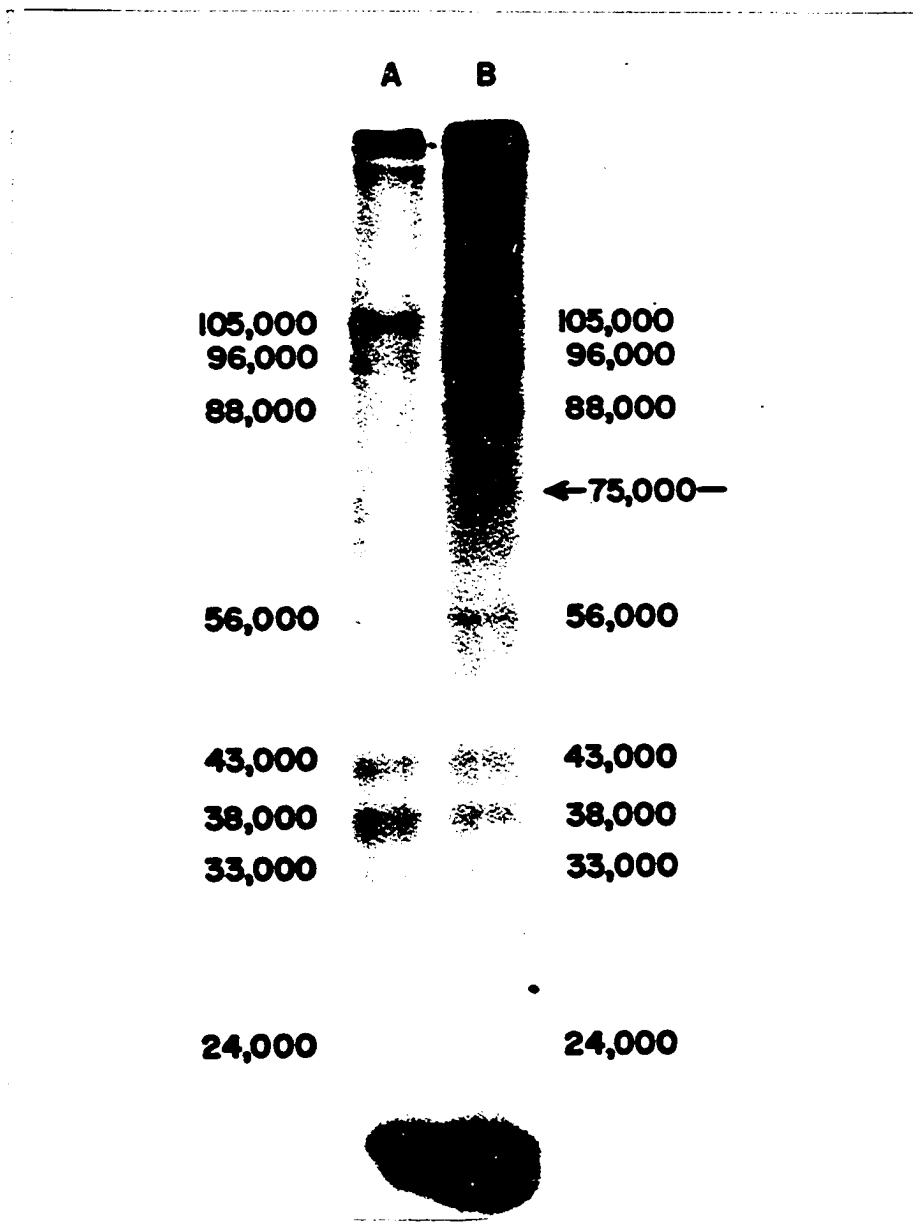
been activated by 10^{-7}M cAMP. These investigators showed that when the activated cytosols were incubated with nuclei from regressing tumors, the major phosphorylation product was a basic non-histone protein with a molecular weight of 76,000, termed regression-associated protein (RAP). The function of the RAP is unknown. The phosphorylation of the RAP is due to the activity of a cAMP-dependent protein kinase, which is translocated into the nucleus from the cytoplasm (Cho-Chung et al, 1979).

In order to correlate the changes we observed in PGE_2 and cAMP with the nuclear phosphorylation, which is a logical progression of action in regressing tumors, we attempted to demonstrate the appearance of the phosphorylated RAP in regressing tumors. Our experimental conditions were more physiological, but less favorable for RAP identification than those used by Cho-Chung. Since we had to compare growing and regressing tumors without prior treatment, tumor slices from growing and regressing tumors were incubated for 1 hour at 37° in the presence of ^{32}P-inorganic phosphate, without pre-activation of the slices with cAMP; then the nuclear proteins were isolated and separated on the basis of molecular weight by SDS-polyacrylamide gel electrophoresis (Laemmli, 1970). Phosphorylated proteins were detected by autoradiography, as shown in Plate II. A band representing a 75,000 MW phospho-protein is evident in nuclei from regressing tumors, but absent in nuclei from growing tumors. This result demonstrated that the degree of phosphorylation of the 76,000 dalton protein, the putative RAP, is indeed greater in regressing tumors than in growing tumors. Thus, phosphorylation of the RAP is correlated with elevated PGE_2 synthesis and binding and with elevated cAMP synthesis in regressing tumors.

PLATE II. Phosphorylated Nuclear Proteins from Growing and Regressing DMBA-Induced Mammary Tumors

^{32}P-Inorganic phosphate was incorporated into tissue slices from growing (A) and regressing (B) tumors. Phosphoproteins from nuclear extracts were separated by SDS-polyacrylamide gel electrophoresis, and were detected by autoradiography as described in Material and Methods. Molecular weights are indicated to the right and left. These results represent one of three experiments which gave similar results.

PLATE II: Phosphorylated Nuclear Proteins from Growing and Regressing DMBA-Induced Mammary Tumors



Furthermore, the phosphorylation of RAP is demonstrated in regressing tumors under the most physiological conditions tested to date. Other ^{32}P-labeled bands which were observed on the autoradiogram were present in the extracts of nuclei from both growing and regressing tumors. The molecular weights of these bands were 105,000, 96-97,000, 88,000, 56,000, 43,000, 38,000, 33,000 and 23-24,000 daltons. The relative intensities of the protein bands at 43,000, 38,000 and 33,000 were quite similar in the autoradiograms from the growing and regressing tumor nuclei. Differences in the intensities of some of the other bands between growing and regressing tumors were observed. For example, the apparent abundancy or phosphorylation state of the 105,000 dalton protein was greater in the growing tumors than in regressing, whereas there was an apparent diminution of the 88,000 dalton protein in growing tumors. The functions of these proteins and the reason for the differences between growing and regressing tumors is unknown. The 56,000 dalton phosphorylated protein appears to be in greater abundance in the regressing tumor nuclei than in the growing tumor nuclei. This band may represent the regulatory subunit of the protein kinase holoenzyme, which undergoes autophosphorylation (Cho-Chung et al, 1979). The greater abundance of this phosphorylated cAMP receptor in regressing nuclei is consistent with the proposal that phosphorylation of RAP is due to a translocated cAMP-dependent protein kinase (Cho-Chung, 1980).

DISCUSSION

The results of these studies demonstrate that during the regression of hormone sensitive mammary tumors an elevation of tumor PGE_2 production is associated with estrogen and prolactin depletion and suggest that PGE_2 may be functionally involved in the tumor regression induced by withdrawal of these hormones. It is proposed that the elevation in PGE_2 synthesis along with the increased PGE_2 binding which follows estrogen/prolactin depletion acts directly to stimulate the synthesis of cAMP which in turn leads to tumor regression. The translocation to the nucleus of the cAMP-dependent protein kinase holoenzyme, as proposed by Cho-Chung (1980; also Cho-Cchung et al, 1979), secondary to the elevation in cAMP content, preceeds an increase in specific nuclear protein phosphorylation within the tumor cells. It is hypothesized that this series of biochemical events may mediate the tumor regression induced by estrogen/prolactin withdrawal.

Tumors were induced by DMBA using both a single dose administration and multiple exposures. A single dose of 20 mg of DMBA given by gastric intubation at 50 days of age resulted in tumors with an average latency of 4.5 months, with the earliest tumors appearing at 55-60 days. In comparison, when rats were exposed to DMBA in 4 weekly doses beginning at 50 days of age (as described in Materials and Methods), the tumors appeared more rapidly. The earliest tumors appeared at 40-45 days and the average latency decreased to 3 months.

Figure 12. Proposed Mechanism of Tumor Regression Due to Hormonal Withdrawal

PL = phospholipid
AA = arachidonic acid
R-C
R-C = cAMP-dependent protein kinase
R = regulatory subunit
C = catalytic subunit
PRL = prolactin
OUX = ovariectomy
RAP = regression associated protein

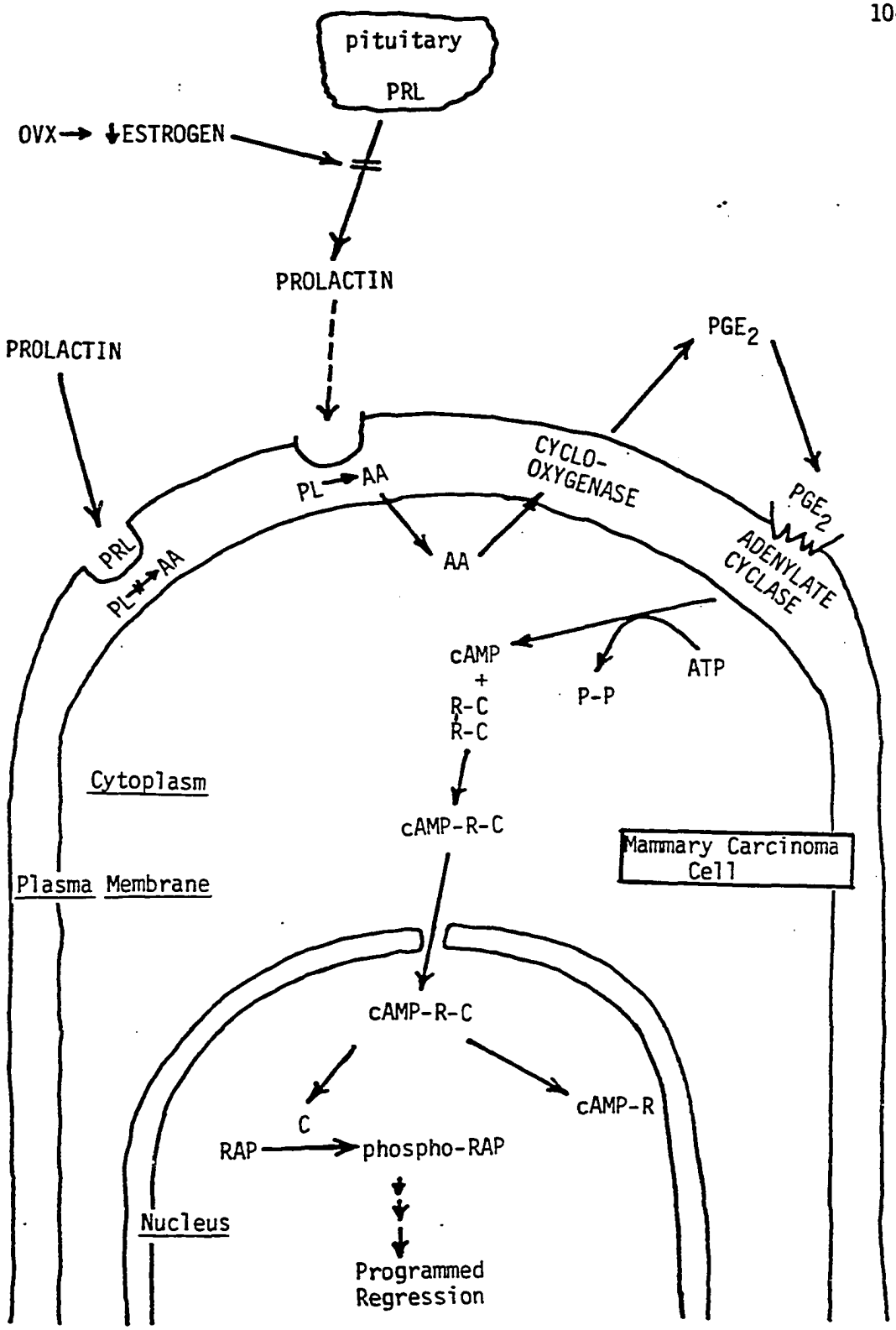


Figure 12. Proposed Mechanism of Tumor Regression Due to Hormonal Withdrawal

Thus, the regimen involving multiple exposure to DMBA was found to be more practical even though the total amount of carcinogen administered was essentially equal in the two regimens. Thus housing costs and lag time are decreased, and more importantly, the appearance of more tumors within a shorter time period allows direct comparison of tumors of similar latency and hormonal dependence. This is important in the design of experiments involving prostaglandin biosynthesis and hormonal dependence, as shown by the data in the Results section.

The results which were obtained in these studies are consistent with the theory that prolactin as opposed to estrogen is of major importance in the control of growth of DMBA-induced mammary tumors, since prolactin overcame the effect of ovariectomy on both tumor growth response, by inducing the tumors to resume growth, and on PGE_2 production, by returning the PGE_2 level to that seen in growing tumors from intact rats.

However, a critical review of the literature suggests that at least a minimal level of estrogen is essential, along with prolactin, for the continued growth of existing mammary tumors. This conclusion is supported by the observation that stimulation of tumor growth by exogenous prolactin in adrenalectomized-ovariectomized hosts is only temporary (Leung and Saski, 1975) and that many tumors do not resume growth if prolactin treatment of adrenalectomized-ovariectomized hosts occurs after a certain delay. Furthermore, the observation that grafting ovaries into ovariectomized hosts with median eminance lesions (and therefore elevated serum prolactin) results in reactivation of tumor growth (Sinha et al, 1973) is also consistent with an estrogen requirement for the growth of these tumors.

The requirement for minimal levels of estrogen may be met in ovariectomized hosts by adrenal steroid sysnthesis. Thus, in adrenalectomized-ovariectomized rats, the support of tumor growth by exogenously administered prolactin, which is temporary, may last just as long as residual estrogen remains in the blood stream of the rat (Welsch, 1972). The requirement for basal estrogen levels may be due to the fact that estrogen is required for maintenance of prolactin receptors.

The situation in which tumor growth is supported only temporarily in rats with median eminence lesions (elevated prolactin) and which have also been ovariectomized, presents a complex problem which may be interpreted as follows. Even though the adrenal glands are intact in these hosts, the requisite minimal level of estrogen cannot be synthesized because of a side effect of the median eminence lesions. The interruption of the median eminence prevents the action of the hypothalamic releasing factors. Thus in the absence of prolactin inhibitory factor, prolactin release is elevated. However, release of all the other pituitary hormones including ACTH is decreased due to the absence of their specific releasing factors. The decrease in ACTH may lead to a decrease in the synthesis of adrenal steroids, including estrogen. Therefore, even the basal source of estrogen is eliminated, explaining why ovarian grafts in the animals are absolutely necessary for the maintenance of tumor growth (Sinha et al, 1973). The studies performed in hypophysectomized-ovariectomized rats also illustrate the importance of prolactin to the growth of these tumors (Manni et al, 1977; Arafah et al, 1980) but fail to take into account the adrenal gland as a source of basal levels of estrogen.

Thus it is concluded that prolactin is an essential stimulant to the growth of DMBA-induced mammary tumors, but prolactin will not support the growth of these tumors in the absence of basal levels of estrogen.

The increase of PGE_2 levels in hormone dependent mammary tumors from ovariectomized rats compared to PGE_2 levels in comparable tumors from intact, non-ovariectomized rats suggests that hormones that are depleted by ovariectomy are involved in the regulation of prostaglandin synthesis in malignant mammary tissue. This postulate is consistent with the work of Knazek et al (1980), who showed that prostaglandin synthesis in normal murine mammary tissue varies with the stage of the estrus cycle.

Although a direct and causal connection between the alteration in hormone levels and the modulation of tumor prostaglandin synthesis cannot be firmly concluded from the present data, there is strong circumstantial evidence that this is so. On the basis of these results, there appears to be a clear inverse relationship between prolactin levels and prostaglandin synthesis. This is the first report that prolactin suppresses prostaglandin synthesis in mammary tumors.

Cho-Chung's (at the NCI, Bethesda, MD) interpretation of the events in growth and regression of hormone-dependent mammary tumors, although similar to the one proposed here, assigns central roles to estrogen and cAMP only, proposing that the estrogen receptor proteins and cAMP binding proteins must both be functional in order for mammary tumor growth to be truly hormone dependent (Bodwin et al, 1978). Thus, Cho-Chung implies that estrogen is of primary importance in the initial sequence of events involved in growth control of DMBA-induced mammary tumors. In contrast, our results suggest that the early events in the sequence which leads to

the phosphorylation of the regression associated protein, which was initially identified by Cho-Chung and her colleagues, may be prolactin withdrawal, followed by an increase in prostaglandin (PGE_2) synthesis, which stimulates an increase in cAMP, leading ultimately to protein phosphorylation.

This study represents one of the first reports of specific binding of prostaglandins to hormone dependent mammary tumors. We have demonstrated specific binding of PGE_2 to DMBA-induced and MTW9-A mammary tumors. Very recently binding of $PGF_{2\alpha}$ and PGE_2 to mammary tumors induced by nitrosomethylurea (NMU) was reported by Liu and Knazek (1982). Based on our results, specific binding of PGE_2 appears to be associated with stimulation of cAMP synthesis. In regressing tumors, in which PGE_2 synthesis is elevated, cAMP synthesis is elevated together with other changes outlined above. The stimulation of cAMP synthesis by prostaglandins in mammary tissue is consistent with the report that arachidonic acid metabolites caused a sharp transient increase in cAMP synthesis in primary epithelial cell cultures of C3H mouse mammary tumors (Burstein et al, 1977).

The cAMP concentrations observed in these studies in growing, stable and regressing tumors are consistent with reports by Matusik and Hilf (1976) and Ip and Dao (1980). Matusik and Hilf (1976) reported that cAMP content was lowest in growing hormone dependent mammary tumors, intermediate in stable tumors and highest in tumors which were regressing subsequent to ovariectomy, ergoline treatment, insulin depletion or spontaneous regression. However, as might be expected, treatment with cyclophosphamide stopped tumor growth without an elevation in cAMP. Thus, it seems that hormonally-induced and spontaneous regression of

DMBA-induced mammary tumors are associated with elevations of cAMP, whereas inhibition of tumor growth by the generalized toxic effects of cyclophosphamide is not.

Ip and Dao (1980) also reported that cAMP content is elevated in regressing and stable tumors compared to growing hormone dependent mammary tumors due to an increase in adenylate cyclase activity as well as decreased phosphodiesterase activity. Based on the observation that the cAMP content was the same in tumors of the same growth status, whether the tumors were obtained from intact or ovariectomized rats, Ip and Dao suggested that cAMP metabolism was independent of the estrogen status of the host (Ip and Dao, 1980). This hypothesis is in contrast with that of Cho-Chung, who suggests that the estrogen and cAMP systems are interdependent and inversely related (Cho-Chung et al, 1978, Bodwin et al, 1978). In mammary tumors which are regressing subsequent to ovariectomy or dibutyryl cAMP treatment, cytosolic estrogen binding activity decreases while cAMP-binding increases. Furthermore, cAMP and estrogen are antagonistic towards one another in the translocation of their respective specific binding proteins to the nucleus (Bodwin et al, 1981).

Our observation that an elevation of cAMP in regressing tumors is associated with a differential phosphorylation of proteins in the nucleus, specifically, an increase in the phosphorylation of the 75,000 dalton regression associated protein (RAP), which was demonstrable in intact tumor tissue, is consistent with the work of Cho-Chung, who identified RAP in vitro. Cho-Chung has extensively studied the relationsip of the elevation of cAMP to tumor regression in the mammary tumor system.

The protein kinase activity responsible for the phosphorylation of RAP is a cAMP-dependent protein kinase (Cho-Chung et al, 1979). This conclusion is supported on the basis of *in vitro* substrate specificity (the preferred *in vitro* substrate is histone) and on the basis of inhibition by a natural inhibitory protein which is specific for the catalytic subunit of the cAMP-dependent protein kinase (Ashby and Walsh, 1972).

Cho-Chung proposes on the basis of her data that cAMP dependent protein kinase is translocated into the nucleus as a ternary complex consisting of cAMP, the cAMP receptor protein and the catalytic subunit of protein kinase. This occurs concommitant with phosphorylation of the regression associated protein (Cho-Chung et al, 1979; Cho-Chung, 1980). The translocation of the cAMP-receptor ternary complex may be a triggering event in tumor regression (Cho-Chung, 1980).

The autoradiogram (Results, Plate II) also showed that a 56,000 dalton phosphoprotein is in much greater abundance in the nuclei of regressing than growing mammary tumors. This band may represent the regulatory subunit of the cAMP-dependent protein kinase type II; the subunit has a reported molecular weight of 56,000 (Corbin et al, 1978). Cho-Chung has reported that it is the cAMP dependent protein kinase type II which undergoes translocation into the nucleus (Cho-Chung et al, 1979). The band observed at 38,000 daltons in Plate II may in turn represent the proteolytic fragment of the 56,000 dalton receptor from protein kinase type II (Cho-Chung et al, 1981).

Both protein kinases type I and type II are present in DMBA-induced tumors, as evidenced by the identification of their regulatory subunits in these tumors. More specifically, cAMP receptor proteins of 56,000,

48,000 (which is the regulatory subunit of protein kinase type I (Hoffman et al, 1975)), and 39,000 daltons are present in DMBA-induced mammary tumors (Cho-Chung et al, 1981). However, only phosphoproteins of 56,000 and 38,000 daltons are detectable in the nuclei of these tumors. There is no evidence for the presence of the 48,000 dalton type I regulatory subunit (which would only be detected if it were phosphorylated) in the nuclei of regressing tumors.

The auto-phosphorylation of the 56,000 dalton protein occurs by phosphorylation of the regulatory subunit by the catalytic subunit, and is essential for translocation of the holoenzyme into the nucleus (Cho-Chung et al, 1981).

The function of the 76,000 dalton RAP is unknown, but its phosphorylation is always observed in regressing tumors. Since phosphorylation of a protein is usually associated with a modulation of the protein activity (frequently an increase in activity), it is quite probable that the alteration of the activity of RAP plays some role in the regression process.

The knowledge of the role that prostaglandins play in the control of cell proliferation may assist in the design of effective therapeutic protocols. The results of this dissertation support the role of prostaglandins, especially PGE_2, as negative modulators of hormone dependent mammary tumor growth. Several studies which were referred to in the Introduction did in fact demonstrate that non-mammary tumor growth and cell proliferation could be inhibited by administration of prostaglandins, generally of the E and A families.

The results of our <u>in vivo</u> studies complement the studies associating growth inhibition with exogenous prostaglandin administration

well as other studies which demonstrate that inhibition of non-mammary tumor or cell growth is dependent on endogenous prostaglandin synthesis. Thus, an important conclusion of these studies is that PGE_2 functions as an endogenous mediator of tumor growth inhibition in hormone dependent mammary cancer. An example in agreement with our studies on the action of endogenously produced PGE_2 as a negative modulator of cell replication, but in a system other than hormone dependent mammary cancer has been reported by Claesson and coworkers. Addition of exogenous arachidonic acid leads to a stimulation of prostaglandin and cAMP synthesis in normal 3T3 cells (Claesson et al, 1977; Claesson, 1980). The cyclooxygenase product responsible for the cAMP stimulation in untransformed 3T3 cells is prostacyclin (PGI_2). In 3T3 filbroblasts which have been transformed by polyoma virus, the major prostaglandin product is PGE_2. PGE_1 and PGI_2 are synthesized at one-tenth the rate of PGE_2. Since the potency of stimulation of cAMP synthesis by the three prostaglandin species when added exogenously is approximately equal, the stimulation of cAMP synthesis is primarily attributed to endogenous PGE_2 in the polyoma virus transformed 3T3 cells (Goldyne et al, 1980). The endogenously produced PGE_2 acts as a negative modulator through cAMP stimulation on growth rate, as evidenced by the fact that indomethacin treatment of the py3T3 cells prevented synthesis of PGE_2, lowered cAMP levels and stimulated the growth rate of the transformed cells (Goldyne et al, 1980).

As stated earlier, the experimental results in this dissertation lend support to an inhibitory role for PGE_2 in mammary tumor growth. This information may assist in the eventual design of therapeutic protocols for diagnosed hormone dependent mammary carcinoma where it is

desirable to induce regression of a locallized tumor. To examine the possibility that the inhibitory effects of PGE_2 might be used therapeutically, future investigations into the regression of DMBA-induced rat mammary tumors might include attempts to modify the growth of established tumors by perfusion directly into the tumor of PGE_2 or a more stable analogue, such as 16,16-dimethyl-PGE_2. To further establish that the increase in prostaglandin synthesis is essential to hormone-induced regression, the growth response of the tumors could be monitored after treatment of ovariectomized rats with a prostaglandin synthesis inhibitor, such as indomethacin or aspirin. However, there are complications involved in the interpretation of experiments involving the administration of non-steroidal anti-inflammatory agents. First of all, these inhibitors suppress prostaglandin synthesis in all tissues, not just tumor tissue. Secondly, these inhibitors may act at biochemical sites other than cyclooxygenase, complicating the interpretation of experimental results.

As Gullino and co-workers (NIH, Bethesda, Md.) have established, regression of DMBA-induced mammary tumors is an endocellular event. Whereas the immune system had to be subverted in the establishment of these tumors, the action of the immune system plays only a minor role in the regression of existing DMBA-induced mammary tumors, as evidenced by the fact that no mobilization of lymphocytes or macrophages into regressing tumors is observed (Gullino and Langerotti, 1972). Cell lysis in regressing tumors is not due to the action of degradative enzymes of extracellular origin (Gullino and Lanzerotti, 1972). The synthesis of new protein, and especially the identification of a specific protein modification (phosphorylation) within the tumor cells, lend strong

support to the conclusion that regression of DMBA induced mammary tumors is an endocellular event. It is on this premise that Cho-Chung (at NCI) has also based the design of her experiments on the molecular basis of hormone-induced tumor regression. It is also the basis of the interpretation of the results of this study.

Some discussion of non-mammary tumors is in order, since several investigators had earlier sought to modify the rate of tumor growth by modulating tumor prostaglandin synthesis. Attempts to modify tumor growth by the use of inhibitors of prostaglandin synthesis in tumor systems other than the hormone dependent mammary carcinoma have given conflicting results. Thus, attempts to control the growth of existing tumors by treatment with inhibitors of prostaglandin synthesis have resulted in either stimulation of tumor growth (cf. Introduction), no effect on the course of tumor growth, or tumor growth inhibition (see below). This apparent discrepancy may be reconcilable. In some cases the inhibition of tumor growth may be explained by the inhibitory effect of indomethacin on prostaglandin production by the tumor with subsequent effects on the immune system. There is evidence that some of the immunosuppressive effects of tumors are due to prostaglandin production by the tumors.

There are several examples where treatment with indomethacin was associated with inhibition of non-mammary tumor growth <u>in vivo</u>. Tashjian et al, (1973) reported that administration of indomethacin to mice bearing $HSDM_1$ fibrosarcomas diminished tumor size, in contrast to their previous studies which showed that indomethacin had no effect on the growth of $HSDM_1$ cells <u>in vitro</u>. Along the same line Humes and co-workers (1974) reported that indomethacin suppressed the growth of

Moloney sarcoma virus-induced tumors *in vivo*. Indomethacin or aspirin administration also inhibited growth of the P815 ascites tumor and Lewis lung carcinoma in mice (Hial et al, 1976).

The growth-modulating effect of indomethacin which results in tumor growth inhibition can be understood in light of a decreased ability of tumor cells to subvert immune surveillance through immunosuppression as a result of decreased prostaglandin production (Plescia et al, 1975). That the immunodeficiency which is often associated with tumorigenesis is a direct action of the tumor is suggested by the report that tumor cells can directly inhibit the formation of plaque-forming cells by sheep red blood cell stimulated spleen cells in vitro (Plescia et al., 1975). At least some of this inhibition of the immune system appears to be due to prostaglandin output by the tumor, since prostaglandins of E series have been shown to be negative modulators of humoral and cellular immunity (Goodwin and Webb, 1980; Goodwin et al, 1977, Plescia et al, 1975). Indomethacin blocks the immunosuppression caused by tumor cells *in vitro*, lending further support to the action of PGE_2 as a possible mechanism of tumor suppression (Plescia et al, 1975). Strausser and Humes (1975) also reported that indomethacin counteracts the prostaglandin-induced suppression of the immune system *in vivo*. Furthermore, indomethacin treatment also enhances the immunotherapeutic effects of *C. parvum* and BCG in tumor-bearing mice (Lynch and Salomon, 1979). Therefore, it appears that PGE_2 is one of a number of potent immunosuppressive products of tumors.

Thus, in the use of inhibitors of prostaglandin synthesis *in vivo*, it must be kept in mind that prostaglandins act to inhibit host immunity as well as inhibiting tumor proliferation. The benefits obtained from

removing the PG-induced suppression of the immune system may be overcome by the fact that the tumor itself will proliferate more successfully in the presence of lowered prostaglandin levels. The balance which is achieved between the two opposing actions on tumor growth will determine what the final outcome of treatment with inhibitors of prostaglandin synthesis will be.

Plescia and others (1975) proposed that the immune system may prevent the development of strongly antigenic tumors and slow the growth of weakly antigenic tumors. The immune system may be of lesser consequence in tumors of very low antigenicity.

There are other reports of inhibition of cell replication by non-steroidal anti-inflammatory agents which cannot be explained by interactions with the immune system. Hial et al (1977) reported that aspirin or indomethacin inhibited the proliferation of two cell lines in vitro, a rat hepatoma cell line and a human fibroblast culture. That the cytostatic action of non-steroidal anti-inflammatory agents on these cells was not due to inhibition of prostaglandin synthesis was proposed by de Mello et al (1980). The evidence supporting their proposal was the following: i) the concentration of indomethacin required to inhibit growth of rat hepatoma cells and human fibroblasts was 30-500 times that required to inhibit prostaglandin synthesis, ii) prostaglandins and arachidonic acid did not reverse the effects of indomethacin, and iii) the degree of cytostatic activity of the anti-inflammatory agents correlated poorly with their ability to inhibit prostaglandin synthesis. However, the most convincing evidence that inhibition of PG synthesis was not involved was the fact that the rat hepatoma cell line lacked the capacity for prostaglandin synthesis. Thus, the inhibition of cell

growth by indomethacin, and probably other anti-inflammatory agents as well, may be mediated by pathways not involving prostaglandins.

Whenever an inhibitor is used to identify the function of a biological component or activity by correlating absence of synthesis or activity with the absence of a subsequent actions, it must be kept in mind that the inhibitor may have more than one site of action. Thus, each of the non-steroidal anti-inflammatory agents may have biological actions other than inhibition of cyclooxygenase activity. When using a PG synthetase inhibitor to demonstrate a specific prostaglandin function, one should probably test two or more structurally unrelated inhibitors, and in addition, demonstrate restoration of activity by adding back the appropriate prostaglandin or intermediate.

For example, in addition to its action as a cyclooxygenase inhibitor, indomethacin has been reported to inhibit cyclic nucleotide phosphodiesterase activity (Ciosek et al, 1974; Flores and Sharp, 1972), phospholipase A_2 activity (Kaplan et al, 1978) and deacylation of cellular lipids (Ohuchi and Levine, 1978). Indomethacin also inhibits cAMP-dependent protein kinase activity in submicromolar concentrations (Kantor and Hampton, 1978). All these other possible actions of indomethacin must be considered in the interpretation of experimental results regarding indomethacin and alterations of cell replication.

In conclusion, the results of this dissertation represent an integrated study of the actions of prostaglandins, cAMP and nuclear protein phosphorylation in regressing hormone dependent rat mammary cancer. It is proposed on the basis of these results that the elevation of PGE_2 production associated with estrogen and prolactin withdrawal is an early event in the sequence of biochemical events which results in

regression. Within the framework of this hypothesis, the elevation in PGE_2 and the increased PGE_2 binding which follow depletion of estrogen and prolactin act directly to stimulate an elevation in cAMP content, leading to tumor regression. The translocation to the nucleus of the cAMP-dependent protein kinase holoenzyme, as demonstrated by Cho-Chung (1980; Cho-Chung et al, 1979), secondary to the elevation in cAMP content, preceeds an increase in specific nuclear protein phosphorylation within the tumor cells. It is hypothesized that this series of biochemical events may mediate the tumor regression induced by withdrawal of estrogen and prolactin.

APPENDIX

CAFFEINE AND UNSATURATED FAT DIET SIGNIFICANTLY PROMOTES DMBA-INDUCED BREAST CANCER IN RATS

J.P. Minton, M.D., Ph.D., M.K. Foecking, M.S.,
M. Bajorek, B.S., and H. Abou-Issa, Ph.D.

Supported by The Ohio State University Development Fund 532809, The Order of the Eastern Star of Ohio, Forty Six Club, Phi Beta Psi Sorority and the National Institutes of Health Grant CA 16058-08.

Submitted to Cancer, April 1982

A B S T R A C T

The following study was carried out to determine the possibility of caffeine being a promoter of breast cancer development in animals consuming vegetable fat. A controlled study, comparing the time of tumor development and the number of tumors as well as the growth rate of breast cancer in Sprague-Dawley rats (which had been given 50mg of DMBA at seven weeks of age) evaluated the tumor promotional effects of caffeine alone, caffeine and unsaturated fatty acids in combination, unsaturated fatty acids alone, and a standard rat chow diet. The results show that the rats which consumed caffeine and unsaturated fatty acids had the earliest development of tumor and the most multiple tumor occurrence. Average time to development was 95 days. The fat and water rats had an average time to development of 134 and 140 days respectively. The caffeine-alone rats had a mean time to tumor development of 188 days. The combination of an unsaturated fat diet and caffeine significantly shortened the time to tumor development when compared with the other three groups.

Dietary factors had been shown to modulate the incidence of breast cancer in humans as well as chemically-induced mammary tumors in animals. Several studies have implicated fat in the enhancement and development of mammary carcinogenesis (1-6). We have also reported previously that caffeine consumption is an etiological factor in the development of fibrocystic breast disease (7-9), some forms of which are characterized as being premalignant because of proliferative epithelial histology (10-12). Although the effects of fat have been extensively studied in relation to mammary tumor development, the effect of caffeine and/or caffeine and fat on the progression of breast disease to cancer has not been reported. Like fat, caffeine alone is not carcinogenic but has been shown to be a promoter of cancer induction and development. Several workers reported that caffeine potentiates the effect of alkylating agents on DNA (13-15) and enhances both virus and chemical carcinogen-induced cell transformation (16-18).

Both caffeine and fat appear to affect the tumorigenesis process at the promotional stage. In view of the well known effects of caffeine on fat mobilization and deposition, the interaction of caffeine and fat may act synergistically in the promotional development of breast cancer. Caffeine-induced lipolysis may continuously increase the free fatty acid pool possibly from the adjacent adipose tissue in the extracellular fluid surrounding transformed breast epithelial cells. Free fatty acids have been shown to stimulate mammary epithelial proliferation and growth (19). The lipolytic effects of caffeine on fat may be of significant importance in the promotion of breast cancer especially in view of the fact that the mammary gland in the human is encased in a matrix of adipose cells (20) providing an immediate source of free fatty acids. The increased availability of the free unsaturated fatty acids through caffeine lipolysis and their proliferative effects on tumor cell growth may relate to the well documented selective effects of unsaturated fatty acids on enhanced mammary carcinogenesis in experimental animals (1,4-6,21).

In view of these considerations and the possible effects of caffeine on fat *in vivo*, we were prompted to investigate how caffeine interacts with endogenous and exogenously supplied fat on mammary tumor development in carcinogen-treated rats. The experiments were designed to study the effect of caffeine alone, caffeine and fat, and fat alone on tumor production, as measured by tumor latency, in carcinogen-treated rats.

MATERIALS AND METHODS

Mammary tumors were induced in female Sprague-Dawley rats by gastric intubation of 20mg of dimethylbenz(a)anthracene (DMBA) in 1 ml of sesame oil at 50 days of age. Eighty rats were randomly divided into four groups of twenty. One group was given standard rat chow and tap water *ad lib*. The second group received standard chow, but the only source of fluid available to the rats was a caffeine solution. The amount of caffeine which each rat received per day was equivalent to 500 mg of caffeine in a 50 kg woman, based on surface area. Since surface area is proportional to weight (SA $W^{2/3}$), the basis for calculation was:

$$\frac{500 \text{ mg Caffeine}}{(50{,}000 \text{ g})^{2/3}} = \frac{\text{mg Caffeine (rat)}}{(\text{W rat (g)})^{2/3}}$$

The concentration of caffeine in the water was calculated based on the volume of fluid consumed daily per rat. The concentration of this solution was adjusted weekly, as the body weights and fluid consumption of the rats changed. A third group of rats received tap water along with a diet which was 20% vegetable fat (ICN Nutritional Biochemicals, Cleveland, Ohio). The composition of the 20% vegetable fat diet is shown in Table 1. The composition of the vegetable fat is shown in Table 2. The fourth group of rats was maintained on the 20% vegetable fat diet and the caffeine solution. The rats were weighed and examined for tumors weekly. When tumors appeared, the rate of growth was measured by vernier calipers in two dimensions.

RESULTS

Tumor latency, that is, the time between exposure to the carcinogen and the appearance of a tumor, was compared among the four treatments groups. Table 3. The group exposed to both caffeine and fat had the shortest tumor latency. The mean latency of the group on fat alone was shorter than the control group, but the difference was not significant, while the group maintained on caffeine alone had the longest tumor latency and the lowest tumor incidence.

The combination of caffeine with a high fat diet not only promoted the early appearance of mammary tumors in the rodent model but also resulted in a higher incidence of multiple tumors per rat (Table 4), compared to the control group or the groups fed fat alone or caffeine alone.

Our results on the effect of high fat diet on tumor production, as measured by tumor latency, is similar to the results reported by Hopkins

et al (22), where latency was shorter than the control group but not statistically significant.

DISCUSSION

Concern that caffeine consumption could influence the development of human cancer has received some attention in recent years. Hardly any commonly consumed drugs affect the genetic material in as many different ways as caffeine. It not only produces mutational and chromosomal aberrations, but also strongly enhances the lethal mutagenic and chromosome damaging effects of other agents. The potentiating effects of caffeine have been reported to be the result of its direct binding to DNA, as well as its ability to inhibit repair of the damage caused by the other agents to chromosomal DNA (23-27).

Indirect evidence linking caffeine with human cancer came from a number of case-control studies on the epidemiology of cancer that suggest an association between coffee drinking and cancer (28-32). On the other hand, some investigators (33,34) were unable to correlate the occurrence of cancer with the consumption of caffeine-containing beverages.

Although the literature is replete with information on the mutagenic effects of caffeine, hardly any experimental evidence links caffeine alone with human or animal cancer. However, evidence that caffeine may act as a promoter of cancer development or induction came from several studies of *in vitro* cell transformation. Several workers (16,17,18,35) reported that caffeine enhances both virus and chemical carcinogen-induced cell transformation. The general properties of the transformed cells correlate well with malignant growth. Other workers (13-15) have also shown that the effect of alkylating agents on DNA is strongly potentiated by the simultaneous administration of caffeine. These studies suggest that although caffeine by itself is not a carcinogen, it is capable of enhancing the carcinogenic effect of other agents.

Our initial results showed that caffeine consumption enhances the epithelial proliferation of the breast tissue and the development of fibrocystic breast disease. We have also found that fibrocystic breast disease, deemed premalignant by its proliferative histology, completely resolves with total abstention from caffeine consumption (7-9). This disease resolution by dietary modification alone decreased the need for surgical and radiological procedures and may reduce the risk of breast cancer development. It has been reported that breast cancer occurs in patients with fibrocystic breast disease 4 to 6 times more often than in women with normal breasts (10-12).

Several mechanisms exist which may explain the potentiating role of caffeine as a promoter of breast cancer development. In addition to its known effects on DNA damage and inhibition of DNA repair, most of its biochemical and endocrine effects *in vivo* point to a possible role in the proliferation and growth of mammary epithelial cells. Thus, if the lDNA of mammary epithelial cells is damaged by physical or chemical agents,

and then induced to proliferate in response to caffeine consumption, there may be no chance for an appropriate DNA repair and a great risk of cancer development. This process of enhancing the proliferation of DNA-damaged mammary epithelial cells, together with caffeine's effect on inhibition of DNA repair, may explain the potential role of caffeine as a promoter of human or animal breast cancer. Several reports show that the tumorigenicity of chemical carcinogens is enhanced by the stimulation of target tissues to proliferate (36-38).

Several biological actions of caffeine may be directly involved in the promotion of epithelial proliferation in the breast. Recent reports indicate that the release of certain serum-circulating hormones is stimulated by caffeine consumption and some of these hormones are involved in the pathogenesis of breast disease. Serum studies in animals have shown that caffeine causes an increase in serum prolactin (39), insulin (40), and corticosterone (41) levels, as well as a decrease in TSH, T_3 and T_4 (39). In other studies, these hormones are reported to play a role in the pathogenesis of breast cyst formation and in the growth of the mammary gland (42-44). There are no reports on the effect of caffeine on estrogen, progesterone, FSH, or LH, which are key hormones in the development of mammary epithelium. Other authors have shown increased release of catecholamines (45-47) in response to caffeine consumption. Catecholamines can modify the intracellular metabolic activities by the stimulation of the production of cAMP and cGMP (48,49). Patients with benign breast disease and breast cancer have higher urine levels of catecholamines than normal woemn (50). We have documented a progressive increase in cAMP and cGMP levels from normal breast tissue to fibrocystic disease to breast cancer (8,51,52). Because of these endocrine effects, we believe that the caffeine-catecholamines and other hormone relationships are the key to understanding breast epithelial proliferation.

In addition to these endocrine effects, the lipolytic effects of caffeine can also enhance the proliferation of mammary epithelial cells. Several studies showed a significant release of free fatty acids (46,53,54) in response to caffeine which, in turn, have been shown to stimulate the proliferation and growth of normal and neoplastic mammary cells (19). These lipolytic effects may be of significant importance, especially in view of the fact that the mammary gland of humans is encased in a matrix of fat that provides close proximity of adipocytes to the gland (20). Such an anatomical structure makes the free fatty acids from caffeine lipolysis of the breast adipose tissue readily available to the mammary epithelial cells. The availability of free unsaturated fatty acids and their effects on tumor cell growth may relate to the well documented selective effects of unsaturated fatty acids on enhanced mammary carcinogenesis in experimental animals (1,4,6,21) as well as to the epidemiologically defined role of dietary lipids in human mammary cancer (2,3,55).

We believe that caffeine in the presence of excessive unsaturated fat has an enhancing effect on breast cancer development. Indirect support to our experiments comes from epidemiological studies which have

shown independently a correlation with breast cancer production in countries where there is documented high caffeine consumption (56,57) or high fat consumption (58). Our analyses of their independent reports show that the countries reporting the highest incidence of breast and other cancers are countries where both high fat diets and high caffeine consumption have occurred simultaneouslsy.

Table 1: Composition of 20% Vegetable Fat Diet

Casein	20%
Methionine	0.3
Corn Starch	15
Sucrose	35
Fiber	5
Mineral Mix	3.5
Vitamin Mix	1
Choline Bitartrate	0.2
Vegetable Fat	20

Table 2: Composition of Vegetable Fat

Oleate	43-47%
Linoleate	39-42
Palmitate	7-8
Stearate	3-4
Arachidonate	0.5

Table 3: Effect of Dietary Modifications on Latency of DMBA-Induced Rat Mammary Tumors

Group	Avg. Latency (days)* mean ± SD
Standard Chow	140 ± 63
20% Fat Diet	134 ± 44
Caffeine + Standard Chow	188 ± 58 ($p<0.05$)
Caffeine + 20% Fat Diet	95 ± 32 ($p<0.05$)

*Tumor latency is the time between exposure to the carcinogen and appearance of a tumor.

Table 4: Effect of Dietary Modifications on Mammary Tumor Incidence in Rats

Group	Tumor-bear. Rats/ Total No. Tumors	Tumor/ Rat	Tumor-bear. rats with mult. tumors
Standard Chow	12/15	1.2	16%
20% Fat Diet	9/11	1.2	22%
Caffeine + Standard Chow	5/8	1.6	40%
Caffeine + 20% Fat Diet	11/26	2.4	73%

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