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Mode and mechanism of inhibition of mammary cancer by retinoids

Duruibe, Valentine AnayoChukwu, Ph.D.
The Ohio State University, 1987

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MODE AND MECHANISM OF INHIBITION OF MAMMARY CANCER
BY RETINOIDS

DISSERTATION

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

By

Valentine AnayoChukwu Duruibe, B.S., M.S.

*****

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DEDICATION

They worked so hard, not because they had to. They loved so much, generation upon generation. They new little of the golden fleece, yet their hearts would have rejoiced the most. They learned to love and loved till eternity. They are the source of energy and love. Like old soldiers they never died; just faded away.

To them I dedicate this work:

Mrs. Elizabeth Nweke Okafor

Mrs. Maria Uzoka
ACKNOWLEDGEMENT

I am greatly indebted to Dr. Hussein Abou-Issa for permitting me to work in his research area in his laboratory. He generously offered the suggestion and the supervision, and his invaluable advise, continuous guidance and financial support were available throughout the whole work. My gratitude goes to Dr. Daniel Couri for accepting me as his graduate student under some unusual circumstances and for his invaluable advice, friendly cooperation, and for selflessly giving his time so patiently to see to the progress of this study. I also extend my gratitude to Dr. John P. Minton for his invaluable help, kind and friendly cooperation and to Dr. Thomas Webb for his invaluable help and cooperation, for teaching me the autoradiography procedure and for the use of his laboratory space and materials for this process. I also thank the above professors and Dr. Sarah Tjioe for their suggestions as members of my reading committee. My thanks also go to Dr. Ralph Stevens for the use of his tissue culture laboratory, materials and equipments, Dr. Richard Fertel for his generous supply of $^{125}$I-cAMP and antisera for cAMP assay, Dr. John Merola for the use of his densitometer, and Dr. Gopi Tejwani for introducing me to the department of pharmacology.
For their friendly help and advice during the tougher periods of my study, I thank Dr. Norton Neff, Ms. Carol Jones, Dr. Ladi Oredipe, Mr. Alvin Jackson (medical student), I also appreciate the corporation and friendship of fellow students in the department of Pharmacology, and college of Medicine in general.

My gratitude and love go to my family.

This work would never have been presented without the patronage of St. Jude and the intervention of our saviour Jesus Christ. To them my thanks can only represent an insignificant recognition. Therefore I can only hope that this work represents to its reader a testimony to the success of their client and to their blessedness 2010.
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Hussein M. Abou-Issa, Valentine A. Duruibe, John P. Minton, Saroj Larroya, Chandradhar Dwivedi, and Thomas E. Webb: Metabolites Derived from Dietary Combination of N-(4-Hydroxyphenyl)retinamide and Calcium Glucarate Act Synergistically to inhibit the Induction of Rat Mammary Tumors by 7,12-Dimethylbenz(a)anthracene. Manuscript in preparation for submission to PNAS.

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INTRODUCTION

In addition to its role in growth promotion (Olson et al. 1981), visual process (Shichi, H. 1983) and reproduction (Howell et al. 1963), vitamin A is also important for the maintenance of normal cellular differentiation and cellular proliferation (Jetten 1981). It is also very active in the reversal of malignant transformation (Harisiadis et al. 1978; Todaro et al. 1978; Merriman and Bertram 1979; Bertram, 1980) and in the inhibition of carcinogenesis (Moon et al. 1976; Brown et al. 1977; Bollag 1979; Meyskens et al. 1982; Sporn and Roberts 1984).

The first experiments on the modulatory effects of vitamin A on growth process were conducted at the beginning of this century. It was first discovered that mice fed a lipid-free diet did not survive unless a fat-soluble extract from egg yolk was added to the diet (Stepp 1909). Soon after this, the active principle was also extracted from butter fat and cod liver oil, and was for the first time identified as an entity named fat-soluble accessory factor or "fat-soluble A" (McCollum and Davies, 1915; McCollum 1920). The name "Vitamin A" was suggested as an alternative in 1920 (Drummond, 1920).
CHAPTER I

CHEMISTRY OF RETINOIDS

Following the initial total synthesis of retinol (Isler et al. 1947), there was a massive increase in the industrial synthesis of several analogues similar to retinol in function and/or structure (Newton et al. 1980). The name "retinoid" was later introduced to describe vitamin A and its synthetic analogues (Sporn 1976).

The parent structure of retinoids is that of retinol (Figure 1), which has a hydrocarbon ring, a polyunsaturated hydrocarbon (polyprenoid/polyene) side chain and a polar terminal group. This structure is also intrinsic in the pro-vitamin A carotenoids such as beta-carotene.

A. Naturally occurring retinoids: The structure of some naturally occurring vitamin A-like compounds are shown in figure 2. The oxidative cleavage of beta-carotene at the 15-15' position yields two molecules of retinal, which can be reduced to retinol or irreversibly oxidized to vitamin A acid (retinoic acid). Retinol is stored in the liver in its esterified forms (Mahadevan et al. 1963; Huang and Goodman 1965), especially as vitamin A palmitate (retinyl
palmitate). These esters can be rehydrolyzed to retinol in the body. Due to their easy synthesis, retinyl palmitate and retinyl acetate (figure 3) are the major commercial forms of vitamin A.

B. **Synthetic retinoids**: Since the first synthesis of vitamin A by Isler and coworkers (Isler et al. 1947), over one hundred retinoids have been synthesized and tested for their potency in preventing or reversing preneoplastic and neoplastic changes in vitro (Newton et al. 1980) and in vivo (Mayer et al. 1978; Moon and McCormick 1982). Several retinoids have been produced by modification of the retinol structure. Some retinoids resulting from these structural alterations are shown in figure 3.

The activities of many of the synthetic amide derivatives of retinoic acid were reported to be almost as high as those of the parent compounds (Newton et al. 1980).

![Chemical Structure](image)

**Figure 1**: Structures of Retinol (a) and Beta-carotene (b).
Figure 2: Structures of some naturally occurring retinoids. Retinal (a), all-trans-retinoic acid (b), retinyl palmitate (c)

Figure 3: Structures of some synthetic retinoids. Retinyl acetate (a), 13-cis-retinoic acid (b), N-(4-Hydroxyphenyl)-retinamide (c), retinyl methylether (d)
CHAPTER II

PHARMACODYNAMICS OF RETINOIDS

A. Absorption of retinoids:

Vitamin A activity in the body is maintained mainly through the unscheduled dietary consumption of provitamin A carotenoids from plant sources and long-chain retinyl esters from animal tissues. Plant carotenoid pigments such as beta-carotene are mostly converted to retinol during intestinal absorption, in the mucosa cells. Only about 30% of dietary beta-carotene is absorbed unchanged (Goodman et al. 1966). Also, 13-cis-retinoic acid is not completely absorbed when given as a capsule (Brazzell and Colburn 1982). The absolute bioavailability of 13 cis-retinoic acid as determined in the dog was approximately 25% (Brazzell and Colburn 1982).

B. Distribution of Retinoids:

Retinoids are distributed to various sites in the body as retinoid-protein complex. Retinyl esters in the liver are redistributed to other sites of action as the unesterified retinol bound to a specific 21,000 dalton transport protein, retinol binding protein (RBP), synthesized
in the liver (Kanai et al. 1968; Goodman 1984). This redistribution is stringently regulated by the amount of the RBP present in the blood circulation. Retinoic acid is transported in the blood circulation, as the anion bound to serum albumin. This is perhaps the mode of transport for retinoic acid analogues. Some specific retinol and retinoic acid binding proteins have also been detected inside some normal and transformed cell lines (Chytil and Ong 1984; Goodman 1984).

Chronic dietary administration of 4-HPR results in a high level of retinoid in the mammary gland, with little accumulation in the liver (Moon et al. 1979).

C. Metabolism and Excretion of retinoids:

The oxidative cleavage of beta-carotene to two molecules of retinal, which can be reduced to retinol, occurs in the mucosa, liver and kidney. This biosynthesis of retinol from beta-carotene is catalyzed by two soluble enzymes; beta carotene 15,15'-dioxygenase which catalyzes the oxidative cleavage step, and retinaldehyde reductase which catalyzes the subsequent reduction of retinal to retinol (Goodman and Olson 1969).

Retinyl esters in the diet are hydrolyzed to retinol by retinyl ester hydrolases in the duodenum and retinol is readily absorbed into the mucosal cells. Approximately 75% of the retinol generated in the intestinal mucosa is esterified with long-chain fatty acids, to form mainly
retinyl palmitate and small quantities of other fatty acyl esters (Huang and Goodman 1965). This esterification which is catalyzed by retinol acyltransferase involves the reaction of retinol with a fatty acyl-CoA ester (Helgerud et al. 1983). The formed retinyl esters are released into the lymph by solution in the triglyceride phase of chylomicrons (Blomhoff et al. 1982), while some unesterified retinol is rapidly transported to the liver through the portal circulation (Yeung and Veen-Baigent 1974). The absorbed retinyl esters accumulate in the Kupffer cells of the liver. This hepatic retinyl ester usually represents over 90% of the total body reserve of vitamin A. Retinyl esters are constantly being hydrolyzed and re-esterified in the liver, while the parenchymal cells export free retinol in combination with specific transport proteins.

Some possible steps in the metabolism of retinol are shown in figure 4. Retinol and retinal are usually equilibrated in the body, by an NAD-coupled alcohol dehydrogenase present in most tissues (Emerick et al. 1967). The retinal formed by the reversible oxidation of retinol by alcohol dehydrogenase, can be irreversibly oxidized to retinoic acid (Deshmukh et al. 1965; Lotan 1980). Thus, low concentrations of retinoic acid can be found as endogenous retinoid in several tissues. Since retinol naturally occurs as an all-trans isomer, the major
oxidation product of retinol is all-trans retinoic acid. However, it has been suggested that 13-cis-retinoic acid may also be formed during retinol metabolism (Zile et al. 1967; Brazzell and Colburn 1982). Studies on the metabolism of all-trans- and 13-cis-retinoic acid in rodents (Swanson et al. 1980) and 13-cis-retinoic acid in man (Brazzell and Colburn 1982), showed that both of these retinoids are rapidly metabolized in the body.

The plasma elimination half life for 13-cis-retinoic acid was determined to be approximately 4 hours in the rat (Swanson et al. 1980) and 10 to 20 hours in man (Brazzell and Colburn 1982). However, this half life is longer than that of the all trans-isomer, which is approximately 20 min in the rat (Swanson et al., 1981a) and 6 to 13 hours in man (Brazzell and Colburn 1982).

Some of the detected metabolites of retinoic acid are 5,6-epoxy derivatives, beta-glucuronides and 4-hydroxy derivatives (figure 4). At physiological doses, both isomers are metabolized to 13-cis-4 hydroxy-retinoic acid (Roberts, 1981). The 4-hydroxy-retinoic acid can be further oxidized to 4-keto retinoic acid, which can be further metabolized to more polar metabolites (Roberts 1981). The NADPH-dependent metabolism of retinoic acid to 4-hydroxy RA and of 4-oxo-RA to more polar metabolites are inducible by pretreatment with retinoids (Roberts 1981). The major
metabolite of 13 cis-retinoic acid in human plasma is 4-oxo-13-cis-retinoic acid (Colburn et al., 1983; Chytil, 1984).

HPR has a longer plasma elimination half life (12 hours) in rats, than retinoic acid (Sani and Meeks 1983; Swanson et al. 1980; 1981a). Although the major metabolite of HPR is not yet fully identified, some studies suggested that HPR may be metabolized to more lipophilic compounds (methoxy-HPR and an unidentified metabolite) and several polar metabolites, including HPR-O-glucuronide and possibly HPR-SO₄ (Swanson et al. 1980; 1981b).

In general, retinoids with intact carbon chain are excreted in the feces, whereas the acidic chain shortened products are excreted in the urine (Olson 1981).

D. Toxicity:

The clinical application of retinoids for the treatment of epithelial cancers was hindered by the hypervitaminosis-A syndrome associated with high doses of retinol and its natural analogues. In humans, this syndrome manifests as alterations of the skin (erythema, hair loss, desquamation), and mucus membrane (stomatitis, cheilitis, conjunctivitis), headache, nausea, vomiting, abdominal cramps (Cassidy et al., 1981), and in more serious cases hepatic damage (Smith and Goodman 1976). The symptoms observed in laboratory animals include weight loss, skin
Figure 4. Pathways of retinol and retinoic acid metabolism.
desquamations, hair loss, and bone fractures (Sani and Meeks 1983). The problem of hypervitaminosis A, led to many attempts to synthesize retinoids with high activity, high tumor specificity and low toxicity (Mayer et al., 1978, Newton et al., 1980).

Like all trans-retinoic acid, 13-cis-retinoic acid does not accumulate in the liver (Dowling and Wald 1960, Swanson et al. 1980). It is also less toxic than both all trans-retinoic acid (Sporn et al. 1977) and retinyl acetate (Hixon and Denine, 1978, 1979; Hixon et al., 1979). Its LD$_{50}$ (145 mg/kg i.p.) is much higher than that of all trans-retinoic acid (33 mg/kg) in mice (Swanson et al., 1981a).

N-(4-Hydroxyphenyl)retinamide is one of the retinamides identified in the attempt to improve upon the therapeutic index obtained with 13 cis-RA (Moon and McCormick, 1982). They are also less toxic than all-trans-retinoic acid and possess appreciable anticarcinogenic properties in experimental animals (Hixson and Denine 1979; Moon et al. 1979; Thompson and Becci 1979). HPR has the additional advantages of higher LD$_{50}$ (500 mg/kg i.p.) than retinoic acid.
CHAPTER III

PHARMACOLOGY OF RETINOIDS IN RELATION TO CANCER

A. Effects on cancer:

Even before the name "vitamin A" was introduced, there were attempts to determine the effect of fat-soluble A on carcinogenesis. Although Passey excluded vitamin A as a modifier of experimental soot cancer, their study revealed a slight delay in tumor development in animals fed vitamin A-rich diet (bread soaked in butter) as compared to the controls given bread alone (Passey, 1922). It is possible that Passey expected this growth promoting factor to enhance, rather than inhibit cancer. At about the same period, a study using in vitro organ culture, revealed that vitamin A deficiency induced hyperkeratosis and also caused metaplastic changes in the epithelia of several tissues (Mori 1922). It was later reported that an adequate level of vitamin A was necessary for the control of normal cellular differentiation and proliferation (Wolbach and Howe, 1925). In the above classical study, vitamin A deficiency was associated with failure of normal differentiation from stem cells to mature epithelial cells in the rat.
It was also associated with excessive epithelial cell proliferation and some abnormal cellular differentiation, characterized by hyperkeratosis. The first direct link between vitamin A and cancer in vivo was made in the mid 1920s. In 1926, a study by Fujimaki, revealed a high incidence of stomach carcinoma in rats fed vitamin A deficient diet. During the same period, it was hypothesized that cancer was a result of agents that deplete vitamin A and cause local vitamin A imbalance (Burrows, 1926). The connection between vitamin A and the inhibition of carcinogenesis gained more attention in the early 1950s, when another classical study showed that the differentiated phenotype of chick epidermis in organ culture, could be changed from keratinized to mucous-producing, by treatment with either retinol or retinyl acetate (Fell and Mellanby, 1953). The above study, which presented the mirror image of the 1925 classical experiments of Wolbach and Howe, was perhaps the first application of vitamin A and its analog in the reversal of altered differentiation of the epithelium. As suggested by Sporn (1976), all preneoplastic lesions of the epithelia from different organs represent altered states of epithelial differentiation and it could be said apriori that retinoids can reverse the metaplasia due to carcinogens.

Subsequent studies revealed some similarities between
the metaplasia due to vitamin A deficiency and those due to carcinogens (Moore 1967; Harris et al. 1972). The above reports, coupled with the fact that one of the physiological roles of vitamin A is the maintenance of normal phenotypic expression in epithelial tissues, led to considerable attempts to prevent or reverse tumorigenesis with retinoids.

In 1964, some investigators detected that the induction of squamous cell tumors of the esophagus and forestomach of hamsters by polycyclic hydrocarbons, was inhibited by concomitant administration of retinyl palmitate with the carcinogen (Chu and Malmgren, 1965). Soon after, it was shown that retinyl palmitate delayed the appearance and inhibited the growth of virus-induced papillomas in the rabbit (McMichael, 1965), DMBA-induced papillomas in the mouse skin (Davies, 1967), and benzo(a)pyrene-induced cancer of the respiratory track in hamsters (Saffiotti et al., 1967). Another study in the mid 1960s suggested that retinol enhanced the inhibitory action of cytoxan on mammary cancer in mice (Brandes and Anton, 1966).

More recent studies have confirmed that retinoids inhibit epithelial cancers of several sites, including the skin (Bollag, 1975), urinary bladder (Sporn et al., 1977), lung (Nettesheim et al., 1976), prostate (Lasnitzki, 1976), and mammary gland (Moon et al., 1976). Also, some
recent studies showed that retinoids caused the differentiation of fully transformed, invasive, neoplastic cells to more benign, nonneoplastic phenotypes (Strickland and Mahdavi, 1978; Breitman et al., 1980, 1981).

B. **Effect on cancer incidence in humans:**

Epidemiological studies have associated low serum vitamin A levels with cancer incidence in humans (Kark et al. 1981). However, another study suggested that the low serum vitamin A observed in the cancer patients may be a consequence rather than a cause of the cancer (Wald et al. 1986). It should however be noted that most primary cancers in humans occur in the epithelial tissues that depend on retinoids for normal cellular differentiation (Moore 1975; Verna et al. 1979).

Thus retinoids have been successfully used to promote the differentiation and inhibit the growth of human melanoma cells (Meyskens and Fuller, 1980). They are also effective in the chemoprevention and therapy of other human skin basal cell carcinomas (Peck et al., 1982) and keratoacanthomas (Haydey et al. 1980).

C. **Effect on breast cancer:**

As mentioned above, several retinoids have been tested for their potencies in the inhibition of carcinogenesis, including mammary cancer (Moon et al.).
1983). However, this section will consider only a few retinoids relevant to the present study.

1. **Retinyl Acetate:** A decade after the suggestion that retinol may potentiate the inhibitory effect of cytoxan on mammary cancer (Brandes and Anton, 1966), Moon and coworkers demonstrated the inhibition of DMBA-induced mammary carcinogenesis by retinyl acetate (Moon et al., 1976). In their experiments, 50 days old virgin female Sprague-Dawley rats treated intragastrically with a single dose of 2.5 or 15 mg DMBA, were started 7 days later on a diet containing either retinyl acetate (1 to 2.5 mg/day) or a placebo. Retinyl acetate inhibited the occurrence of both DMBA-induced and spontaneous mammary tumors in the rats. The above investigators later showed that retinyl acetate can also inhibit MNU-induced mammary tumors (Moon et al., 1977). More recently, retinyl acetate was also shown to have little antiproliferative effect on human mammary carcinoma MCF-7 cell line (Ueda et al., 1980).

However, like retinol and other esters of retinol, the accumulation of retinyl acetate in the liver and the associated hypervitaminosis A syndrome prohibits its use at doses high enough to maintain plasma levels needed to suppress mammary cancer in humans.

2. **Retinyl Palmitate:** The effectiveness of retinyl palmitate in the inhibition of mammary cancer was suggested
by its ability to inhibit the growth of transplanted syngeneic mammary adenocarcinoma in C3H/HeJ mice (Rettura et al. 1975). Retinyl palmitate was shown to be more potent than retinyl acetate in the inhibition of MCF-7 cell growth in culture (Ueda et al. 1980).

Like retinyl acetate and other retinol esters, the incidence of hypervitaminosis A syndrome prohibits the use of retinyl palmitate in the prevention of mammary and other epithelial cancers.

3. **13-Cis-Retinoic acid:**

Studies with low doses of 13-cis-retinoic acid have shown no inhibition of MNU-induced mammary tumors (Thompson et al., 1978) or advanced, hormone-resistant, human mammary cancer (Cassidy et al. 1981). However, it has been shown to be more effective than retinol, retinyl palmitate or retinyl acetate in the inhibition of growth of human mammary carcinoma, MCF-7 cells in culture (Ueda et al., 1980). Its effect on the DMBA-induced mammary tumor has not been demonstrated.

Some investigators have suggested that 13-cis-retinoic acid may be effective in the inhibition of mammary cancer, if administered more frequently than at the rates used in previous studies (Kerr et al., 1982). This requirement for more frequent administration of 13 cis-retinoic acid is a result of its pharmacokinetic
properties. It is not completely absorbed when given as a capsule (Brazzell and Colburn 1982).

It has been reported that 13-cis-RA is equivalent to all-trans-RA in biological activity, in vitro (Frolik, 1981) and in vivo (Zile and De Luca 1968).

4. **N-(4-hydroxyphenyl)retinamide**: Attempts to synthesize retinoids with high tissue selectivity and high anticarcinogenic activity in the mammary gland, as well as low liver accumulation, resulted in the synthesis of many retinamides (Moon et al. 1979).

Of all the retinamides synthesized and tested, **N-(4-hydroxyphenyl)retinamide (HPR)** was the most active for the inhibition of chemical carcinogenesis (Moon et al. 1982). This is also perhaps the most promising retinoid for the chemoprevention of breast cancer, due to its pharmacokinetics and tissue distribution (Swanson et al. 1980). Like all-trans-retinoic acid, HPR has been shown to inhibit prolactin-induced end bud differentiation, while also causing thin ducts and reduced ductal branching, in the mouse mammary gland (Mehta et al. 1983).

D. **Effects on organ culture**:

The first report of the effects of retinoids on organ culture was perhaps the study of Fell and Mellanby (1953),
using chicken epidermis. The ability of various retinoids to reverse hyperkeratosis in organ culture is now well established. Thus, the reversal of hyperkeratinosis in the hamster tracheal organ is now widely used as a test for the potency of new retinoids (Newton et al. 1980). Retinoids have been shown to inhibit the prolactin-induced end bud differentiation in the mammary gland (Mehta et al. 1983). They have also been shown to prevent mammary gland transformation by carcinogenic hydrocarbons in whole organ culture (Dickens et al. 1979).

E. **Effects on cell systems:**

As previously mentioned, vitamin A is essential for normal growth and differentiation of the epithelium. Retinoic acid at nanomolar concentrations promotes the growth of epithelial cells (Ueda et al. 1980), whereas micromolar concentrations inhibits epithelial cell growth and proliferation (Lotan et al. 1978; Ueda et al. 1980). It has been shown that retinoic acid restores density-dependent inhibition of growth in mouse L-929 cells (Dion et al., 1977). The inhibitory effect of retinoids on human breast cancer cells has also been reported (Ueda et al., 1980; Wetherall and Taylor 1986). Retinoic acid was more effective than retinol or retinyl esters, retinyl palmitate and retinyl acetate in the inhibition of MCF-7
cell growth. However, there is no report on the effect of retinamide on the growth of these cells.

Retinoids can also induce the terminal differentiation of many cell types. Relative to this, F-9 embryonal carcinoma cells were induced to differentiate into parietal endodermal cells following treatment with retinoic acid (Plet et al., 1982; Strickland and Mahdavi, 1978). Also, treatment with retinoids caused HL-60 promyelocytic leukemia cell line to differentiate into functional granulocytes capable of phagocytosis (Breitman et al., 1980). It has been reported that melanoma cell lines growth inhibited by retinoids produced more melanin and had increased tyrosinase activity (Lotan et al., 1978). The inhibitory effect of retinoids is sometimes associated with morphological changes in the cell. Thus, cell fattening (Chu-Chong 1982) and increased glycosylation of membrane proteins (Moore et al., 1981) have been detected following treatment with retinoids. However, various investigators have reported marked differences in the susceptibility of various untransformed and transformed cells in vitro, to growth inhibition by retinoids (Chytil and Ong, 1976; Lotan and Nicolson, 1977).
CHAPTER IV
MECHANISM OF ACTION OF RETINOIDS ON CANCER

Several studies have shown that retinoids inhibit the process of carcinogenesis in vivo and inhibit malignant transformation, reverse altered differentiation, and inhibit cellular proliferation in vitro in several experimental systems. These compounds represent one of the more well defined areas of biological modifiers, and an elucidation of their mode and mechanism of action should prove important to the nontoxic control of cancer.

The mechanism by which retinoids prevent or inhibit carcinogenesis is presently unknown. Retinoids have been shown to inhibit end bud proliferation in the mammary gland of rats (Moon et al., 1979) and to inhibit areolar nodulogenesis in mice mammary gland (Moon et al., 1983). The inhibition of DMBA-induced mammary cancer and other epithelial cancers by retinoids is generally believed to involve the inhibition of growth or differentiation of premalignant lesion (Nettesheim et al., 1976; Sporn 1976; Grubbs et al., 1977). However, other mechanisms mediated by the endocrine system (Sporn and Harris 1981) or the immune system
(Grubbs et al. 1977; Lotan 1980; Tannock et al. 1972; Meltzer and Cohen 1974) have also been suggested (Mayer et al. 1978). Some investigators also suggested that retinoids may be acting through their labilization of lysosomes, leading to the release of lysosomal enzymes, which may be cytotoxic (Jarrett and Spearman, 1970). Although other biological effects have also been considered (Kensler et al. 1978), this section will be limited to the more popular proposed mechanisms.

A. Labilization of lysosomes: This mechanism assumes that the anticarcinogenic action of retinoids is a function of their labilizing effects on membranes. Retinoids have been shown to exert some labilizing effects on membranes in vitro (Fell et al. 1962). This led to the suggestion that similar labilization of lysosomes, leading to the release of lysosomal enzymes such as cathepsin and other proteases (Lucy et al. 1961) and the subsequent cytotoxicity may be involved in retinoid inhibition of carcinogenesis (Jarrett and Spearman, 1970).

However, this mechanism cannot explain several effects of non cytotoxic levels of retinoids in the reversal of altered differentiation and prevention of premalignant transformations. Also, it has been shown that retinoids can inhibit cellular proliferation in the presence of membrane stabilizing agents such as corticosteroids (Lotan et
al. 1978). Other studies have shown decreased amounts of lysosomal enzymes after treatment with retinoids in vivo (Peck et al., 1982) and in vitro (Camisa et al. 1982). There was also no evidence of cytotoxicity in the above patients. An objective exclusion of lysosomal labilization related cytotoxicity as a mechanism of retinoid inhibition of cell growth, was evidenced by the high cell viability and lack of retinoid-induced lysosomal enzyme release during treatment of MCF-7 cells with growth inhibiting concentration of retinoids ($10^{-7} - 10^{-4}$M) (Ueda et al., 1980). Therefore, this mechanism can only explain some toxic effects of retinoids (Meeks et al., 1981).

B. Induction of necrosis: The induction of necrosis was suggested as a possible mechanism of action of retinoids in the regression of skin papillomas (Mayer et al., 1978). The above authors suggested that retinoids may induce necrosis in papillomas by increasing the synthesis and release of glycoproteins and/or mucopolysaccharides into the intercellular spaces, with subsequent disruption of cell contact and anchorage. Thus, necrosis was suggested to be the cause of regression of these tumors.

It is unlikely that this can be a generalized mechanism, since it cannot explain several effects of retinoids which do not involve any evidence of necrosis. For example, it cannot explain the in vitro antiproliferative and
differentiating actions of retinoids, nor can it explain the retinoid induced inhibition of premalignant transformation. Also, not all cases of retinoid-induced regression is associated with necrosis. However, the increase in glycoprotein synthesis may be one of several results of retinoid-induced differentiation (Peck et al. 1982).

C. Immunological effects: The suggestion that retinoids may have some anticarcinogenic effects mediated through the immune system was based on some of the following effects of retinoids on the immune system. Retinoids are known to promote the rejection of skin allografts (Jurin and Tannock 1972) and to also show some immune mediated effects on some tumor systems (Tannock et al. 1972; Seifter et al. 1973). It has been suggested that retinoids act as adjuvants and non-specifically increase the host immune system. Thus, retinoids were used experimentally as adjuvants for the treatment of malignant tumor (Meyskens et al. 1982).

The differentiating action of retinoids has been associated with the regulation of interferon production (Blalock and Gifford, 1977). In this respect, immune stimulation in combination with retinoid administration in the diet, synergistically inhibited the incidence of DMBA-induced mammary tumors in the Sprague-Dawley rat (Welsch and DeHoog 1983). Immune stimulation alone was ineffective in the inhibition of these tumors. The effects of retinoids
on the immune system may result from the induction of
differentiation by retinoids and cannot explain the in
vitro effect of retinoids on malignant cells.

D. Endocrine System alteration: There were suggestions that
retinoids may effect cell growth by regulating the action
of some growth hormones and growth factors (Jetten 1981;
Mehta et al. 1983). In the case of mammary tumors, it was
suggested that retinoids may inhibit estrogen which is
necessary for the growth of mammary tissues (Mehta et al.
1983).

However, various studies have shown that the latter
mechanism is unlikely. For instance, there was no change in
the oestral cycle of rats whose mammary tumors were
inhibited with retinoids (Mehta et al. 1976; Moon et al.
1976 & 1979). Also, the combination of retinoids and
hormonal manipulations such as ovariectomy, or an
antiestrogen such as tamoxifen, was synergistic in the
inhibition of mammary tumor induction (Moon and McCormick,
1982; Welsch and DeHoog 1983). An action through hormonal
alteration can also be excluded in explaining the
antiproliferative effects of retinoids in vitro.
E. **Enhancement of differentiation:** The most important rational for the use of retinoids in chemoprevention of cancer is their physiological role in maintaining the normal differentiation of epithelial and some mesenchymal tissues (Wolbach and Howe, 1925; Sporn and Roberts, 1984; Breitman et al., 1981; Strickland and Mahdavi, 1978). As pointed out above, retinoids can influence several systems through their induction of differentiation. The ability of retinoids to maintain a normal level of differentiation in untransformed epithelia and also prevent and reverse carcinogenesis in several systems has led to studies directly aimed at elucidating the underlying mechanism of these effects of retinoids. It is commonly agreed that retinoids can regulate cellular differentiation by modulation of gene expression.

F. **Cellular retinoid binding protein dependent action:**

A plausible hypothesis suggested that retinoids act like steroid hormones by binding to specific intracellular proteins, the complex of which is transported into the nucleus where they bind to the DNA and regulate gene expression (Chytil and Ong 1976; Saini and Hill 1976). This hypothesis was supported by the detection of specific binding proteins for retinol (CRBP) and retinoic acid (CRABP) in the cytoplasm and nuclei fractions of some
transformed cell lines (Libby and Bertram, 1982).

However, there are reports of retinoid-inhibited cells which lack either of these specific retinoid binding proteins. For example, retinoid binding protein was not detected in the C3H/10T1/2 C18 cell line which was sensitive to the anticarcinogenic action of several retinoids (Libby and Bertram, 1982). Also, another study found no correlation between the level of CRABP or CRBP and the extent of inhibition of several malignant cell lines by either retinoic acid or retinyl acetate (Lotan et al., 1980). In the above study, neither the MSV3T3 cells with CRABP, nor the RAW8 cells with both CRABP and CRBP were growth inhibited by retinoic acid or retinyl acetate. On the other hand, L1210-A5 leukemia cells, whose proliferation was inhibited by both retinoids, had no detectable retinoid binding protein. Thus, the presence of retinoid binding protein does not appear to be a prerequisite for the differentiating and antiproliferative effect of retinoids.

Although the presence of retinoid binding protein may aid the intracellular transport of retinoids, it does not appear to be a universal property of all retinoid-sensitive cells. Therefore, the mechanism based on steroid hormone-like regulation of gene expression can not explain the diverse action of retinoids.
G. Modulation of the cyclic AMP system:

An alternative mechanism by which retinoids can control gene expression and cellular proliferation in a more universal manner is via the cyclic AMP (cAMP) system (Kuo and Greengard 1969). The cAMP system (figure 5) is a universal and ubiquitous secondary mediator for most hormonal actions (Sutherland, 1972). It has been implicated in the regulation of cell growth and differentiation (Pastan et al., 1975; Costa 1978; Parasad et al., 1980).

\[
\begin{align*}
A \text{ cyclase} & \\
4\text{ATP} & \rightleftharpoons 4\text{cAMP} + 4\text{ADP} \\
\text{R} \quad \text{R} & \\
\text{C} \quad \text{C} & \\
\text{inactive cAMPdPK} & \\
\text{R} \quad \text{R} & \\
\text{C} \quad \text{C} & \\
\text{active cAMPdPK} & \\
\text{Mg}^{2+} \text{ATP(Y-P)} & \rightarrow \text{Y-P} \\
\text{ADP} + \text{Protein} & \\
\text{Response} & \\
\rightarrow 2\text{R}\text{+4cAMP} &
\end{align*}
\]

Figure 5: Schematic representation of the Cyclic AMP system. cAMPdPK; cAMP-dependent protein kinase, R; regulatory subunit. C; catalytic subunit. / \; cAMP
CHAPTER V

THE CYCLIC AMP SYSTEM

A. Cyclic AMP-dependent Protein kinase:

In every system studied, cAMP has been shown to activate a specific protein kinase, which in turn phosphorylates specific proteins that regulate cellular processes (Walsh et al. 1968; Kuo and Greengard 1969). Cyclic AMP-dependent protein kinases have been purified from a number of tissues. The holoenzyme consists of two catalytic and two regulatory subunits (Gill and Garren, 1970; Brostrom et al., 1970; Tao et al., 1970; Kumon et al., 1970). More detailed studies have shown that there are two monomeric catalytic subunits and one dimeric regulatory subunit, containing a high affinity and a low affinity cAMP binding site per monomeric chain (Corbin et al. 1978). The regulatory subunit exerts an inhibitory action on the catalytic subunit; thus the holoenzyme has low activity. Binding of two molecules of cAMP to each of the two monomeric regulatory subunits allows the catalytic subunits to dissociate (Brostrom et al. 1971), activating the
enzyme (Swillens, 1978) according to the following equation.

\[ R_2C_2 + 4cAMP <---------> R_2.4cAMP + 2C \ldots \ldots \ldots \ 1. \]

The holoenzyme in vivo is very sensitive to changes in the levels of cAMP. Thus, the amount required for 50% stimulation; \( S_{0.5} \) for cAMP is 10nM at enzyme concentrations of 0.2-0.7 uM (Hemmings 1985).

B. cAMP dependent protein kinase subtypes:

Two isozymes of cAMP-dependent protein kinase have been shown to exist in tissues. These isozymes called type I and type II cAMP dependent protein kinase differ only in their regulatory subunits (Corbin et al. 1975, Hofmman et al. 1975), referred to as \( R_I \) and \( R_{II} \) respectively. The reactions catalyzed by these enzymes can be generally represented by the following equation:

\[ NTP.Mg^{++} + Protein \rightarrow Protein-P + NDP.Mg \ldots \ldots 2. \]
CHAPTER VI

THE CAMP SYSTEM IN CANCER CONTROL

A. cAMP in cellular proliferation and cancer control:

Contact inhibited cells in culture have higher cAMP contents than growing cells, and several transformed cell lines have lower cAMP levels than their normal cells of origin (Pastan et al., 1975). Some investigators have suggested that increase in cellular cAMP is a prerequisite for contact inhibition of cell growth (Bannai and Shephard 1974). However, increased cAMP was also observed after 3T3 cells stopped dividing, regardless of density (Siefert and Paul, 1972). Analogues of cAMP such as dibutyryl cAMP have been shown to inhibit the growth of human breast cancer cells in vitro (Cho-Chung et al., 1981). Thus, the intracellular accumulation of endogenously generated or exogenously supplied cAMP has been shown to result in the inhibition of growth of both normal and transformed cells in vivo and in vitro. There is an inverse relationship between the amount of intracellular cAMP and the rate of cell growth in many cases. Although unrestrained growth which is characteristic of neoplastic cells is not always
associated with diminished levels of cAMP, it is generally believed that cAMP plays an important role in the control of cellular proliferation and malignancy. Several studies have shown that cAMP, its analogues or agents that elevate cAMP induce cellular differentiation in many systems (Cho-Chung, 1982). The results of this differentiation can be observed as changes in the morphology (Fayet and Lassitsky, 1970; Johnson et al ., 1971; Cho-Chung et al ., 1981), ultrastructure (Klein and Loizzi, 1977), and function (Wicks, 1974; Cho-Chung and Gullino, 1974; Pastan and Johnson, 1974; Prasad, 1975) of the cells.

B. **cAMP-dependent protein kinase in cancer control:**

An increase in cAMP dependent protein kinase activity by activation with cAMP or by addition of the catalytic subunit, inhibited, where as addition of the regulatory subunit induced cell division (Maller and Sadler 1981).

Some studies have involved cAMP-dependent protein kinase in the inhibition of mammary tumor growth following ovariectomy or treatment with dibutyryl cAMP (Cho-Chung, 1980; Foecking *et al* ., 1983). Subcellular redistribution of cAMP-dependent protein kinase occurs during the inhibition of mammary tumors by dibutyryl cAMP treatment or ovariectomy (Cho-Chung and Redler, 1977) and upon treatment of F9 embryonal carcinoma cells with retinoic acid (Plet *et al* ., 1982).
C. **Cyclic AMP system in retinoid action on cancer:**

Some studies have suggested the involvement of cAMP-dependent protein kinase as a possible modulator of the antiproliferative and differentiative action of retinoids.

1. Priming of human myeloid leukemic cell lines with retinoic acid potentiated the antiproliferative and differentiating action of dibutyryl cAMP and other cAMP-inducing agents (Olsson et al. 1982).

2. Both cAMP and retinoic acid did not inhibit the growth of protein kinase-deficient cell lines. Thus, cAMP was unable to inhibit the growth of a protein kinase deficient variant of $S_{49}$ lymphoma cells (Coffino and Gray, 1978), and retinoic acid could not inhibit the growth of MR-4 cells, a protein kinase deficient variant of $B_{16}$-$F_{1}$ cells (Ludwig et al., 1980). The growth of the original cells were inhibited in both cases.

3. The modulation of cAMP dependent protein kinase and phosphorylation of their substrates have recently been associated with the extent of differentiation induced in HL60 cells and human myeloblast cell line RDFD by retinoids (Fontana et al. 1986).
CHAPTER VII

STATEMENT OF THE PROBLEM

Various investigators have attempted to elucidate the mechanism of action of retinoids on processes involved in carcinogenesis. However, most of the suggested mechanisms cannot explain the diverse effects of retinoids on malignant transformation, altered differentiation, cellular proliferation, tumor inhibition and tumor regression in several epithelial systems.

The most likely explanation of this diversity is that retinoids act through a universal system to regulate gene expression which in turn influences all the above processes. Of all the mechanisms proposed, the action modulated by the cyclic AMP system is the best to account for the diverse action of retinoids on several systems. As previously mentioned, treatment with analogs of cAMP induced the redistribution of cAMP-dependent protein kinase of 7,12-dimethylbenz(a)anthracene-induced mammary tumors (Chochung and Redler 1977; Foecking et al. 1983). It is possible that retinoids also cause a redistribution of cAMP-dependent protein kinase activity into the nucleus, causing the phosphorylation of specific nuclear proteins that regulate gene expression and cause a selective increase or
decrease in synthesis of some specific proteins that regulate cellular differentiation, cell growth and tumor development. Retinoids have been shown to modulate RNA synthesis in the epithelium (Zachman 1967). It is possible that the retinoid-induced RNA is messenger RNA coding for specific proteins or enzymes. One of these specific proteins may be the 75,000 dalton regression associated protein (RAP), shown to be phosphorylated during dibutyryl cAMP-induced regression of DMBA-induced mammary tumor (Cho-Chung et al., 1978).

However, most of the currently available literature suggests that the action of retinoids in the inhibition of malignant cell growth in culture is mainly related to the increase in the membrane rather than nuclear protein kinase activity (Plet et al., 1982). Whether or not a similar mechanism is involved in the inhibition of mammary cancer cell growth by retinoids is still unknown.

A. **Working Hypothesis:** The working hypothesis will investigate the mode of action of retinoids in the inhibition of mammary cancer. The following studies will test the hypothesis that retinoids inhibit mammary cancer by increasing the activity and redistribution of cAMP-dependent protein kinase, causing the phosphorylation of some specific cellular proteins, that may regulate the expression of the genes involved with cellular differentiation, cellular proliferation and mammary tumor development.
A. Experimental Design:

1. Chemoprevention of mammary cancer: Female Sprague-Dawley rats were treated with 7,12-dimethylbenz(a)anthracene (DMBA) and fed retinoid supplemented diets beginning one day after DMBA intubation, to study the chemopreventive effect of retinoids. The rats were checked for mammary tumors for 150 days. Tumor sizes were measured and the number of tumors counted.

2. Chemotherapy of established mammary cancer: Rats treated with DMBA were allowed to develop mammary tumors before starting retinoid treatment, to investigate the effect of retinoids on the established mammary tumors. Tumor sizes were determined periodically, for ten days before retinoid treatment, 13 days during retinoid treatment and some tumors for ten days after stopping retinoid treatment.

3. Changes in the cAMP system in vivo: The tumors from each of the above studies were harvested and the effect of retinoid treatment on the tumor cyclic AMP system determined.
4. In vitro effects on mammary cancer cells: Human mammary cancer cells, line MCF-7 were treated with retinoids to determine the early effect of retinoids on mammary cancer cells in vitro.

5. Changes in the cAMP system in cells: The activity and distribution of cAMP dependent protein kinase of MCF-7 cells, after treatment with retinoids, was determined at various times before growth inhibition was apparent.

6. Macromolecular synthesis in cells: The effects of retinoids on DNA, RNA, and protein synthesis were investigated in the MCF-7 cells.

7. Endogenous protein phosphorylation: Endogenous phosphorylation was determined in the subcellar fractions and the phosphorylated protein bands determined by sodium dodecyl sulfate - polyacrylamide gel electrophoresis and autoradiography. The extent of phosphorylation was determined by densitometric scanning of the autoradiographs.

B. In vivo studies

1. Animals: Virgin, female Sprague-Dawley rats at 50-52 days of age were purchased from Harlan Industries, Indianapolis, Indiana.
2. **Retinoids:** 13-Cis-Retinoic Acid (RA) and N-(4-Hydroxyphenyl) Retinamide (4-HPR; HPR) were generously provided by Roche Pharmaceutical Co., Rahaway, New Jersey and the National Cancer Institute, Bethesda, MD. respectively. All-trans- Retinyl acetate (RAc) and Retinyl palmitate (RP) were purchased from Sigma Chemical Co., St. Louis, MO.

3. **Diet Preparation:** Each retinoid (1.5 or 2 mmol / kg diet) was first dissolved in 15ml of a vehicle consisting of ethanol and tricaprylin (1:4), containing 6% alpha-tocopherol as an antioxidant (Moon et al. 1979). It was necessary to warm this vehicle in order to completely dissolve the retinoids. The retinoid solution was thoroughly mixed with ground chow. The mixed diet was stored in plastic bags, protected from light with aluminum foil and stored at -20°C.

4. **Retinoid toxicity check:** The animals were observed daily and weighed weekly to ascertain the absence of retinoid toxicity. Rats found to be moribund were sacrificed in a carbon dioxide chamber. Infected rats were quarantined in a separate cage. Dead animals were incinerated.
5. **Tumors**

   a. **Production:** Rat mammary tumors were induced with 7,12-Dimethylbenz(a)anthracene (DMBA) (Sigma Chemical Co.). Fresh DMBA was dissolved in sesame oil at a concentration of 15mg DMBA/ml sesame oil (Moon et al. 1976). The solution was heated and stirred in a ventilated hood to completely dissolve the DMBA.

   The 50-52 days old rats were fasted for four hours to empty their stomachs before DMBA administration. Each rat was mildly anesthetized with an inhalation anesthetic, to relax the esophagus and was intubated once with 1ml of the DMBA solution (15mg DMBA/ml sesame oil/rat). After DMBA administration, the rats were given regular unground chow ad libitum, until they were started on the experimental diets. The initiation of carcinogenic response to DMBA is complete within 24 hours of the i.g. administration (Moon et al. 1976).

   b. **Detection and Measurement:** New tumor production was determined by palpating the rats along the mammary gland areas, at least twice a week. The date of appearance of each new tumor nodule was recorded and the nodule checked each time until it is of measurable size.

   Tumor size was determined by measuring two perpendicular diameters of the tumor, using a pair of
venier calipers. Tumor volume was calculated using the mean
diameter as follows:

\[
\text{Tumor volume} = \frac{4}{3} \pi R^3 
\]

where \( R \) is one half of the mean diameter of the tumor
(Steel 1977).

Relative tumor volume was computed as the ratio
between the tumor volume on any particular day and the
volume just before the animals were started on the
experimental diet. This parameter \((\text{volume} / \text{volume day 0})\)
was used to determine the growth rate of the established
tumors.

c. **Harvesting**: Rats were sacrificed by decapitation,
using a guillotine and the tumors quickly excised and
frozen in liquid nitrogen-cooled plastic vials, until they
were processed for biochemical analysis.

A fresh piece of some of the tumors was taken prior to
freezing, for tissue staining. The fresh piece was immersed
in phosphate buffered formalin (10%) and later cut for
staining.

d. **Processing**: Each of the frozen tumors was
pulverized at liquid nitrogen temperature.
A fraction of the pulverized tumor was weighed and processed variously as described in Methods section; Biochemical analysis (D;1a, 2a, and 3a).

C. In vitro studies

1. Human Breast Cancer MCF-7 Cells: The human breast cancer cell, MCF-7, was supplied by Dr. Ralph Stevens, Department of Pathology, The Ohio State University, Columbus, Ohio. The cells were retrieved from liquid nitrogen containers, where they were stored in vials in minimum essential medium (MEM) containing 0.1% DMSO.

2. Media: Medium B-10, IX consisted of Minimum essential medium (MEM; Earle's base), supplemented with MEM nonessential amino acids (1 mM), L-glutamine (2 mM), 100 units each of penicillin, streptomycin and fungizone, and 10% fetal calf serum (Ueda et al. 1980).

3. Cell culture: MCF-7 cells were routinely grown as monolayers in T-25, T-75 or T-150 plastic flasks (Falcon, Oxnard, CA). The B-10, IX medium was maintained at approximately 5 ml, 10 ml or 20 ml for the T-25, T-75, and T-150 flasks, respectively. Half of the medium was replenished every 4 days.
4. **Retinoid treatment:** The retinoid (RA or 4-HPR) was dissolved in ethanol so as to obtain a final concentration of 0.1% ethanol in the culture medium. Retinoid solution was added to culture medium one day after cell plating, while the control cultures were treated with 0.1% ethanol. The cells were fed every four days by adding one half volume of fresh medium containing the same amount of retinoid and 0.1% ethanol.

5. **Cell harvesting:** The cells were thoroughly washed with phosphate buffered saline (PBS) and then detached from the flasks with 0.5% trypsin containing 0.1% EDTA. The detached cells were centrifuged at 800 x g for 10 min, resuspended in PBS and recentrifuged at 800 x g for 10 min.

For cell counting, the cells resuspended in PBS were counted with a Coulter counter and/or a hemocytometer. Viability was determined by means of a dye exclusion test using trypan blue. Cells for other analysis were resuspended in the homogenization buffer, described in Methods sections 1a, 2a (buffer A) and 3a (buffer B).

6. **Cell preparation:** Cells resuspended in 2.5ml of buffer A or buffer B containing 0.25M sucrose (buffer B') were homogenized at 4°C and the necessary fraction isolated for biochemical analysis as described below (figure 6).
Homogenize cells (60 million cells / 2.5ml Buffer A or B')
Centrifuge (800xg, 10min, 4°C)

Supernatant
pellet

centrifuge (18,000xg, 15min)
Supernatant
pellet (discard) 18,000xg 15min Pellet

Resuspend in 2ml buffer C
Layer on 10ml of buffer C

Pellet

Resuspend in 10ml buffer A/B'
Centrifuge (8000xg, 15min, 4°C)

pellet

Resuspend in 10ml buffer A/B'
Centrifuge (80,000xg 1hr, 4°C)

Pellet

discard

Resuspend in 10ml buffer (buffer A/B) (A-T/B'-T)
Centrifuge (18,000xg, 15min, 4°C)

Pellet
discard

Pellet
discard

RESUSPEND in Buffer A-T/B-T
over night, 4°C on ice

Centrifuge (104,000xg, 30min, 4°C)

Supernatant (membrane fraction)
pellet (discard)

Supernatant (nuclear fraction)
discard

Figure 6. Flow diagram for Isolation of subcellular fractions.
Buffers: A; 0.3M sucrose, 0.02M Tris-HCl pH 7.4, 10mM KCl, 2mM MgCl₂, 0.4mM CaCl₂.
B; 0.05M KPO₄ pH 6.5.
a. **Nuclei isolation:** Nuclei isolation was carried on by a modification of the method of Chauveau et al. (1956). Cells were homogenized with a Potter-Elvehjem homogenizer at 4°C. The homogenate was centrifuged at 800 x g for 15 min at 4°C to sediment crude nuclear fraction. The crude nuclear pellet was resuspended in 2ml of 2.2M sucrose buffer, containing 1 mM ethyleneglycoltetraacetic acid (EGTA), and 10 mM MgCl₂ (buffer C). The nuclear suspension was layered on 8ml of the above sucrose buffer and centrifuged at 80,000 x g for 1 hr at 4°C. The supernatant was carefully aspirated and the nuclear pellet resuspended in 10 ml of buffer A or B', containing 0.1% triton X-100 as a detergent (buffer A-T or B'-T). The resuspended pellet was centrifuged at 18,000 x g for 15 min, at 4°C. The supernatant was discarded and the pellet resuspended in 3ml of either buffer A-T or B'-T. The resuspended nuclear fraction was left overnight at 4°C. After solubilization, the nuclei fraction was centrifuged at 104,000 x g for 30 min at 4°C and the supernatant collected for analysis.

b. **Membrane isolation:** The 800 x g supernatant obtained from the initial cell homogenate above was centrifuged at 18,000 x g for 30 min at 4°C to sediment crude membranes. The membrane pellet was resuspended in 10ml of buffer A or B and centrifuged at 8,000xg for 15min.
The 5,000xg membrane supernatant was centrifuged again at 18,000xg for 15 min. To ensure the purity of the membrane fraction used for endogenous phosphorylation, the 8,000xg supernatant was centrifuged over 10% Ficoll (Plet et al. 1982) bed and the upper layer collected. The membrane obtained was centrifuged at 18,000xg for 15min. The resulting pellet was resuspended in buffer A and centrifuged at 18,000xg for 15min. Afterwards, the pellet was resuspended in 0.5ml of buffer A-T (or B-T) and left overnight at 4°C. The solubilized membrane fraction was centrifuged at 104,000 x g for 30 min at 4°C and the supernatant used as membrane fraction.

c. Cytosol isolation: The initial 18,000xg supernatant from the cell homogenate was recentrifuged at 104,000 x g for 30 min at 4°C. The 104,000 x g supernatant used as the cytosol fraction.

D. Biochemical Analysis

1. Cyclic AMP:
   a. Extraction: Samples for cAMP extraction were homogenized in 5% TCA. Pulverized tumors were homogenized in 10 Vol of 5% TCA, whereas the MCF-7 cells were homogenized in 2 ml of 5% TCA. All homogenization was done on ice, using a polytron at speed 7 for 30 sec.
The homogenate was centrifuged at 104,000 x g for 30 min at 4°C. The 104,000 x g pellet was resuspended in 1 ml, 1N NaOH for protein estimation. The supernatant was transferred into a 16x125 mm test tube for further processing.

TCA was removed from the supernatant by mixing it with 3 ml of water saturated ether (12% water in diethyl ether). The mixture was vortexed and allowed to settle for 2-3 min on ice and the upper ether layer aspirated and discarded into an ether waste container. This cleaning process was repeated twice and the remaining ether removed by applying vacuum to the cAMP solution. Increasing the temperature slightly also helped facilitate the evaporation of ether from the solution. The pH was adjusted to 6.5 with 0.5M sodium acetate pH 5.3 (50-100ul). The extracted cAMP was stored at -20°C and was stable for up to 4 weeks at this condition.

b. Assay: Cyclic AMP was assayed by the radioimmunoassay method of Steiner (1974). The cAMP antibody used was generously provided by Dr. Richard Fertel, Department of Pharmacology, The Ohio State University, Columbus, Ohio. The cross reactivity of this antibody was determined to be less than 0.001% for adenine and guanine nucleotides and the interference from cGMP was
0.1%. The antisera was diluted 50 times with 0.05M acetate buffer pH 6.2, containing 1% bovine serum albumin. The \( ^{125}\text{I}\)cAMP used was purchased from Amersham Nuclear, and diluted in 0.05M acetate buffer pH 6.2 containing 0.5% bovine gammaglobulin.

The cAMP standards consisted of 0.001 to 5 pmol cAMP diluted in 0.05M acetate buffer pH 6.2. The samples were diluted 10 and 50 or 100 times with 0.05M acetate buffer pH 6.2 and assays ran from each stock dilution. The acetylation reagent consisted of 5 parts triethylamine and 2 parts acetic anhydride. The acetylating reagent was added at 4°C, in a ventilated hood and the tube vortexed immediately after the addition. Acetylation was carried on at room temperature, for 10min.

After addition of all the reagents, the assay mixtures were vortexed and incubated for 18 hr at 4°C. The antibody-cAMP complex was then precipitated with 2ml of 60% ammonium sulfate solution for 20min at 4°C, followed by centrifugation at 800xg for 10min. The supernatant was discarded into a radioactive waste container and the tubes allowed to dry while inverted on a rack padded with paper towels. Bound radioactivity was determined in a Beckman model GAM 7000 gamma counter.

The amount of cAMP in the samples was interpolated from a plot of known standards versus the radioactivity
expressed as a percent of the trace as shown in figure 6. The graphical amounts were adjusted for stock dilution and the cAMP content expressed as pmoles cAMP per mg protein (from the 104,000 x g pellet digested in 1N NaOH).

2. Cyclic AMP binding:
   a. Sample preparation: Samples for cAMP binding assay were homogenized in buffer A, consisting of 0.3M sucrose, 20 mM Tris- HCl pH 7.4, 10 mM KCl, 2 mM MgCl2, 0.4 mM CaCl2, at 4°C.

   (3H)cAMP (36 Ci/mmol) was purchased from ICN Pharmaceuticals and diluted to approximately 8 nmol/ml with 1mM cAMP and distilled water.

   b. Binding assay in isolated fractions: Cyclic AMP binding was assayed by filtration technique (Gilman, 1970), using 1 umolar 3H cAMP in a final volume of 0.2 ml. The assay mixtures consisting of 0.05M potassium phosphate buffer pH 6.5, 10mM theophylline, 1uM 3H-cAMP ± 1 mM cAMP were incubated at 37°C for 2 hr. The reaction was stopped by dilution with 1 ml of cold 0.05M KPO4 pH 6.5. This was followed by filtration through a 0.45um cellulose filter type H, placed on a Millipore filtration manifold (Millipore Corp., Bedford, MA). Each filter was rinsed twice by passing 2 ml of 0.05M KPO4 buffer through it.
The filters containing the retained \(^{3}H\)cAMP-protein complex were then dissolved overnight in 1 ml of ethyleneglycol monomethyl ether (2-methoxyethanol) (Fisher Scientific co.), in 8ml plastic scintillation vials and reconstituted to 6ml with liquid scintillation cocktail (Dupont, New England Nuclei).

The radioactivity retained on the filters was counted in a Beckman LS6800 liquid scintillation counter (Beckman Instruments Inc., Fullerton CA.). The nonspecific activity was subtracted from the total activity to obtain the activity due to specific binding of \(^{3}H\)cAMP to the regulatory subunits of cAMP-dependent protein kinase. Cyclic AMP binding was expressed as pmoles of \(^{3}H\)cAMP bound per mg protein.

c. Binding in intact cells: Equal amount of \(^{3}H\)cAMP ± cAMP (2mM) was added to the culture medium of MCF-7 cells and the cells incubated at 37°C in 5% CO\(_2\) for 20 hours. The cells were then harvested as previously described and washed twice with PBS (2ml). The cells were resuspended in 1ml PBS and sonicated for 30 min at 37°C. Nuclear and cytoplasmic fractions were separated by centrifuging at 800xg for 10min. The nuclear pellet was resuspended in 2ml of cold 0.05M phosphate buffer pH 7.0. Both fractions were passed through millipore filter papers as previously described.
The filters were transferred into scintillation vials and dissolved in 5ml of aquasol scintillation cocktail. The radioactivity associated with each fraction was determined in a liquid scintillation counter. The same amount of \((^{3}\text{H})\text{cAMP}\) used in the assay was counted to determine the total count and was used to calculate the amount of \((^{3}\text{H})\text{cAMP}\) bound. Specific binding was determined by subtracting the radioactivity in the presence of 2uM cAMP from the radioactivity in the absence of exogenous cAMP.

3. Protein Kinase Activity

a. Sample preparation: Samples for protein kinase assay were homogenized in 0.25M sucrose containing 0.05M potassium phosphate buffer pH 6.5 (buffer B). Cell fractions were isolated as previously described.

b. Enzyme assay: Protein kinase activity was determined by measuring the incorporation of \(^{32}\text{P}\) from ATP(\(Y^{32}\text{P}\)) into histone protein, using filter separation technique (Corbin \textit{et al}. 1975). The incubation mixture contained 0.05M potassium phosphate buffer pH 7.5, 10mM \(\text{MgCl}_2\), 1mM theophylline, 0.6mg histone type II (Calf thymus; Sigma Chemical Co.), 1mM ATP together with ATP(\(Y^{32}\text{P}\)) (20Ci/mmole; ICN pharmaceuticals, Irvin CA.), and 0.05 or 0.1ml of cellular fraction (enzyme source), in a final
volume of 0.2ml (see table 1). All assays were performed in the presence and absence of 1mM cAMP.

All incubations were carried on at 30°C for 5-10 min, in a shaking water bath. The reaction was stopped by precipitating the proteins with 1ml of cold 20% TCA for 10min. The mixtures were then vortexed gently and passed through 0.45um membrane filters in a Millipore filtration manifold. The filters were washed twice by passing 2ml of 5% TCA through it. Each washed filter was treated as described above for cAMP binding assay and the retained radioactivity counted at $^{32}$P window (LL=400; UL=1000), in the Beckman LS6800 liquid scintillation counter.

Three filters, each containing the same amount of ATP(Y$^{32}$P) used for the assay were dissolved similarly and counted to determine the total radioactivity in the assay mixture. This was used to calculate the amount of ATP consumed in the process of protein phosphorylation.

Endogenous phosphorylation was adjusted for by running parallel assays in the absence of exogenous histone. Protein kinase activity was expressed as the amount of ATP consumed per min per mg protein. Cyclic AMP dependent activity was determined by subtracting the enzyme activity in the absence of exogenous cAMP from the activity in the presence of 1mM cAMP.
Table 1

Protein kinase activity assay mixtures

<table>
<thead>
<tr>
<th>Reagent</th>
<th>-Histone</th>
<th>-cAMP</th>
<th>+cAMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5M KPO₄</td>
<td>0.02</td>
<td>0.02</td>
<td>0.02</td>
</tr>
<tr>
<td>0.02M Theophylline</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>0.2M MgCl₂</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>30mg/ml Histone</td>
<td>----</td>
<td>0.02</td>
<td>0.02</td>
</tr>
<tr>
<td>20uM cAMP</td>
<td>----</td>
<td>----</td>
<td>0.01</td>
</tr>
<tr>
<td>0.02M ATP</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>5uCi/ml ATP(Y-³²P)</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>DD. H₂O</td>
<td>0.09</td>
<td>0.07</td>
<td>0.06</td>
</tr>
<tr>
<td>Enzyme source</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
</tr>
</tbody>
</table>
c. Subtypes (I & II) assay: Cytosols were prepared from a pool of tumor samples. Aliquots of 10mg of cytosol protein were chromatographed on identical 1cm x 10cm DEAE-cellulose columns as previously described (Hoffman et al. 1975). The elution was carried on with a linear KCl gradient (0 - 0.4M) in 0.01M Tris-HCl, pH 7.4. Fractions of 2ml were collected and aliquots of each fraction assayed for protein kinase activity and cAMP-Binding.

E. Determination of the effects of retinoids on Macromolecular synthesis:

1. DNA synthesis: Effect on DNA synthesis was determined by the incorporation of $^3$H-Thymidine into TCA and ethanol insoluble materials (Ueda et al 1980). MCF-7 cells were plated in 35cm$^2$ wells and treated after one day with retinoids. $^3$H-Thymidine was added either together with the retinoid or at the end of retinoid treatment. Following the incorporation period, the medium was discarded and the cells washed twice with cold PBS. Cells were then harvested as previously described and resuspended in 10% TCA / 70% ethanol. The resuspended cells were sonicated at 4°C for 30 min to ensure lysis. Following cytolysis, the suspension was allowed to sediment for 10 min, and then centrifuged at 800xg for 10 min. The supernatant was aspirated and discarded and the pellet washed twice with 10ml of 10% TCA
/ 70% ethanol. The insoluble pellet was digested with 0.5ml 1N NaOH and mixed with 5ml Aquasol and counted in a Beckman liquid scintillation counter.

2. **RNA synthesis:** Effect on RNA synthesis was determined by the incorporation of $^{14}$C-Uridine into TCA and ethanol insoluble materials (Ueda et al. 1980). MCF-7 cells were plated in 35cm$^2$ wells and treated after one day with retinoids. $^{14}$C-Uridine was added either together with the retinoid or at the end of retinoid treatment. The remaining procedure was exactly as described above for the assay of DNA synthesis. In some experiments double label incorporation of $^{14}$C-uridine and $^3$H-thymidine were studied.

3. **Protein Synthesis:** Protein synthesis was determined by measuring the amount of $^3$H-Leucine incorporated into newly synthesized proteins in MCF-7 cells in culture, using a modification of previously described methods (Ueda et al. 1980). Cells were plated in 35cm$^2$ wells and treated after one day with control medium or media containing cycloheximide (CX), HPR or CX+HPR (CX was added 30 min before the addition of HPR). The $^3$H-leucine was then added to the culture media and the cells incubated at 37°C in 5% CO$_2$ atmosphere.
At the end of the incubation period, the medium was discarded and cells rinsed twice with cold PBS. The cells were harvested as previously described and washed again with PBS. After centrifuging at 800xg for 10min, the PBS was discarded and the sedimented cells resuspended in 1ml of 10% TCA. The TCA suspension was sonicated for 30min, vortexed, allowed to sit for 10min and centrifuged at 800xg for 10min. TCA was aspirated, leaving behind the TCA-insoluble pellet. The pellet was washed twice with 5ml of 10% TCA, then resuspended in 0.5ml of 1N NaOH and hydrolyzed by heating in a hot water bath.

The radioactivity associated with the TCA-insoluble protein was determined in a liquid scintillation counter and taken as a measure of the amount of leucine incorporated during denovo protein synthesis.

F. **Effect of protein synthesis inhibition with Cycloheximide on the induction of Protein Kinase activity by retinoids:** MCF-7 cells were plated for one day and then treated with CX (10^{-5}M), HPR (10^{-7}M) or CX (10^{-5}M) + HPR (10^{-7}M) in the culture medium containing 0.1% ethanol. The control medium contained 0.1% ethanol only. Cells were cultured in CX containing medium for 30 min, before treatment with CX + HPR medium. The cells were then cultured for 40 hours and harvested as previously described.
The cells were homogenized in 5ml of 0.02M KPO$_4$ buffer pH 6.5 using Potter-Elvehjem homogenizer (5 strokes) on ice. Cellular fractions were isolated as previously described.

G. **Protein Estimation:** Protein assay was performed by the phenol reagent method, using bovine serum albumin (BSA) as a standard (Lowery et al. 1951). The amount of protein in the samples were interpolated from a graph of the known BSA standards (20-160ug) versus the absorbance readings.

H. **SDS-Polyacrylamide gel electrophoresis:**

All chemicals used for gel electrophoresis were purchased from Sigma Chemical company, St Louis, Mo. The electrophoresis was done in a Pharmacia electrophoresis unit with a constant current of 80mA per 3mm slab gel for 6 hours.

1. **Gel buffer:** The gel buffer consisted of 0.2M sodium phosphate pH 7.0, containing 0.2% sodium dodecyl sulfate (SDS).

2. **Acrylamide solution:** Stock solution of acrylamide / bisacrylamide (37:1) was prepared by dissolving 37g acrylamide and 1g methylene bisacrylamide in 200ml of
double distilled water. The solution was filtered through a Whatman no. 1 filter and stored at 4°C in a dark bottle.

3. **Running Gel:** Running 10% gel was prepared by mixing 20 parts of deaerated gel buffer with 18 parts of acrylamide solution, 2 parts of freshly prepared ammonium persulfate (15mg / ml), and 0.025 part of TEMED. The gel polymerized within 30 to 40 min.

4. **Running buffer:** The gel buffer was diluted with an equal volume of distilled water and used as the running buffer.

I. **Endogenous Protein Phosphorylation:**

1. **Sample Preparation:** Samples used for electrophoresis were prepared as previously described under cell preparation (p. 42). The protein concentration of the samples were adjusted to 1 mg / ml using 0.05M potassium phosphate buffer pH 6.5.

2. **Phosphorylation Assay:** Endogenous protein phosphorylation was determined by assaying protein kinase activity in the absence of exogenous protein. The reaction mixture contained 50mM potassium phosphate pH 6.5, 10mM MgCl₂, 1mM theophylline, 2uM (γ-^{32}P)ATP (12Ci/mmol) (Liu and Greengard
1976), ± 1μM cAMP, in a final volume of 0.1ml. The reaction was started by adding 60μg of sample protein (1mg/ml) and incubating at 30°C for 5min.

The reaction was stopped by adding 100 μl of electrophoresis sample-buffer, containing 2% sodium dodecyl sulfate (S.D.S), 50mM sodium phosphate pH 7.4, 20% glycerol, and 2% 2-mercaptoethanol (Fontana et al. 1986). The mixture was heated at 37°C for two hours, and subjected to S.D.S-polyacrylamide gel electrophoresis on 10% gel, by the method of Weber and Osborn (1969).

3. Gel Drying: Gels were soaked in two changes of 40% methanol / 7% acetic acid solution for 8 hours to remove excess ³²P from the gel. They were then soaked in 2% glycerol overnight, to prevent it from cracking during drying. The gels were washed twice with distilled dimineralized water, and left in 1% glycerol solution for 1 hour. The soaked gels were then transferred unto a filter paper and dried under vacuum in a gel dryer for 4 hours.

4. Autoradiography: Autoradiography was carried on by exposing an X-ray film (Kodak XR-5; Sigma Chemical co. St. Louis, Mo) on the dried gel for 48-72 hours in the dark. The X-ray film was then developed either manually or in an automatic film processor.
5. **Densitometry:** Each lane on the autoradiographs was scanned with a densitometer and the areas under the peaks at corresponding positions compared for the various durations of retinoid treatment.

### J. Molecular weight determination:

1. **Protein visualization:** A duplicate gel containing a lane of molecular weight markers and lanes for the sample proteins was run parallel to the gels used for autoradiography. The gel was fixed in 40% methanol / 7% acetic acid overnight and stained with 0.2% coomassie brilliant blue dye for at least 2 hours. Following destaining of the gel, the molecular weights of the marker proteins were plotted against the log of their relative mobilities. The molecular weights of the unknown proteins extrapolated from this curve, based on their relative mobilities.

2. **Marker proteins:**

   a. **Prestained Marker Proteins:** The prestained marker protein used include: a$_2$-macroglobulin (180,000d), B-galactosidase (116,000d), fructose-6-phosphate kinase (84,000d), pyruvate kinase (58,000d), fumarase (48,000d), lactate dehydrogenase (36,000d), and triose phosphate isomerase (26,600d) (Sigma Chemical Co. St. Louis, MO).
K. **Data analysis:** The significance of the observed changes were determined by the Student's T-test.
CHAPTER IX.

RESULTS

A. Chemopreventive effect of Retinoids on DMBA-induced Mammary Tumor in Rats

Intragastric administration of a single dose of DMBA (20mg/ml sesame oil/rat) to 50-52 days old virgin female Sprague-Dawley rats resulted in mammary tumor production in all the treated rats within 150 days. Similar administration of 15mg DMBA per rat resulted in mammary tumor production in approximately 80% of the treated rats within 150 days. The latter rate of tumor production was considered to be comparable to the rate of mammary cancer incidence in women (Grubbs et al., 1977).

1. Tumor induction: As shown in figure 7, administration of 13-cis-retinoic acid (RA), N-(4-Hydroxyphenyl)-retinamide (HPR), retinyl palmitate (RP) or retinyl acetate (RAC) in the diet (1.5mmol/Kg diet) starting a day post DMBA, resulted in the inhibition of tumor production by 40-60% within 150 days as compared to the controls. At 150 days, the mammary tumor production in the control group (15mg DMBA + vehicle) was 80%, whereas the HPR, RAC, RA and
RP fed groups had only 38%, 41%, 48% and 60% tumor production respectively, within the same period.

2. Latency period to tumor development: Most of these retinoids also prolonged the latency period to tumor development. The control rats developed first palpable tumor within 45-55 days post DMBA, whereas the first palpable tumors were observed after 60 days for RA- or RAc- and after 90 days for HPR-fed rats (figure 7). This delay was not observed with RP which was also least effective in preventing tumor production.

3. Tumor frequency:

Another important effect of retinoids was the inhibition of multiple tumor production on the individual rats (figure 8). The control rats developed an average of 1.1 tumors per rat, while the retinoid groups developed 0.3 - 0.6 tumors per rat at 150 days post DMBA.

4. Tumor Growth: All the retinoids used in this study inhibited the growth of the DMBA-induced mammary tumors. As shown in figure 9, within 4 weeks after their production, the control tumors increased in volume by almost 70-fold, whereas the retinoid-treated rat tumor volumes increased by less than 10-fold. RAc was the most effective in the inhibition of tumor growth, with the tumor volumes remaining at no more than 3-fold of the original volume.
FIGURE 7

Retinoid-induced inhibition of mammary tumor production in rats treated with DMBA. Each group consisted of 12 rats. DMBA intubation and retinoid preparation were as described in Materials and methods. Retinoids were administered in the diet (1.5mmol / kg diet), beginning a day after intubation with DMBA. HPR; N-(4-Hydroxyphenyl)retinamide, RA; 13-cis-Retinoic acid, RAc; Retinyl acetate, RP; Retinyl palmitate.

*; p < 0.05 as compared to the retinoid-treated groups.
Figure 7: Graph showing the number of rats with tumors (percent of rats in group) over weeks after DMBA intubation. The x-axis represents weeks after DMBA intubation, ranging from 0 to 24. The y-axis represents the number of rats with tumors, ranging from 0 to 80. The graph includes lines for different groups: Control, RP, RA, and RAc, with asterisks indicating significant differences.
Inhibition of mammary tumor frequency in rats treated with DMBA by retinoids. Rats treated as described in the legend to figure 7, were checked for tumors at least twice a week, by palpating along the mammary gland areas. The total number of tumors per group was divided by the number of rats in the group, as a measure of tumor frequency.
The effects of RA and HPR on tumor growth were almost the same, with the tumor volumes increased by 9-fold within 4 weeks. RP group with a 10-fold increase in tumor volume was again the least effective of the four retinoids.

The above inhibition in tumor growth may reflect an increase in the doubling time and/or induction of terminal differentiation of the mammary tumor cells by retinoids.

B. Effect of retinoids on the weight of DMBA treated rats:

In order to ascertain that the anticarcinogenic effects of retinoids was not due to retinoid toxicity, the control and retinoid treated rats were weighed at least once a week.

As shown in table 2, there was no significant difference in the weight gains of control and retinoid-treated rats after 12 weeks of retinoid consumption. Thus the observed effects of retinoids on the DMBA-induced mammary tumors were not due to retinoid toxicity.

However, the weight gains of rats fed RP or RAc containing diets lagged by 10% and 24% of the control weights after 24 weeks (data not shown), during which the control rat tumors had also grown significantly, and may have contributed to the total change in weights of the control rats.
FIGURE 9

Retinoid-induced inhibition of growth of DMBA-induced mammary tumors in the rat. Tumor sizes were measured with a pair of calipers as described under methods. The week of first observation of each tumor was noted and used as week one for that tumor. The mean volumes of tumors at the same age for each group were calculated. The values indicated are the means ± S.E.M. for at least five tumors.

*; p < 0.05 as compared with the retinoid-treated tumors.
FIGURE 9

Tumor volume (cm$^3$) vs. Tumor age (Weeks)

- Control
- RP
- HPR/RA
- RAc
### TABLE 2

Effect of retinoids on the weight gain of rats treated with DMBA.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Weight</th>
<th>Percent of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>240 ± 18</td>
<td>100</td>
</tr>
<tr>
<td>HPR</td>
<td>229 ± 13</td>
<td>95</td>
</tr>
<tr>
<td>RA</td>
<td>234 ± 10</td>
<td>97.5</td>
</tr>
<tr>
<td>RAC</td>
<td>220 ± 9</td>
<td>91.7</td>
</tr>
<tr>
<td>RP</td>
<td>231 ± 11</td>
<td>96.2</td>
</tr>
</tbody>
</table>

Rats were fed either the control diet or a diet containing a retinoid (1.5mmol / kg diet) and weighed at least once a week. The values indicate the mean ± S.D. for at least ten rats, weighed at 12 weeks of retinoid treatment.
C. **Changes in the cAMP system of DMBA-induced mammary tumors produced during retinoid treatment:**

1. **Cyclic AMP content:** Since tumor and cell growth rates are known to be inversely related to their cAMP contents (Cho-Chung, 1982), the above DMBA-induced tumors were analyzed to determine the effect of retinoids on their cAMP content.

   The control tumor cAMP content was approximately 6 pmoles/mg protein whereas the cAMP content of tumors from retinoid-treated rats were 10-18 pmoles/mg protein (figure 10). Thus, retinoids increased the cAMP content of DMBA-induced mammary tumors by 2 to 3-fold. Although there was no statistically significant difference in the cAMP content of tumors from rats treated with the various retinoids, there was a trend towards increase in the tumor cAMP content with increase in the anticarcinogenic potency of these retinoids. Thus RP with the least anticarcinogenic potency also caused the lowest increase in tumor cAMP content. On the other hand, RAc which was most effective caused the greatest increase in tumor cAMP content. Next to RAc was HPR, followed by RA in both antitumor effectiveness and increase in tumor cAMP content.
FIGURE 10

Effect of retinoids on the cyclic AMP content of DMBA-induced mammary tumors in rats. Tumor preparation and cAMP determination were as described under materials and methods. The values indicated are the means ± S.E.M. for at least five tumors assayed in quadruplicates.

* $p < 0.05$, ** $p < 0.01$ as compared to the control.
FIGURE 10

The graph illustrates the levels of cAMP (pmoles/mg protein) for different treatments: C, RP, RA, HPR, and RAc. The treatments show varying levels of cAMP, with HPR and RAc having significantly higher values compared to the control (C) and other treatments. The asterisks (*) indicate significant differences compared to the control, while the double asterisks (**) indicate even more significant differences.
2. **Cyclic AMP Binding and Protein kinase activity:**

Treatment with RA or HPR resulted in a marked increase in cAMP binding (2 to 3-fold) in the cytosol of DMBA-induced mammary tumors in rats (table 3). The increase in cAMP binding due to HPR (3-fold) was more than that due to RA (2-fold).

As also shown in table 3, tumors from RA or HPR treated rats showed 2 to 3-fold increase in histone phosphorylating activity as compared to the control tumors. This increase in protein kinase activity was mainly due to increase in the cAMP-dependent component of the enzyme. The increased cAMP activated component in the presence of already increased (2 to 3-fold) intracellular cAMP, suggests an increase in the total amount of cAMP-dependent protein kinase in the retinoid-treated tumors.

3. **Binding Properties of cAMP dependent protein kinase:** The concentration-dependent binding of \(^{3}H\)cAMP into cytosol proteins of control and retinoid-treated rat tumors was studied to determine if the increase in cAMP binding was due to an increase in the amount of the binding protein, or due to an increase in the affinity of the protein for cAMP. As shown in the Scatchard plots (figure 11), the apparent dissociation constants (Kd) for
TABLE 3

Increases in Cytosolic cAMP Binding and Protein kinase activities in DMBA-induced mammary tumors of retinoid-treated rats

<table>
<thead>
<tr>
<th>TREATMENT</th>
<th>$^{3}$HcAMP BOUND (pmoles / mg protein)</th>
<th>PROTEIN KINASE ACTIVITY (pmoles /min /mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-cAMP</td>
<td>+cAMP</td>
</tr>
<tr>
<td>DMBA only</td>
<td>3.3 ± 0.7</td>
<td>24.5 ± 2.6</td>
</tr>
<tr>
<td>DMBA + RA</td>
<td>7.1 ± 0.7*</td>
<td>28.2 ± 6.4</td>
</tr>
<tr>
<td>DMBA + 4-HPR</td>
<td>10.8 ± 1.7*</td>
<td>33.7 ± 5.5</td>
</tr>
</tbody>
</table>

Rats were given retinoids daily in the diet (1.5mmol/kg diet) beginning a day after DMBA intubation.

The results shown represent the mean ± S.E.M. for five tumors, each assayed in duplicate.

*; p < 0.05, **; p < 0.01 as compared to the control (DMBA only).
the high and low affinity binding of \(^3\text{H}\)cAMP to cytosol proteins of tumors from the RA-treated \((1.4 \times 10^{-8} \text{ and } 1.1 \times 10^{-7}\text{M})\) and the control \((1.2 \times 10^{-8} \text{ and } 1.0 \times 10^{-7}\text{M})\) tumors were not significantly different. However, at saturating concentrations of \(^3\text{H}\)cAMP, the retinoid-treated tumors \(^3\text{H}\)cAMP binding \((15.4 \text{ pmoles/mg protein})\) was 2.5-fold greater than that of the control tumors \((6.2 \text{ pmol/mg protein})\). These results indicate that the retinoid-induced increase in cAMP binding was due to increase in the amount of the binding protein rather than due to alteration in the binding properties of the protein. It has been shown that most of the cAMP binding is on the regulatory subunit of the holoenzyme (Taylor et al. 1981).

4. Activity of cAMP-dependent protein kinase Subtypes (I & II): To determine if there was a selective increase in one of the cAMP dependent protein kinase subtypes (type I and type II) cytosol preparations were chromatographed on DEAE-cellulose columns. The elution profiles of protein kinase activity and \(^3\text{H}\)cAMP-binding of control and retinoid-treated tumors, from this study is shown in figure 12. The two peaks of cAMP-dependent protein kinase, referred to as type I and type II based on their order of elution from the DEAE-cellulose column with increasing salt concentration (Hoffman et al., 1975), were observed.
Retinoid treatment preferentially increased (3-fold) the type II cAMP-dependent protein kinase (peak II). A minor increase (20%) in type I cAMP dependent protein kinase was also observed. These increases were paralleled by similar increases in the cAMP binding activities of the fractions. Thus the cAMP binding increased by approximately 3-fold in the fractions corresponding to the type II isozyme (peak II) of cAMP-dependent protein kinase.

These findings support the involvement of the cAMP system in the chemoprevention of mammary tumors by retinoids. They further suggest that the type II cAMP-dependent protein kinase may play a pivotal role in the anticarcinogenic effect of retinoids on the DMBA-induced mammary tumors.
FIGURE 11
Scatchard plots of cAMP binding in control (o) and 13-cis-retinoic acid-treated (●) rat DMBA-induced mammary tumors. Each point represents the mean of duplicate assays using cytosols from pools of at least five tumors.
FIGURE 12

Increase in cAMP-dependent protein kinase subtypes in DMBA-induced mammary tumors of rats treated with 13-cis-retinoic acid (1.5 mmol/kg diet). Cytosols were prepared from pools of three tumors and aliquots of the cytosols chromatographed on identical DEAE-cellulose columns as described in Materials and Methods. Protein kinase activity and cAMP binding were assayed as previously described in Material and Methods.
FIGURE 12
D. Chemotherapeutic effects of retinoids on already established DMBA-induced mammary tumors:

1. Effect on tumor growth:

   a. Growth before treatment: As shown in figure 13a, all the tumors applied in this study were actively growing prior to retinoid treatment.

   b. Growth during treatment: The administration of RA or HPR at 1.5 mmol/kg diet to Sprague-Dawley rats with established, actively growing DMBA-induced mammary tumors, resulted in the arrest of growth of these tumors. The control tumors continued to grow and increased in volume by approximately 2-fold within 5 days and by 3.5-fold within 13 days, while the retinoid-treated tumors did not significantly change in volume within the same period, as compared with their initial volume on day 0.

   When the rats bearing DMBA-induced mammary tumors were fed a diet containing 2 mmole RA or HPR/kg, approximately 60% of the tumors (table 4) showed 20-30% decrease in tumor volume (figure 13b).
FIGURE 13

Effect of retinoids on the growth of established DMBA-induced mammary tumors in rats. Tumors were induced as described in methods. The tumor bearing rats were distributed into a control group, RA-fed group or HPR-fed group.

(A) All the rats were fed regular chow and tumors measured for 10 days before starting the rats on experimental diets.

(B) Rats were fed control (o), RA (▲) or HPR (<>), at 1.5mmol (open) or 2 mmol (closed) per kg diet. Each group contained at least 15 rats.

(C) Rats were fed control diet.

* p < 0.05 as compared to the control.
FIGURE 13
As shown in Table 4, only 6 of 23 tumors in the control group stopped growing and these included 2 spontaneous regressions.

However, in the groups treated with RA (24 tumors) or HPR (22 tumors), 21 and 18 tumors respectively, stopped growing, with 15 and 11 of these tumors regressed by approximately 20-30% as compared to their initial volumes (on Day 0).

c. Growth after withdrawal of retinoids: Some of the rats were withdrawn from retinoid diet to ascertain that the growth inhibition was due to retinoid treatment.

As also shown in Figure 13, retinoid withdrawal resulted in the resumption of growth of the previously regressing tumors. The tumors withdrawn from retinoid treatment grew at the same rate (17% per day) as the rate observed for the control tumors during the first 7 days of treatment (19% per day). However, a few of the tumors which completely regressed as a result of retinoid treatment did not regrow following discontinuation of retinoid treatment for 7 days.

Thus, the above retinoids had some antitumor effects besides their well established anticarcinogenic effects on the DMBA-induced mammary tumors.
TABLE 4

EFFECT OF RETINOID TREATMENT ON ESTABLISHED DMBA-INDUCED MAMMARY TUMORS IN RATS

<table>
<thead>
<tr>
<th>GROUP</th>
<th>TOTAL GROWING**</th>
<th>STATIC REGRESSING</th>
<th>RESPONDING</th>
<th>NET**+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>23</td>
<td>17</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>RA</td>
<td>24</td>
<td>3</td>
<td>6</td>
<td>15</td>
</tr>
<tr>
<td>HPR</td>
<td>22</td>
<td>4</td>
<td>7</td>
<td>11</td>
</tr>
</tbody>
</table>

+ Each group consisted of 15 rats with DMBA-induced mammary tumors. Retinoids were given at the dose of 2 mmol/kg diet for 13 days.

** Growing (>20% increase); static (<20% change), regressing (>20% decrease) in tumor volume within 10 to 13 days.

++ Net; total responding minus spontaneous inhibition and regression as determined in the control group. RA; 13-cis-retinoic acid. HPR; N-(4-hydroxyphenyl) retinamide.
E. **Stimulation of the cAMP System of Established mammary tumors by retinoids:**

1. **Cyclic AMP content:** Tumor cAMP measured after 10 days of the treatment, revealed only a slight increase in the tumor cAMP content of retinoid-treated rats as compared to the control tumor cAMP level (Table 5). This slightly elevated cAMP level in the retinoid-treated tumors dropped to the control level in the tumors of rats withdrawn from retinoids for 7 days (Table 5).

2. **Cyclic AMP Binding and Protein kinase Activity:** The activity of cAMP-dependent protein kinase was determined to investigate the involvement of the cAMP system in the antitumor effects of these retinoids.

As shown in table 6, the cAMP-binding and cAMP-dependent protein kinase activity were increased by approximately 2-fold, only in the tumors that were growth arrested or regressed by retinoids, as compared to the control or retinoid-treated growing tumors. There were also no significant increases in the cAMP-binding and cAMP-dependent protein kinase activity in the control tumors which were spontaneous growth inhibition (data not shown).

These results suggest that the antitumor effects of retinoids, like the anticarcinogenic effects may be mediated through the cAMP system.
TABLE 5

Effect of Retinoids on the Cyclic AMP Content of Established DMBA-Induced Mammary Tumor in the Rat.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day 10# Cyclic AMP (pmoles/mg protein)</th>
<th>Withdrawal## Cyclic AMP (pmoles/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (vehicle)</td>
<td>8.94 ± 1.35</td>
<td>8.94 ± 1.35</td>
</tr>
<tr>
<td>RA</td>
<td>11.17 ± 2.16*</td>
<td>8.17 ± 3.0</td>
</tr>
<tr>
<td>HPR</td>
<td>12.82 ± 1.74*</td>
<td>6.47 ± 1.7*</td>
</tr>
</tbody>
</table>

Retinoids were given in the diet (2 mmol/kg diet).

# Tumors were obtained from rats fed retinoid-supplemented or control diet for 10 days.

## Tumors obtained from rats after 7 days of withdrawal from retinoid-supplemented diet.

The values shown are the means ± S.E.M. for at least five tumors.

* 0.5 <p <0.1 compared to control.
TABLE 6

Increases in cAMP-Binding and cAMP-Dependent Protein Kinase Activity in Established DMBA-Induced Mammary Tumors Responding to Retinoid Treatment of the Host

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Growth status</th>
<th>cAMP-Binding (pmoles/mg protein)</th>
<th>Protein Kinase Activity (pmoles/min/mg Protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>-cAMP</td>
<td>+cAMP</td>
</tr>
<tr>
<td>Vehicle only Growinga</td>
<td>4.53 ± 0.7</td>
<td>48.8 ± 4.2</td>
<td>134.1 ± 12.3</td>
</tr>
<tr>
<td>RA</td>
<td>Growing</td>
<td>4.40 ± 1.1</td>
<td>50.5 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>Regressingb</td>
<td>9.7 ± 2.0*</td>
<td>54.0 ± 2.6</td>
</tr>
<tr>
<td>HPR</td>
<td>Growing</td>
<td>2.30 ± 0.6</td>
<td>70.0 ± 4.5</td>
</tr>
<tr>
<td></td>
<td>Regressing</td>
<td>11.2 ± 2.4*</td>
<td>68.1 ± 4.4</td>
</tr>
</tbody>
</table>

The values indicate the means ± S.E. for five tumors assayed in duplicates.

a. Growing; >20% increase in tumor volume as compared to the initial volume on day 0.

b. Regressing; >20% decrease in tumor volume.

* p<0.05 when compared to the control values.
F. **Effect of retinoids on Human mammary carcinoma, MCF-7 cells:**

1. **Effects on cell growth:**

   a. **Concentration dependent effects:** As shown in figure 14, RA (10^{-7} - 10^{-5}M) or HPR (10^{-8} -10^{-6}M) inhibited the growth of MCF-7 cells in a concentration dependent manner. As in the DMBA-induced mammary tumor system, HPR was more potent than RA in the inhibition of MCF-7 cell growth. Addition of either 10^{-7}M HPR or 10^{-6}M RA inhibited the growth of MCF-7 cells to 40% of the control growth within seven days.

   b. **Time dependent effect:** Retinoid induced inhibition of MCF-7 cell growth was apparent after three days of retinoid treatment (figure 15 and 16).

   c. **Effect of retinoid withdrawal:** Discontinuation of retinoid treatment resulted in the resumption of cell growth. The cells previously treated with 0.1uM HPR or 1uM RA, regrew from the inhibited rate of 40% to 60-70 % of control rate within seven days of retinoid withdrawal (figure 16). This regrowth was confirmed when the retinoid treated cells were harvested and replated at the same density as the control cells (figure 17).
As shown in figure 17, the RA pretreated cells regrew at approximately 60% of the control rate, whereas the HPR pretreated cells regrew at the same rate as the control cells. This pattern of regrowth was previously observed in the established DMBA-induced mammary tumors.

2. Effect on DNA and RNA synthesis: Treatment with either RA (1μM) or HPR (0.1μM) resulted in the inhibition of $^3$H-thymidine and $^{14}$C-uridine incorporation into TCA and ethanol-insoluble materials in MCF-7 cells (Table 7). These inhibitions were observed after 20 hours of continuous retinoid treatment. As shown in table 7a, the inhibition of DNA/RNA synthesis due to 0.1μM HPR treatment (74%/30%) was greater than that due 1μM RA treatment (48%/21%). Also both DNA inhibitions were greater than the respective RNA inhibitions.

The inhibitions of DNA and RNA synthesis were also dependent on retinoid concentration (Table 7b).

G. Stimulation of the cAMP system of MCF-7 cells by Retinoids:

In order to determine the involvement of the cAMP system in the inhibition of MCF-7 cells by retinoids, the control and retinoid-treated cells were analyzed for cAMP content, cAMP binding, and cAMP-dependent protein kinase activity.
TABLE 7

Effect of retinoids on DNA and RNA synthesis in MCF-7 cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Nucleotide incorporation (cpm /1.6 x10⁵ cells)</th>
<th>³H-Thymidine</th>
<th>¹⁴C-Uridine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(A)</td>
<td>(B)</td>
</tr>
<tr>
<td>Control</td>
<td>4824 ± 103</td>
<td>96 ± 17</td>
<td>104 ± 2</td>
</tr>
<tr>
<td>RA (10⁻⁶M)</td>
<td>2523 ± 325**</td>
<td>76 ± 4*</td>
<td>69 ± 20*</td>
</tr>
<tr>
<td>HPR (10⁻⁷M)</td>
<td>1258 ± 215**</td>
<td>67 ± 2*</td>
<td>49 ± 4**</td>
</tr>
</tbody>
</table>

MCF-7 cells were treated with RA or HPR for 20 hours (A) or 40 hours (B) in the presence of labeled nucleotide. The control cells were treated with 0.1% ethanol. Assays were done as described in Materials and Methods. The values indicated are the means ± S. E. for two experiments done in triplicate.

*, P <0.05 as compared to the control.

**, P <0.01
MCF-7 cells were treated with various concentrations of RA or HPR for a total of 7 days, and the number of viable cells counted with hemocytometer by the trypan dye exclusion method. The values shown represent the mean ± S.E.M for three experiments ran in triplicates and counted in triplicates.

* p <0.05 as compared with control (0.1% ethanol only) cells.
FIGURE 14

Cell Growth (% of Control cells on day 7) vs. Log Retinoid concentration (nmolar)

RA
HPR
Concentration and Time dependent inhibition of MCF-7 cells by 13-cis-retinoic acid. MCF-7 cells were treated with various concentrations of RA and the number of viable cells counted after various times, with hemocytometer by the trypan blue dye exclusion method. The values shown represent the mean ± S.E.M for three experiments done in triplicate and counted in triplicate.

* p <0.05 as compared with control (0.1% ethanol-treated) cells.
FIGURE 16

Time related inhibition of MCF-7 cell growth by retinoids. MCF-7 cells were treated with 1 μM RA or 0.1μM HPR, and the number of viable cells counted after various times, with hemocytometer by the trypan blue dye exclusion method. The values shown represent the mean ± S.E.M for three experiments done in triplicate and counted in triplicate. * p <0.05 as compared with control (0.1% ethanol-treated) cells.
FIGURE 16

CELL GROWTH (% OF CONTROL)

DURING TREATMENT  AFTER WITHDRAWAL

-D-RA (10^-6M)

HPR (10^-7M)

*D*
1. Cyclic AMP content: Addition of RA in the medium of MCF-7 cells resulted in a concentration dependent increase in cellular cAMP content (figure 18). This increase reached a plateau within 72 hours, and was sustained through the experimental period (7 days).

2. Cyclic cAMP Binding:

There was also an increase (2-3 fold) in the cAMP binding in the retinoid treated cells (Table 8). The increase in cAMP binding occurred mainly in the cytosol and membrane fractions of the retinoid treated cells.

3. Activity and distribution of cAMP-dependent Protein kinase:

Treatment with either RA (10^{-6}M) or HPR (10^{-7}M) resulted in time-dependent increases in the cytosolic and membranous cAMP-dependent protein kinase activity. As shown in Figure 19, the increased activity due to these retinoids was apparent in the cytosol after 40 hrs of continuous retinoid treatment. Both HPR at 10^{-7}M and RA at 10^{-6}M increased the cytosolic cAMP-Pk activity by approximately 2.4-fold at 40 hr and by approximately 1.5-fold at 72 hr.
Growth of MCF-7 cells previously treated with retinoids. The cells were treated with retinoids, harvested and counted as described in Materials and methods.

Cells treated with 0.1% ethanol (o-o), 1uM RA (△-△) or 0.1uM HPR (x-x) for ten days were harvested and replated at equal density (1500 cells per well) in medium free of the above treatments.

Each point represents the mean ± S.E.M. for three experiments counted in triplicates.

* p <0.05 compared to control.
NUMBER OF VIABLE CELLS X 1000

DAYS AFTER RETINOID WITHDRAWAL

FIGURE 17
FIGURE 18

Effect of retinoid treatment on the cAMP content of MCF-7 cells. Cells were treated with 1uM RA as described in Materials and methods. At various durations after the addition of RA to the medium, cells were harvested and resuspended in 10% TCA. Cellular cAMP content was determined as described in Methods.

* p <0.05 as compared to the control (0 hour).
TABLE 8

Effect of retinoid-treatment in the culture medium on the cAMP binding of subcellular fractions of MCF-7 cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>3H-cAMP Binding (pmoles / mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duration (hours)</td>
<td>0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>0.04±0.02</th>
<th>0.28±0.14</th>
<th>0.96±0.17</th>
<th>2.64±0.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytosol:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Membrane:</td>
<td>1.03±0.2</td>
<td>1.13±0.27</td>
<td>3.1±1.25</td>
<td>2.81±0.6</td>
</tr>
<tr>
<td>Nuclei:</td>
<td>3.31±1.33</td>
<td>3.42±0.91</td>
<td>4.30±5.0</td>
<td>2.56±1.38</td>
</tr>
</tbody>
</table>

HPR (10^-7M) was added to the culture medium as previously described in Materials and Methods. Also cell fractions were isolated as described in Materials and Methods. The results indicate the means ± S.E. for two studies using 6 flasks per treatment period.

* p <0.05 as compared to the values at time 0.
However, the membrane effect of HPR (10^{-7}M) occurred earlier than that of RA (10^{-6}M). HPR increased membrane cAMP-Pk activity by 72% within 12 hr, during which time RA had no effect on the enzyme activity as compared to the control (Figure 19). A similar increase was induced by RA (10^{-6}M) after 40 hr of continuous treatment. At 40 hr, HPR (10^{-7}M) increased membrane cAMP-Pk activity by 2.5-fold, and continued to increase the activity to almost 4-fold of the control level following 72 hours of treatment. RA (10^{-6}M) increased the membrane enzyme activity to 3-fold of control level within 72 hr. Thus the increase in cAMP dependent protein kinase activity in the membrane and cytosol of MCF-7 cells occurred prior to the observed inhibition of cell growth.

These increases did not extend to the nuclei, where retinoid treatment either had no significant effect or caused a decline in cAMP-Pk activity (Table 9).

The above results support the early involvement of cAMP-dependent protein kinase in the inhibition of mammary cancer by retinoids. It further shows that this effect is primarily due to increase in the membranous and cytosolic enzyme activities.
FIGURE 19

Increase in cytosolic and membranous cAMP-dependent protein kinase activity of MCF-7 cells by retinoids. Cells were treated with retinoids $10^{-6}$M RA (△) or $10^{-7}$M HPR (x) for various time intervals and the enzyme activity determined in the membrane and cytosolic fractions. The values shown represent the mean ± S.E.M. for three experiments done in duplicates.

* $p < 0.05$ as compared to the control.
Specific Activity (pmoles / min / mg protein)

Membrane
Cytosol

Duration of treatment (hours)

FIGURE 19
TABLE 9

Effect of Retinoids on Protein kinase activity in the cytosol, nuclear and membrane of MCF-7 cells

<table>
<thead>
<tr>
<th></th>
<th>HPR (10^{-7}M)</th>
<th>RA (10^{-6}M)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Specific Activity (pmoles / min / mg protein)</td>
<td></td>
</tr>
<tr>
<td>(Hours)</td>
<td>-cAMP</td>
<td>+cAMP</td>
</tr>
<tr>
<td>0</td>
<td>44 ± 11</td>
<td>72 ± 10</td>
</tr>
<tr>
<td>12</td>
<td>18 ± 0.3</td>
<td>42 ± 0.7</td>
</tr>
<tr>
<td>40</td>
<td>74 ± 15</td>
<td>139 ± 29</td>
</tr>
<tr>
<td>72</td>
<td>60 ± 20</td>
<td>109 ± 21</td>
</tr>
<tr>
<td></td>
<td>CYTOSOL</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>165 ± 42</td>
<td>213 ± 43</td>
</tr>
<tr>
<td>12</td>
<td>194 ± 17</td>
<td>282 ± 22</td>
</tr>
<tr>
<td>40</td>
<td>184 ± 51</td>
<td>318 ± 55</td>
</tr>
<tr>
<td>72</td>
<td>427 ± 7</td>
<td>622 ± 6</td>
</tr>
<tr>
<td>84</td>
<td>132 ± 18</td>
<td>373 ± 69</td>
</tr>
<tr>
<td></td>
<td>MEMBRANE</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>182 ± 25</td>
<td>271 ± 37</td>
</tr>
<tr>
<td>12</td>
<td>98 ± 44</td>
<td>186 ± 10</td>
</tr>
<tr>
<td>40</td>
<td>132 ± 23</td>
<td>228 ± 48</td>
</tr>
<tr>
<td>72</td>
<td>257 ± 8</td>
<td>346 ± 13</td>
</tr>
<tr>
<td>84</td>
<td>247 ± 55</td>
<td>383 ± 77</td>
</tr>
<tr>
<td></td>
<td>NUCLEI</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>182 ± 25</td>
<td>271 ± 37</td>
</tr>
<tr>
<td>12</td>
<td>98 ± 44</td>
<td>186 ± 10</td>
</tr>
<tr>
<td>40</td>
<td>132 ± 23</td>
<td>228 ± 48</td>
</tr>
<tr>
<td>72</td>
<td>257 ± 8</td>
<td>346 ± 13</td>
</tr>
<tr>
<td>84</td>
<td>247 ± 55</td>
<td>383 ± 77</td>
</tr>
</tbody>
</table>

The values shown indicate the Mean ± S.E. for at least 5 plates assayed in duplicates.
H. Effect of cycloheximide on the early action of retinoids on the cAMP system of MCF-7 cells:

1. Effect on protein synthesis: Treatment with 10uM cycloheximide (CX) inhibited \(^{3}\text{H}\)-leucine incorporation to 45% of control (0.1% ethanol-treated) within 20 hours (Table 10). HPR (0.1uM) inhibited leucine incorporation by only 23% of the control level within the same period and this inhibition was not statistically significant. However, the combined inhibition due to CX and HPR was additive.

2. Effect on cAMP Binding: Addition of CX (10uM) to the medium of MCF-7 cells inhibited HPR induced-increase in cAMP binding in the cells (figure 20). CX alone had no significant effect on cAMP binding in the cells. However, it reduced the increase due to HPR by approximately 70%. HPR alone increased cAMP binding by 3 to 4 fold as compared to the control cells.

3. Effect on cAMP dependent Protein kinase: As shown in figure 21, addition of CX (10uM) to the medium, prevented the HPR-induced increase in cytosolic and membrane cAMP-dependent protein kinase activity. These findings suggest that the early retinoid-induced increase in cAMP-dependent protein kinase activity is due to an increase in the amount of enzyme through denovo synthesis.
TABLE 10

Effect of N-(4-hydroxyphenyl)retinamide and cycloheximide on the incorporation of 3H-leucine in MCF-7 cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>$^{3}$H-Leucine (cpm / min / 100,000 cells)</th>
<th>Percent of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>11.0 ± 3</td>
<td>100</td>
</tr>
<tr>
<td>CX</td>
<td>5.0 ± 1.7*</td>
<td>45</td>
</tr>
<tr>
<td>HPR</td>
<td>8.4 ± 2.4</td>
<td>77</td>
</tr>
<tr>
<td>CX+HPR</td>
<td>2.5 ± 2*</td>
<td>25</td>
</tr>
</tbody>
</table>

CX; cycloheximide (10uM) was added 30 min before addition of HPR (0.1uM) to the medium. The values indicate the means ± S.D. for three assays ran in duplicates.

* p <0.05 compared to the control
FIGURE 20

Inhibition by cycloheximide (CX) of retinoid-induced increase in cAMP binding capacity of MCF-7 cells. CX (10uM) was added to the medium of MCF-7 cells, 30 min prior to addition of HPR (0.1uM) to the CX+HPR medium. Approximately 0.2uCi $^3$H-cAMP (36 Ci/mmol) ± 2uM cold cAMP was then added to the medium.

Cyclic AMP binding was determined in the intact cells as described in the Materials and Methods. Each bar represents the mean ± S.E. for three experiments ran in duplicates.

* p <0.05 as compared to the control (0.1% ethanol-treated).
FIGURE 20

$^{3}H$-cAMP BOUND (CPM / 10^5 CELLS)

TREATMENT

C  CX  HPR  CX+HPR

*
FIGURE 21

Effect of cycloheximide (CX) on the induction of cAMP-dependent protein kinase by HPR. CX (10uM) was added 30 min before the addition of HPR (10^-7M). The cells were allowed to grow in the experimental medium for 40 hours and were harvested as previously described in methods. Cyclic AMP dependent protein kinase was assayed in cell fractions. The values indicated are the means ± S.E.M. for three experiments assayed in duplicates. Cytosol (open bars); Membrane (solid bars).

* p < 0.05 as compared to the control (0.1% ethanol-treated).

** p < 0.01 as compared to the control (0.1% ethanol-treated).
FIGURE 21

SPECIFIC ACTIVITY
(pmoles / min / mg protein)

TREATMENT

C

HPR

CX+HPR

0 20 40 60 80 100 120 140 160 180 200
I. **Effect of retinoid treatment on the phosphorylation of endogenous proteins in MCF-7 cells:**

1. **Cytosolic proteins:** Two major bands of phosphorylation were observed in the cytosol of MCF-7 cells (Plate I). However, the extent of phosphorylation of only one of these bands with apparent Mwt. of 56,000 dalton was increased in retinoid treated cells as compared to the control cells.

2. **Membrane Proteins:** Retinoid treatment resulted in the increase in phosphorylation of three membrane proteins (Plate 2). The major phosphorylation occurred on the 52,000 dalton membrane protein. This phosphorylation increased with time, up till 40 hours. Phosphorylation of three other membrane proteins, with apparent Mwt. of 79,000, 98,000 and 108,000 daltons occurred at slower rates (Table 11). The increase in membrane phosphorylation was confirmed in 10% ficoll purified membrane. Phosphorylation of the 52,000 dalton phosphoprotein increased by 2-3 fold, within 40 hours of retinoid treatment (figure 22). At this time, the phosphorylation of the 79,000, 98,000 and 108,000 dalton proteins had increased by only 50%, 27% and 56% respectively, due to retinoid treatment (Table 11).
However, there was no further increase in phosphorylation of the 52Kd and 79Kd proteins beyond the 40 hour period, whereas phosphorylation of the 98Kd and 108Kd proteins continued to increase up to 69% and 82% respectively as compared to time 0. Thus treatment with retinoids resulted mainly in an early increase in the phosphorylation of the 52 Kd membrane protein and a late increase in the 98Kd and 108Kd proteins in a time dependent manner.

Plate III, are representative pictures of the stained gels ran parallel to those used for autoradiography.
<table>
<thead>
<tr>
<th>Mol.WT. (Daltons)</th>
<th>Area under curve (arbitrary units)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 hour</td>
</tr>
<tr>
<td>52,000</td>
<td>1.25</td>
</tr>
<tr>
<td>79,000</td>
<td>0.8</td>
</tr>
<tr>
<td>98,000</td>
<td>0.8</td>
</tr>
<tr>
<td>108,000</td>
<td>0.55</td>
</tr>
</tbody>
</table>

The values indicated were obtained by scanning the autoradiographs represented in plate 2, using a densitometer.
Autoradiograph of phosphorylated endogenous cytosolic proteins of MCF-7 cells treated with HPR (0.1uM) for 0, 12, 40, and 72 hours. Cells were treated with HPR as previously described and harvested after the indicated durations of retinoid treatment. The cells were then fractionated and cytosolic endogenous protein phosphorylation determined as described in Materials and Methods. Each column represents a sample of cytosol phosphoprotein prepared from approximately 60 million cells pooled from 6 different flasks.
PLATE I

Mol. Wt. (daltons)

DURATION OF RETINOID TREATMENT (hours)
PLATE II

Autoradiograph of phosphorylated endogenous membrane proteins of MCF-7 cells treated with HPR (0.1μM) for 0, 12, 40, and 72 hours. Cells were treated with HPR as previously described and harvested after the indicated durations of retinoid treatment. The cells were then fractionated and membrane endogenous protein phosphorylation determined as described in Materials and Methods. Each column represents a sample of membrane phosphoproteins prepared from approximately 60 million cells pooled from 6 different flasks.
PLATE II

Mol. Wt. (daltons)

DURATION OF RETINOID TREATMENT (hours)
FIGURE 22

Densitometric plots of time-response phosphorylation of membrane proteins in MCF-7 cells treated with HPR (0.1 uM). Autoradiographs from retinoid-treated cells described in Plate II were scanned with a densitometer.
Figure 22

Duration of Retinoid Treatment

Density of bands (arbitrary units)

- cAMP  
+ cAMP  
- cAMP  
+ cAMP

0 hour  
12 hours  
40 hours  
72 hours

52kd
Representative Coomasie brilliant-stained gels containing bands of proteins from the cytosol and membrane fractions of MCF-7 cells treated with HPR (10^{-7}M). Cell fractionation and gel electrophoresis were performed as described under materials and methods. Electrophoresis was done with 60ug protein per lane.
PLATE III

DURATION OF RETINOID TREATMENT (hours)

MW (kd)

27 36 48 58 84 116 180

CYTOSOL  MEMBRANE

0 12 40 72 0 12 40 72
A. Inhibition of DMBA-induced mammary tumors in relation to the cAMP system:

This study provided the first comparison of anticarcinogenic effects of various retinoids on mammary cancer in relation to their effects on the cyclic AMP system. The mode of action of retinoids on the DMBA-induced mammary tumor was correlated with the ability to stimulate the cAMP system.

1. Cyclic AMP content: In this regard, a correlation was made between the increase in cAMP level and the prevention of tumor development by various retinoids. The order of increase in cAMP content by retinoids (RP < RA < HPR < RAc) correlated well with their respective abilities to inhibit tumor induction and growth of DMBA mammary tumors in rats. However, HPR rather than RAc was most effective in the prolongation of latency period before tumor appearance. The increase in basal cAMP level by retinoids has also been reported in other cancer cells (Livesey et al. 1985). Therefore the effect is not specific to mammary cancer. 

125
cells. It has been reported that retinoids increased the sensitivity of cells to agents that increase cellular cAMP content (Olsson et al. 1982, Fontana et al. 1985).

2. Cyclic AMP binding

The specific binding of cAMP to the regulatory subunit of cAMP dependent protein kinase is more indicative of increased enzyme activation than cAMP content (Hilz et al. 1978). Only a very small increase in cAMP level is enough for the activation of this enzyme (Hemmings 1985).

The fact that retinoids also increased cAMP binding by 2- to 3-fold in the above tumors strongly indicated that retinoids have a stimulatory effect on the cAMP system. However, an increase in cAMP content as well as cAMP binding affinity would be an inefficient mode of enzyme activation for an endogenous modulator such as retinoic acid. As was revealed by the scatchard plot of the concentration dependent binding of cAMP, retinoid treatment increased the amount of cAMP binding protein, rather than increase its affinity for cAMP.

These multiple effects of retinoids on both cAMP content and the amount of protein kinase, would assure an increase in the activity of cAMP dependent protein kinase in vivo. The increase in enzyme content may explain the synergism between retinoids and agents that increase
cellular cAMP content. Thus, dibutyryl cAMP acts synergistically with retinoids in the inhibition of mammary tumors (McCormick et al. 1982).

3. Cyclic AMP dependent protein kinase:

The increase in cAMP binding induced by retinoids was also associated with a simultaneous increase in the activity of cAMP dependent protein kinase. This 2-3 fold increase in enzyme activity was mostly due to an increase in synthesis of the type II isoenzyme. The increase in cAMP dependent protein kinase peak II has been previously associated with tumor regression (Cho-Chung 1981). Other authors have recently associated an increase in peak II/peak I ratio with the inhibition of cells by retinoic acid (Plet et al. 1981; Livesey et al. 1985). The appearance of this type II isozyme was postulated as a marker of differentiation (Lee et al. 1976).

B. Growth arrest and regression of DMBA-induced mammary tumors by retinoids in relation to the cAMP system:

1. Tumor arrest and regression:

Stimulation of cAMP system by retinoids also caused the growth inhibition and/or regression of established mammary tumors. In this model, the inhibitory effects of RA and
HPR were similar. Administration of 1.5 or 2 mmol retinoid per kg diet to the tumor bearing rats, caused a marked inhibition of mammary tumor growth within 3 to 5 days. The tumors in the control group continued to grow during the same period indicating that the growth inhibition was due to retinoid treatment.

2. Retinoid withdrawal and tumor regrowth:

Discontinuation of retinoid supplementation in the diet resulted in the resumption of growth of the growth-arrested, or partially regressed tumors. Thus the effect of retinoids was more cytostatic rather than cytotoxic in nature and could be eliminated by discontinuation of retinoid therapy. A similar regrowth of retinoid-inhibited tumors was previously demonstrated in the Shope rabbit papilloma (McMichael 1965) and in the rat papilloma (Sporn 1976).

An interesting result of HPR treatment is the more rapid regrowth of mammary tumors, following withdrawal of this retinoid. This type of response could be useful in the treatment of breast cancer. Since the resumption of growth occurred only in growth-inhibited and partially regressed but not in completely regressed tumors, it could be avoided by continuous treatment with HPR until the tumor is completely regressed.
The faster growth of the HPR withdrawn tumors could be used to selectively increase the susceptibility of breast cancer cells to the effect of other chemotherapeutic agents which depend on cell turnover for their action. In this respect, patients can be pretreated for a short period with HPR and then treated with the cell turnover-dependent chemotherapeutic agent, just after retinoid withdrawal. This combination therapy may be more effective and less toxic than chemotherapy alone.

Other investigators have demonstrated the inhibition and regression of skin cancer by retinoids in humans and in experimental animals (Bollag 1971, 1974; Meyskens, 1982). The only previous suggestion that retinoid can inhibit established mammary tumor, was from the report that a moderate dose of retinyl palmitate inhibited the growth of transplantable adenocarcinoma C3HBA in mice (Rettura et al., 1975). During the course of the present study, retinyl palmitate and retinyl acetate were also tested for their effects on the established mammary tumor in rats. These two retinoids slightly inhibited tumor growth. However, they both exhibited toxicity reflected in significant weight loss when administered in large doses (2 mmol/kg diet) to the rats (data not shown).
3. Spontaneous tumor growth and spontaneous regression:

When these retinoids were given to CD8F-1 mice with spontaneous mammary adenocarcinomas, the inhibition of these tumors was apparent after ten days of treatment (see appendix). Thus the response was less dramatic than those observed in the DMBA-induced tumors. However, the CD8F-1 mice were in the late stages of their life span (see Stolfi et al., 1971) and might have responded better if the tumors were treated at an early stage.

The growth arrest and/or regression of DMBA-induced mammary tumors was not due to spontaneous growth arrest or regression. Thus, 60-66% of HPR and RA treated rats responded to treatment, whereas spontaneous growth arrest or regression, as observed in the control group, accounted for the inhibition of additional 20% of the total 80-86% tumor inhibition in the retinoid-treated rats. Resumption of tumor growth upon retinoid withdrawal from the diet, is also a clear indication that the tumor inhibition is due to retinoid treatment.

C. Dose related effects of retinoids relative to retinoid action and toxicity:

The inhibitory effect of retinoids on mammary tumor growth was not due to toxicity at the doses of retinoids used in this study. The results of this study, as well as
others (Moon et al., 1979) showed that chronic treatment of rats with 2 mmol/kg diet of HPR produced no toxicity. In spite of the common concern about the toxicity of retinoids, there was no difference in weight gain between the control rats and rats treated with RA or HPR. In the studies on the chemoprevention of mammary tumor development by retinoids, there was also no overt toxicity even after the chronic treatment of rats with RA or HPR at 1.5 mmol/kg diet for approximately 6 months. Previous studies have shown that 13-cis-RA can be tolerated at p.o. doses as high as 100 mg/kg mouse/day and 40 mg/kg rat/day (equivalent to approximately 2 mmol/kg diet) without evidence of major toxicity (Hixson and Denine 1978; 1979). The above investigators also reported a lack of difference in weight gain and food consumption between the control and RA-treated rats (Hixon and Denine 1979). As suggested by some investigators (Kerr et al., 1982), there may be pharmacokinetic limitations to the use of low doses of 13-cis-RA, which has a short-life, in the treatment of cancer. Thus it was also suggested that RA should be given more frequently (Kerr et al., 1982). This may explain why there was an inhibition of mammary tumor growth with higher doses of RA used in this study, while other investigators (see Hill and Grubbs, 1982) did not induce similar inhibitions with low doses administered less frequently.
D. **In vitro effects of retinoids on MCF-7 cells:**

1. **Cell growth:**

   During the course of this study, a question arose as to whether the inhibition of tumor growth by retinoids was due to cytocidal effect of these retinoids or due to their prolongation of the cell doubling time. In vitro studies with MCF-7 cells, revealed that the control cells doubled in approximately 42 hours, whereas the cells treated continuously with 100 nmolar HPR or 1 umolar RA doubled every 72-80 hours. These concentrations of the above retinoids had no significant effect on cell viability, as determined by the ability of the cells to exclude trypan blue dye.

2. **Retinoid withdrawal and cell regrowth:**

   As in the in vivo experiment, the HPR pre-treated cells grew faster than the control cells after 8 days of discontinuation of treatment. The RA pre-treated cells grew at less than the control rate after 8 days of discontinuation of retinoid treatment. However, cells pre-treated with RA or HPR doubled at the same rate within 8 days of retinoid withdrawal. Similar resumption of growth has also been reported by other investigators using retinoic acid (Udea *et al.*, 1980).
Since the concentration of RA or HPR used did not impair cell viability, and the cells regrew following retinoid withdrawal, it is likely that the effect of retinoids was on cell doubling rather than due to cell death.

The reason for the faster growth of HPR-pretreated cells was not evident in the present study. Since HPR has a longer elimination half-life than RA, it seems unlikely that this withdrawal effect is due to rapid metabolism of HPR. However, the metabolic products of HPR in the cells may be less inhibitory to the cell growth than those of RA. Retinoyl B-glucuronide, a metabolite of retinoic acid, inhibits cell growth (Swanson et al., 1981). Whether or not the corresponding HPR-O-glucuronide is equally inhibitory to cell growth is not clear. It is also not known if HPR-O-glucuronide is a significant metabolite of HPR in mammary cancer cells. Some previous studies suggested that 5-methoxy-HPR is the major metabolite of HPR in rat plasma (Swanson et al., 1981).

3. Effect on Cyclic AMP system:

The antiproliferative action of the above retinoids was preceded by stimulation of the cAMP system. Thus, treatment with RA resulted in a concentration dependent increase in cellular cAMP content within 12 hours.
However, this increase in cellular cAMP plateaued within 72 hours of continuous retinoid treatment. Other investigators have reported an increase in basal cellular cAMP content, during the treatment of rat osteogenic sarcoma cells with retinoic acid (Livesey et al., 1985).

The present study showed that the increase in cAMP occurred very early during retinoid treatment and was sustained through the period of retinoid treatment. Discontinuation of retinoid treatment resulted in the drop of cAMP to or below the control level. This may explain the resumption of growth of the cells or tumors that were previously inhibited by retinoids. At least one other diterpene, forskolin, has been shown to activate almost all adenylate cyclase systems studied (Segmon et al., 1981). Although the mechanism of action of this compound is not clear, it seems to be dependent on a rapidly turning over protein of unknown identity and can be inhibited by protein synthesis inhibitors (Brooker et al., 1983). It is possible that the same mechanism operates in both retinoids and forskolin.

However, as previously stated, the binding of cAMP to cAMP-dependent protein kinase is a better indicator of activation of the enzyme than the level of cAMP. The increase in 3H-cAMP binding during treatment of MCF-7 cells with retinoids indicates an increase in enzyme activation.
by retinoid treatment. The prevention of this increase in cAMP binding by the inhibition of protein synthesis with cycloheximide supports the earlier conclusion that retinoids increased the synthesis rather than the cAMP binding affinity of cAMP dependent protein kinase. This observation represents the first report of the stimulation of cAMP binding in cultured mammary cancer cells by retinoids.

The increase in the amount cAMP dependent protein kinase by retinoids and the prevention of this increase by cycloheximide supports the induction of this enzyme by retinoids. This increase in enzyme synthesis resulted mainly in the increase in specific activity of the membrane associated enzyme. It is possible that retinoids stimulate the phosphorylation of membrane and cytosolic enzymes and receptors that regulate cell growth.

Recently, it was also reported by Plet et al. (1982), that retinoic acid increased membrane and cytosol associated cAMP-dependent protein kinase in F9 embryonal carcinoma cells. In the above study, the increase in activity was also due to increased type II/type I enzyme ratio. This mode of action of retinoids in the inhibition of cell proliferation differed from the mechanism proposed by Cho-Chung et al. (1981), for the inhibition of MCF-7 cells with dibutyryl cAMP. Their study showed a translocation of
cAMP dependent protein kinase from the cytosol into the nucleus following treatment of cells with dibutyryl cAMP. In contrast, retinoids promoted the increase in membrane rather than nuclear cAMP-dependent protein kinase activity. The reason for this discrepancy is not clear. However, it is noteworthy that the translocation of this enzyme into the nucleus is modulated by the amount of cAMP in the cell. Thus a slight elevation in cellular cAMP favors while a large increase inhibits the translocation of the enzyme into the nucleus (Cho-Chung 1981).

4. **Endogenous protein phosphorylation:**

Distribution of phosphorylating activity to either the membrane or nucleus can influence gene expression. Also, protein phosphorylation is a major general mechanism by which intracellular events respond to external physiological stimuli (Cohen 1982). Phosphorylation of some membrane receptors for growth factors can modulate their effects on gene expression. Phosphorylation of tyrosine kinase receptors such as those for insulin, platelet-derived growth factor, and epidermal growth factor, as well as several cellular and retroviral oncogeny proteins results in growth enhancement (Obberghen and Gammeloft 1986).
In contrast, all kinases involved in the control of intermediary metabolism are serine or threonine specific (Cohen 1982, Denton et al., 1981, Obberghen and Gammeltoft 1986). The two phosphoamino acids constitute about 99.97% of all phosphorylated amino acids, while phosphotyrosine accounts for only 0.03% (Hunter and Sefton 1980). Tyrosine residues have high affinity but low capacity for phosphate, whereas serine residues have low affinity but high capacity for phosphate. This difference could explain the ability of retinoids and cAMP to promote cell growth at physiological concentrations and inhibit growth at higher concentrations. Low concentrations of these compounds could increase cAMP dependent protein kinase activity enough to phosphorylate only the high affinity but low capacity tyrosine residues, resulting in growth enhancement. However, at higher concentrations of retinoids or cAMP the cAMP dependent protein kinase activity can be increased enough to phosphorylate the preponderant serine residues, thus leading mainly to metabolic changes and possibly growth inhibition. Phosphorylation of pyruvate kinase will inhibit this enzyme activity and glycolysis. Similarly inhibition of lipogenesis will result from phosphorylation of acetyl coA carboxylase (Krebs and Beavo, 1979) which catalyzes the synthesis of malonyl-CoA from acetyl CoA and CO₂.
Malonyl-CoA is an essential substrate for long chain fatty acid synthesis. Inhibition of its synthesis and thus of lipogenesis will inhibit the alternative source of energy generation for cancer cell growth.

Some investigators have reported the phosphorylation of membrane proteins, which are neither enzymes nor receptors (Hosey and Tao 1977). The observation of at least three membrane proteins, whose phosphorylation was increased in retinoid treated cells, suggests that more than one membrane phosphoprotein may be involved in the mediation of retinoid effects on MCF-7 cells. Similar observation of multiple phosphoproteins have been reported in human and rabbit erythrocyte (Hosey and Tao 1977) and in synaptic (Ueda et al., 1973) membranes. As in the present study, Hosey and Tao also reported a membrane phosphoprotein with apparent molecular weight of 100,000 daltons. A phosphoprotein in the same molecular weight range was implicated in the transport of anions and water in red cells (Hosey and Tao 1977). Phosphorylation of such a protein may be important to the transport of nutrients into the cell.

Retinoids increased thymidine and uridine uptake into MCF-7 cells (data not shown). Whether or not this increase is related to the phosphorylation of the 98,000 dalton membrane protein is not known. However, this increased
uptake was not due to increased macromolecules synthesis, since retinoids inhibited the incorporation of $^3$H-thymidine and $^{14}$C-uridine into MCF-7 cell DNA and RNA respectively. Similar inhibition of macromolecular synthesis was reported by other investigators in the MCF-7 cells (Ueda et al., 1980). The increase in uptake may be a non-specific increase in membrane permeability by retinoids, and could account for the reported increase in cytoplasmic volume of MCF-7 cells treated with retinoids (Cho-Chung 1982).

The effect of retinoids on ion permeability has also been considered in regard to the cellular effects of Ca$^{++}$ (Yuspa et al., 1981). Ionic calcium is a critical regulator of epithelial cell growth and differentiation (Hennings et al., 1980). Retinoic acid was reported to alter the mode of induction of terminal differentiation by a high concentration of Ca$^{++}$ (Yuspa et al., 1981).

Another membrane phosphoprotein whose phosphorylation was progressively increased in retinoid treated cells is the one with apparent molecular weight of 108,000 daltons. The identity or function of this protein is not evident from the present study. However, a 110 kilodaltons (Kd) protein whose phosphorylation is inhibited by insulin, has been demonstrated in the rat liver plasma membrane (Tran and Desbuquois 1980).
Some investigators reported the presence of a 50 Kd and an 80 Kd phosphoserine-containing polypeptide, in immunoprecipitates made from $^{32}$P-labeled transformed chicken cells with antitumor antiserum (Hunter and Sefton 1980). The 50 Kd polypeptide was determined to also contain some phosphotyrosine residues. Its phosphorylation by some viral oncogene products is suspected to be associated with cell transformation (Hunter and Sefton 1980). If this polypeptide is the same observed in the MCF-7 cells, it may be involved in the regulation of proliferation of these cells. The above investigators also reported the existence of a 52 Kd polypeptide related to the Rous sarcoma virus src gene product which is homologous to one or a few genes (sarc) found in all normal vertebrates (Spector et al., 1978).

It was reported that insulin treatment in the presence of cAMP increased the phosphorylation of a 52 Kd protein associated with the peripheral plasma membrane of rat liver (Marchmont and Houslay 1980). Similar treatment, decreased the phosphorylation of an 80 Kd membrane protein. Increase in phosphorylation of the 52 Kd membrane protein was observed in 10% ficoll purified membrane from cells treated with 0.1uM HPR for 12-40 hours. Thus the observed increase in phosphorylation of the 52,000 dalton protein may play a pivotal role in the mediation of retinoid inhibition of mammary cancer by the cAMP system.
In summary, this study demonstrated:

1. The in vivo experiments revealed that retinoids have both anticarcinogenic and antitumor effects on DMBA-mammary tumors in the rat.

2. Retinoids including the novel HPR were shown to reversibly inhibit the growth of human mammary carcinoma MCF-7 cell line in culture.

3. Effects of retinoids on the cAMP system preceded their inhibitory effect on cell growth.

4. The cAMP system response to retinoids in vivo and in vitro, included increases in cellular cAMP content, cAMP binding, and cAMP dependent protein kinase activity. This study also represents the first report of the stimulation of cAMP binding and cAMP-dependent protein kinase activity in cultured mammary cancer cells by retinoids.
5. The increase in cAMP-dependent protein kinase was mostly due to increase in synthesis of the type II isozyme and was inhibited by cycloheximide.

6. Increase in the specific activity of the above enzyme occurred mainly in the membrane fraction and to a lesser extent in the cytosol of the retinoid treated cells.

7. The increase in enzyme activity resulted mainly in an early increase in phosphorylation of a 52,000 daltons membrane protein.

8. These findings indicate that the anticarcinogenic, antitumor and antiproliferative effects of retinoids are mediated by the cAMP system.
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