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# INDUCTION OF HEPATIC ORNITHINE DECARBOXYLASE AND PLASMINOGEN ACTIVATOR AFTER SYSTEMIC ADMINISTRATION OF 12-O-TETRADECANOYLPHORBOL-13-ACETATE IN THE RAT

THE UNIVERSITY OF ARIZONA M.S. 1983

# Induction of Hepatic Ornithine Decarboxylase and Plasminogen Activator After Systemic Administration of 12-O-Tetradecanoylphorbol-13-acetate in the Rat

by

Arthur R. Buckley Jr.

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1983

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12/9/83
DATE

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## ABSTRACT

Induction of ornithine decarboxylase (ODC) and plasminogen activator (PA) have been linked to tumor promotion. Rapid increases of these enzymes following a single systemic dose in rats of 12-O-tetradecanoylphorbol-13-acetate (TPA), a tumor promoter, are reported here. Hepatic ODC activity peaked at four hours, remained elevated at eight hours and returned to control levels by twelve hours. Hepatic PA activity was elevated at two hours, peaked at twelve hours then declined toward control levels. Pretreatment with cycloheximide or actinomycin D ameliorated both enzyme inductions. *In vitro*, ODC and transamidated ODC were found to possess considerable PA activity. We conclude that (1) systemic TPA rapidly induces PA and ODC in rat liver (2) both inductions reflect *de novo* enzyme synthesis (3) PA, like ODC, may be obligatory to tumor promotion (4) post-translationally modified ODC possess PA-like activity *in vitro* and further, *in vivo* modification of ODC may contribute to elevated PA levels following TPA.

# CHAPTER 1

## INTRODUCTION

Since the early 1940's, chemical carcinogenesis has been known to occur as a stagewise process of initiation and promotion (Corasanti et al. 1982). Rous and Kidd (1941), Berenblum (1941) and Mottram (1944) applied hyperplasiogenic (promoting) agents subsequent to exposure of mouse skin to subcarcinogenic doses of polycyclic aromatic hydrocarbons (initiating agents). Their results showed this regimen to be more effective in eliciting tumorigenic responses than the hydrocarbons alone. The agent introduced by Berenblum (1941) in these early experiments was croton oil. This substance is obtained from the seeds of the plant Croton tiglium L. (Euphorbiaceae) which is indigenous to India and Ceylon. In the 1960's, the active tumor-promoting components of croton oil were isolated (Hecker 1968) and partially characterized as phorbol esters (Van Duuren 1969). Subsequently, the actions of phorbol ester tumor-promoting agents were studied primarily in cell culture (Colburn 1980). All components isolated were found to be diesters of phorbol. 12-O-tetradecanoylphorbol-13-acetate (TPA), Chemical Abstracts (CA) Registry Number 20839-11-6, CA name: 5H-cyclopropa [3,4] benz [1,2-e] azulen-5-one, 1, 1a , 1b , 4, 4a, 7a , 7b, 8, 9, 9a-decahydro-4a , 7b , 9b, 9a -tetrahydroxy-3-(hydroxymethyl) -1, 1, 6,

8 -tetramethyl-9a-acetate-9-myristate, was found to possess the most potent tumor promoting characteristics.

Since their discovery, experiments targeted at elucidating cellular and biochemical events associated with tumor promotion by phorbol esters have largely utilized mouse skin and cell culture systems. To date, a large number of biochemical changes have been described and much data generated in attempts to clarify the mechanisms of tumor initiation and promotion. It is hoped that with the elucidation of these biochemical pathways, cancer prevention and therapy might be advanced.

## Tumor Promotion

Although a diverse group of non-phorbol agents including phenobarbital, sodium cyclamate, bile acids, sodium saccharin, thioacetamide and others (Colburn 1980) have been shown to exhibit tumor promoting or co-carcinogenic capabilities in a variety of systems, this report will focus on TPA. TPA and other promoting agents elicit similar multistage cellular and biochemical responses (Colburn 1980) with the major differences between them being potency and their specific target organ selectivity. Indeed, TPA, until recently, was believed to be the most potent natural tumor-promoting agent described (Hirakawa et al. 1982).

In contrast to initiating agents, tumor promoters do not appear to covalently bind to cellular DNA nor are they mutagenic in themselves (Weinstein et al. 1979). Instead, it was shown in the mouse

skin model, that topical TPA produces a local irritation which is essential but insufficient in itself for promotion of tumor development (Berenblum 1969). This irritation is thought to be the result of complement system activation by proteolysis (Troll 1976), which in turn is followed by a series of biochemical events involving cell surface and membrane changes, alterations in growth properties and aberrations in enzymatic regulation. One feature common to promoters is that they inhibit terminal differentiation (Colburn 1980) in a variety of cell culture systems including myogenesis in chick myoblasts (Cohen et al. 1977), erythroid differentiation in Friend erythroleukemia cells (Rovera et al. 1977, Yamasaki et al. 1977) adipocyte induction from 3T3 cells (Diamond et al. 1978), chondrogenesis in chick chondroblasts (Pacifici and Holtzer 1977), neurite induction in neuroblastoma cells (Ishii et al. 1978) and melanogenesis in melanoma cells (Mufson 1978). Boutwell and others (1979) showed that tumor promoters, while inhibiting terminal differentiation, stimulate DNA synthesis and hyperplasia, both of which have long been associated with tumorigenesis and, specifically, promotion (Boutwell et al. 1979, Colburn 1980).

## Ornithine Decarboxylase Induction

Ornithine decarboxylase (EC 4.1.1.17, ODC) has been shown to serve as a biochemical marker of tumor promotion. ODC is the initial and rate-limiting enzyme in the polyamine biosynthetic pathway. L-ornithine is decarboxylated by ODC to form putrescine, the precursor of the other naturally occurring polyamines, spermidine and spermine.

Following decarboxylation of ornithine, a transient rise in putrescine serves as a substrate for S-adenosyl-methionine decarboxylase (SAM) catalyzed spermidine biosynthesis by decarboxylation and propylamine transfer. Spermidine, in turn, undergoes a similar reaction to form spermine.

The naturally occuring polyamines have been shown to be intimately involved in growth regulation and cell proliferation (Russell and Durie 1978). ODC is induced by a variety of stimuli and has been implicated as the labile protein which stimulates RNA polymerase I activity (Russell and Durie 1978). Several hormones have been shown to induce ODC, including glucagon, insulin, growth hormone, choriogonadotropin and nerve and epidermal growth factors (Russell 1980). Trophic hormones are not the only substances known to induce ODC; while a large number of pharmacologic agents have the ability to induce ODC, of particular interest is the ODC response elicited by TPA administration.

O'Brien (1976) has shown that a single topical application of TPA results in a 250-fold increase of ODC activity in mouse skin within 4 hours. This increase is the result of de novo enzyme synthesis since cycloheximide, an inhibitor of protein synthesis, abolishes this effect (Goldfarb and Quigley 1978). Exposure to actinomycin D, an inhibitor of RNA synthesis, likewise inhibits ODC induction. Therefore, the ODC response elicited by TPA is dependent on both de novo protein and RNA synthesis ( O'Brien 1976, Russell and Durie 1978). Klimpel and associates (1979) have also shown this effect in vitro in cell culture systems. The time course for ODC induction by TPA in vitro in chick

embryo fibroblasts is biphasic, reaching a first peak by four hours, followed by a decline in ODC activity to a nadir at twelve hours and rising to a second peak by 24 hours.

O'Brien (1976), has shown in the mouse skin model that the magnitude of enzyme induction (ODC, SAM) correlates well with the promoting efficacy of graded doses of TPA and has suggested that induction of epidermal ODC is a phenotypic change essential for carcinogenesis in mouse skin. These and earlier data correlating induction of polyamine biosynthetic enzymes and concomitant increase in cellular concentrations of polyamines (O'Brien et al. 1975), lead to the hypothesis that induction of ODC is obligatory and always associated with tumor promotion.

In vitro, TPA elicited similar increases in polyamines in chick cells transformed by Rous sarcoma virus, and led Don and others (1975) to conclude that these occurrences were specifically associated with malignant transformation of these cells. Further evidence in support of this hypothesis is that TPA exposure results in DNA synthesis (Williams-Ashman et al. 1972), increases in cGMP (Voorhees et al. 1974), decreased cell volume (Griedger and Blumberg 1977), increased sugar transport and saturation density (Griedger and Blumberg 1977), stimulation of synthesis and phosphorylation of membrane phospholipids (Suss et al. 1971, Rohrschneider et al. 1972), noncompetitive inhibition of epidermal growth factor receptor binding (Lee and Weinstein 1978), elevated levels of sister chromatid exchanges in the absence of DNA damage (Kinsella and Radman 1978) and induction of elevated levels

of plasminogen activator (Troll 1976, Weinstein et al. 1977, Weinstein et al. 1978, Weinstein et al. 1979, Wigler and Weinstein 1976). All of these perturbations have also been found to occur in chick cells after transformation by oncogenic viruses (Weinstein et al. 1977).

## Plasminogen Activator Induction

Plasminogen activator, a family of serine proteases, is physiologically responsible for enzymatic conversion of the inactive zymogen, plasminogen, to the catalytically active form, plasmin, the major enzyme of the fibrinolytic cascade. Plasmin lyses a formed fibrin matrix, or "clot", into its component fibrin-split products. Under normal circumstances, the fibrinolytic and coagulation systems function in concert to maintain hemostasis.

Elevated levels of tissue plasminogen activator (PA) have been reported in a number of disease states, particularly those involving the hepatobiliary system (Putnam and Buckley 1981). Experimentally, administration of hepatotoxic agents such as ethanol (Buckley et al. 1982), galactosamine (Buckley and Putnam 1981) and tumor initiating and promoting agents (Unkeless et al. 1973, Ossowski et al. 1973a, Ossowski et al. 1973b, Katz et al. 1974, Pollack et al. 1974, Rifkin et al. 1974, Troll et al. 1975, Troll 1976, Wigler and Weinstein 1976, Weinstein et al. 1977, Weinstein et al. 1978) results in significant induction of PA compared to vehicle-only treated controls.

To date, investigations relating PA induction to tumor promotion have largely employed tissue culture techniques. In vitro, TPA

concentrations ranging from $10^{-8}$ to $10^{-10}$ molar, results in a PA induction that is detectable within one hour, plateaus at 24 - 48 hours and is reversed when the agent is removed from the medium (Weinstein et al. 1979). Like that of ODC, induction of PA is felt to be the result of de novo protein synthesis. Inhibitors of protein and RNA synthesis have been shown to abolish its induction (Weinstein et al. 1979). In Rous sarcoma virus transformed chick embryo fibroblasts, which synthesize plasminogen activator, exposure to phorbol esters further enhances PA synthesis. Studies with this system showed that induced PA synthesis required continuous expression of the sarcoma (SARC) gene of the Rous sarcoma virus (Weinstein et al. 1977, Weinstein et al. 1978). This observation suggests that there is an interaction between products of the SARC gene and the cellular events triggered by TPA (Weinstein et al. 1979).

That TPA induced proteolytic activity is related to tumor promotion in vivo was shown by Troll et al. (1970) in the mouse skin model. From this model and subsequent studies (Troll 1976) it was concluded that the contribution made by proteolytic activity was, in essence, de-repression of the genome by specific removal of a repressor protein from the chromatin (Troll 1976). This allows the transcriptional and translational events associated with de novo protein synthesis to proceed (Weinstein et al. 1972, Granelli-Piperno et al. 1977, Vassalli et al. 1977). Clinical and experimental data have shown that administration of protease inhibitors, specifically fibrinolytic inhibitory agents, results in either tumor encapsulation (Astedt et al.

1977) or diminished tumorigenicity (Troll et al. 1970, Corasanti et al. 1982). In addition hepatotoxin induced, morphologic alterations to liver cells were significantly reduced (Buckley and Putnam 1981).

## Plasminogen Activator and Ornithine Decarboxylase Induction: Is There a Correlation?

The literature dealing with ODC and PA induction by tumor promoting agents and phosphodiesterase inhibitors is quite exhaustive, with similarities abounding. Synthesis of both enzymes can be profoundly influenced by physiologic concentrations of hormones (Unkeless et al. 1973, Strickland and Beers 1976, Vassalli et al. 1976). The intracellular generation of PA (Toskutoff and Paul 1978) and ODC (Russell and Durie 1978) are both early $G_1$ events in the cell cycle. A close experimental relationship among tumorigenicity, PA and ODC inductions and phorbol ester dosage has been shown in vitro. Both enzyme inductions result from de novo enzyme synthesis. In vitro, chick embryo fibroblasts transformed by Rous sarcoma virus synthesize PA. That TPA treatment of these cultures further increases the level of PA activity was shown by Quigley (1979). The time course of this event and the TPA concentration required are quite similar to those found for ODC induction.

As mentioned above, studies concerning the induction of PA and ODC by phorbol esters have been primarily confined to mouse epidermis and to certain cultured cells. The induction of both PA and ODC have not been examined in the same system under the same protocol. In fact,

with the exception of Weiner and Byus (1980), few investigators have concerned themselves with the biochemical and cellular events that occur following systemic TPA administration. Weiner and Byus (1980) administered TPA intraperitoneally in rats and serially measured ODC levels in liver, lung and brain homogenates. In these experiments they found ODC activity increased 45-fold, 9-fold, and 8-fold in liver, brain and lung respectively. The time of maximal enzyme induction was 5 hours and ODC activity returned to control levels by 12 hours.

Since both enzymes are inducible by TPA and both are intimately involved in the mechanism of tumor promotion, an examination of the effects of systemic TPA administration on both ornithine decarboxylase activity and plasminogen activator activity was made under the same protocol to more precisely establish correlations suspected to exist between these two seemingly unrelated enzyme systems. A second group of experiments, aimed at examining what inherent proteolytic activity ODC might possess, were carried out in vitro. Briefly, ODC itself, as well as ODC primary-amine conjugates, were evaluated for urokinase-like plasminogen activator activity in the same assay system used for PA determinations.

# CHAPTER 2

# METHODS

## Materials

Male Buffalo (mean weight 400g) and Sprague-Dawley rats (mean weight 150g) were purchased from NCI (Frederick Cancer Research Center, Frederick, MD) and housed in wire cages in a controlled environment at 23°C with a 12 hour light/dark cycle. Food and water were available ad libitum. All injections were administered at 8:00 a.m. to minimize diurnal variations in enzyme activities.

Reagents were of analytical grade and obtained from J.T. Baker Chemical Co., Phillipsburg, NJ, except for the following: H-D-isoleucyl-L-prolyl-L-arginine-p-nitroanilide-dihydrochloride, S-2288, from Kabi, Stockholm, Sweden; dextran sulfate, 12-O-tetradecanoylphorbol-13-acetate, cycloheximide, actinomycin D, and E. coli ornithine decarboxylase from Sigma, St. Louis, MO.; flufenamic acid from Aldrich, Milwaukee, WI.; urokinase from Calbiochem, Lajolla, CA.; human fibrinogen from Merck Sharpe and Dohme, West Point, PA.; thrombin from Parke-Davis, Morris Plains, NJ; α-difluoromethylornithine and ornithine decarboxylase conjugated primary amines from Dr. Diane H. Russell, Department of Pharmacology, University of Arizona Health Sciences Center, Tucson, AZ.

## Solutions

EDTA buffer: 0.05 M sodium diethylbarbiturate, 0.10 M NaCl, 0.25 (w/v) gelatin and 2.7 mM ethylenediaminetetraacetate (EDTA) adjusted to pH 7.8 with HCl.

Sodium flufenamate: 0.5 M flufenamic acid in 0.5 M 60°C NaOH, diluted to 14 mM with EDTA buffer.

Dextran sulfate: In flufenamate solution, 175 mg/l (EDTA buffer containing 14 mM sodium flufenamate).

Phosphate buffer: 0.01 mM EDTA, 0.05 M each of $Na_2HPO_4$ and $KH_2PO_4$, pH adjusted to 7.5.

Tris buffer: 100 mM Tris, 106 mM NaCl, pH adjusted to 8.4 with HCl.

Barbital buffer: 0.05 M sodium barbital, 4.4 mM $CaCl_2$, 1.4 mM $MgCl_2$, 0.19 M NaCl, pH adjusted to 7.75 with HCl.

## Promoter and Metabolic Inhibitor Administration

In the first *in vivo* experiment, Buffalo rats received a single intraperitoneal TPA injection (200µg/rat). TPA was dissolved in 40% ethanol and 60%, 0.15 M NaCl. The volume injected never exceeded 0.1 ml. Animals were serially sacrificed by decapitation at 2, 4, 6, 8 and 12 hours post-TPA administration. The livers were rapidly excised, frozen and stored in liquid nitrogen vapor until enzyme analysis.

Spraque-Dawley rats were employed in the experiments involving metabolic inhibitors and in the second experiment targeted at

elucidating the temporal relationship between ODC and PA inductions. Cycloheximide (70 mg/kg dissolved in 0.9% NaCl) was administered intraperitoneally fifteen minutes prior to TPA (100 µg/rat). Dimethylsulfoxide 10% in distilled water was used as the vehicle for i.p. actinomycin D (6 mg/kg) administration fifteen minutes prior to TPA. These animals were than sacrificed at 4 hours, the livers excised and assayed for enzyme activities. Other animals were serially sacrificied at 2, 4, 6, 8, 12, 18 and 24 hours following TPA exposure by decapitation and the livers removed and stored in liquid nitrogen vapor until time of enzyme analysis. All results were compared to vehicle-only treated controls.

## Plasminogen Activator Activity

Direct Assay: In order to investigate the inducing capabilities of TPA on hepatic ODC and PA, a precise and reproducible enzyme assay for PA determination in tissue homogenates was necessary. Most available assays measure PA indirectly; that is, by its interaction with exogenous plasminogen added to the assay mixture. The radiolabeled fibrin plate (Johnson and Mansfield 1978) and caseinolytic method (Lewis et al. 1981) are two common, indirect assays. Both assays measure lysed products after plasmin generation from the interaction of plasminogen activator and plasminogen. Plasmin then catalytically acts on its respective substrate, either a formed fibrin matrix or casein. Radioactivity released in the area of lysis is then counted and employed to determine the quantity or activity of PA present in the sample.

A direct assay, measuring PA activity with a specific substrate is required. The synthetic substrate, H-D-isoleucyl-L-prolyl-L-arginine-p-nitroanilide-dihydrochloride, S-2288, Kabi$^{R}$, was chosen due to its neutral protease specificity (Rijken and Collen 1980). Several buffer systems were examined to determine the one best suited for liver homogenate preparation. PA activity was determined in homogenates utilizing pH 7.4 phosphate buffer, KSCN extraction and euglobulin fractionation with and without addition of sodium flufenamate and dextran sulfate (Kluft 1979). The various conditional perturbations examined to define the optimal assay conditions are listed in Table I.

TABLE 1

Various chemical perturbations examined in determining optimal sensitivity for PA analysis.

| | |
|---|---|
| **Homogenate buffer** | |
| 0.25 mol Sucrose | + |
| 0.2 mol Phosphate | ++++ |
| 100 mmol TRIS, pH 8.4 | +++ |
| 2.0 mol KSCN | ++ |
| 0.05 mol $Na_2HPO_4/KH_2PO_4$ | +++++ |
| 0.05 mol KCl | + |
| **Homogenate Centrifugation Speed** | |
| 9000 x g | ++ |
| 4000 x g | +++++ |
| 2000 x g | +++ |
| **Homogenate Preparation** | |
| Polytron | +++++ |
| Dounce | +++ |
| Shaking $H_2O$ bath | + |
| **Euglobulin Fractionation** | |
| pH 2.5 | + |
| pH 5.9 | +++++ |
| KSCN-$H_2O$ | +++ |
| KSCN-E.F. | + |
| E.F. + dextran sulfate and sodium flufenamate | +++++ |
| 1:2 dilution | ++ |
| 1:10 dilution | +++++ |
| **Substrates** | |
| S-2288 | +++++ |
| S-2251 + plasminogen | ++ |
| S-2251 - plasminogen | + |
| **Assay Conditions** | |
| Kinetic - 5 minutes | |
| 0.175 mmol S-2288 | +++++ |
| Incubation - 30 minutes | |
| 1.0 mmol S-2288 | +++++ |
| 0.5 mmol S-2288 | +++++ |
| Turbidity blanks | |
| urokinase standard | +++++ |

Conditions were graded (+) according to their effect in eliciting optimal urokinase-like PA activity.

In vitro analysis of amine-conjugated ODC was carried out utilizing both the fibrin plate and direct spectrophotometric techniques. The scheme for preparation of the dextran sulfate, euglobulin fraction, determined to be the most sensitive technique for PA analysis, is shown in Figure 1. A 1:10 dilution of the sample (tissue homogenate) was made by the serial addition of 8 ml cold distilled water and 1 ml dextran sulfate solution to 1 ml of sample. Solutions were cooled in an ice bath and, after mixing, the pH was titrated to 5.9 with acetic acid. Following cold centrifugation, the precipitate was redissolved in 4°C Tris buffer.

A 600 µl sample aliquot was then added to 600 µl Tris buffer. To this, 600 µl of 0.5 mM S-2288 was added and the assay mixture incubated at 37°C for 30 minutes. Following incubation, 100 µl 50% acetic acid was added to terminate the reaction.

PA activity was determined by measuring at 405 nm the absorbance of p-nitroaniline (pNA) generated by the breakage of the arginine-pNA bond of S-2288. A calibration curve was prepared using urokinase as the standard and the results expressed in urokinase (Ploug) units (Figure 2).

Fibrin Plate-Indirect Assay: With this method, PA activity is determined in a semi-quantitative fashion. Human fibrinogen (containing plasminogen) was dissolved in barbital buffer to a final concentration of 3mg/ml. A 10 ml aliquot of fibrinogen solution was then pipetted into a 100x15 mm petri dish. A fibrin matrix was precipitated

PLASMINOGEN ACTIVATOR ANALYSIS

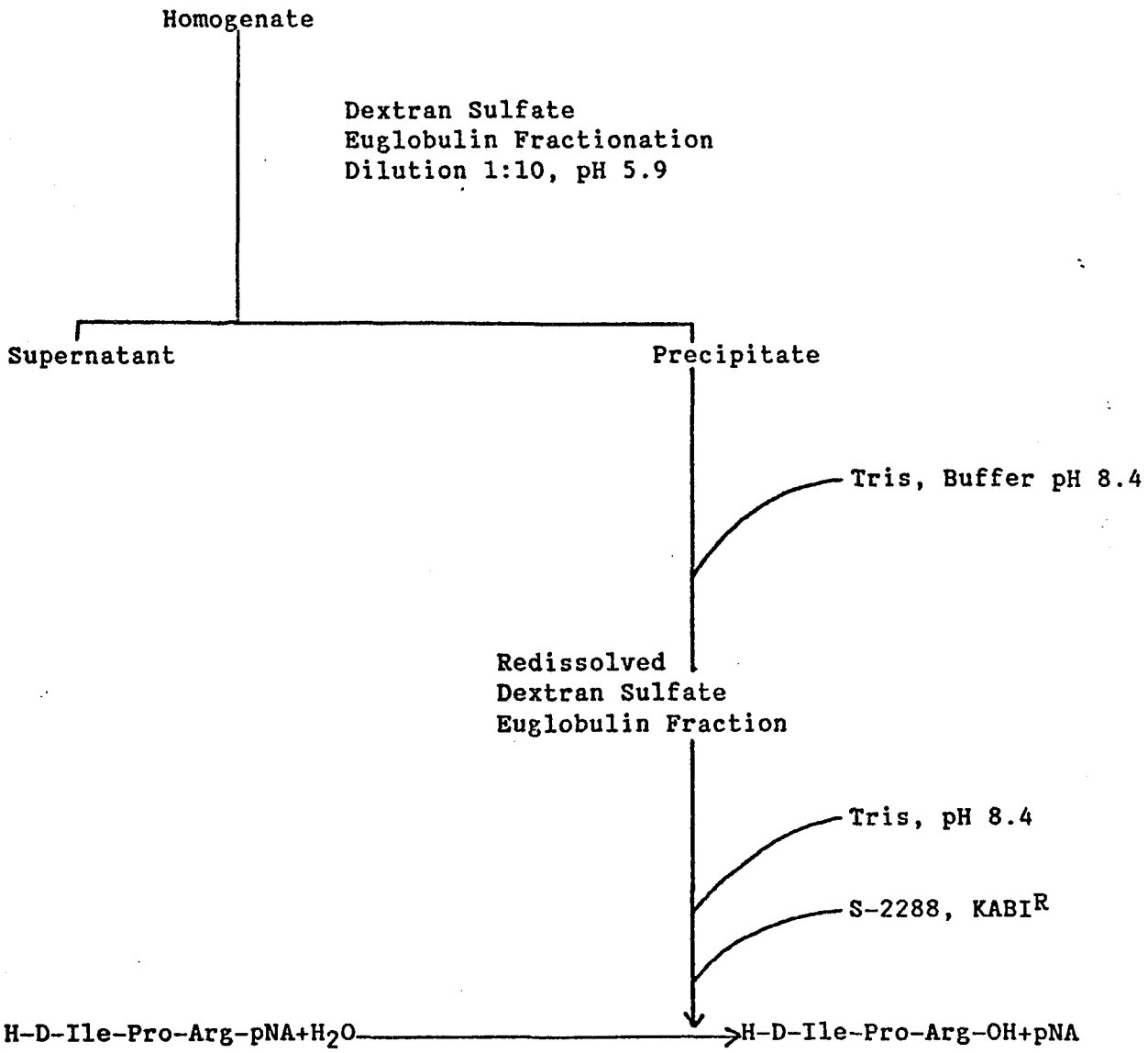


FIGURE 1. Schematic diagram of direct colorimetric PA assay. Amount of pNA generated at 405 nm. is measured following 30 minutes of incubation.

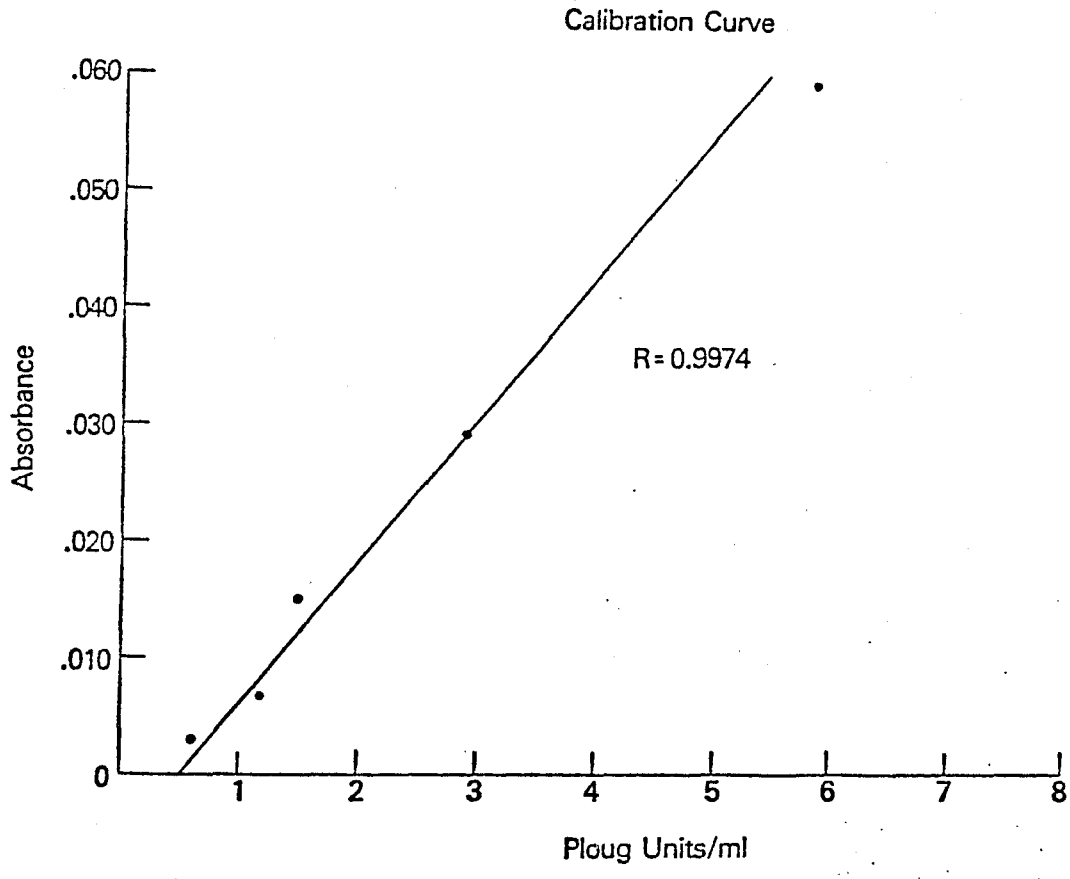


Figure 2. Calibration Curve for Direct measurement of PA Activity. Serial dilutions of urokinase were added to 600 µl of 0.5mM S-2288 and incubated at 37°C for 30 minutes. The amount of pNA liberated was measared spectrophotometrically at 405 nm.

by the addition of 100 µl (6.6 units) bovine thrombin. A 2 x 7 mm punch biopsy of tissue was then placed in the center of the fibrin-layered petri dish and incubated at 37°C in a humidified atmosphere for 24 hours. Following incubation, 5 ml of 1% trichloroacetic acid was added to terminate the reaction. The tissue sample was removed and the area of lysed fibrin determined with an electronic planimeter and expressed in square millimeters.

The technique utilized to determine PA activity in tissue homogenates and ODC-primary amine conjugates was the same as mentioned above with the following changes. A 5mm or 6mm well was cut into the fibrin-layered petri dish and aspirated free of fibrin. A 5 to 25 µl sample aliquot was pipetted into the well, and the plate incubated as indicated. Following the addition of trichloracetic acid, the total area of lysis was measured by electronic planimetry and the area of the well subtracted from the total lytic area. The resulting area of lysis was expressed as $mm^2$. A comparison of the direct, colorimetric assay versus the fibrin plate assay technique is shown in Figure 3. Briefly, calibration curves were prepared for the two techniques using urokinase as the standard. Note that the direct assay is linear with regard to urokinase concentration in contrast to the fibrin plate method.

## Ornithine Decarboxylase Determination

ODC was assayed according to the technique of Russell and Snyder (1968). Livers obtained at sacrifice were bisected and immediately frozen and stored in liquid nitrogen until time of enzyme analysis.

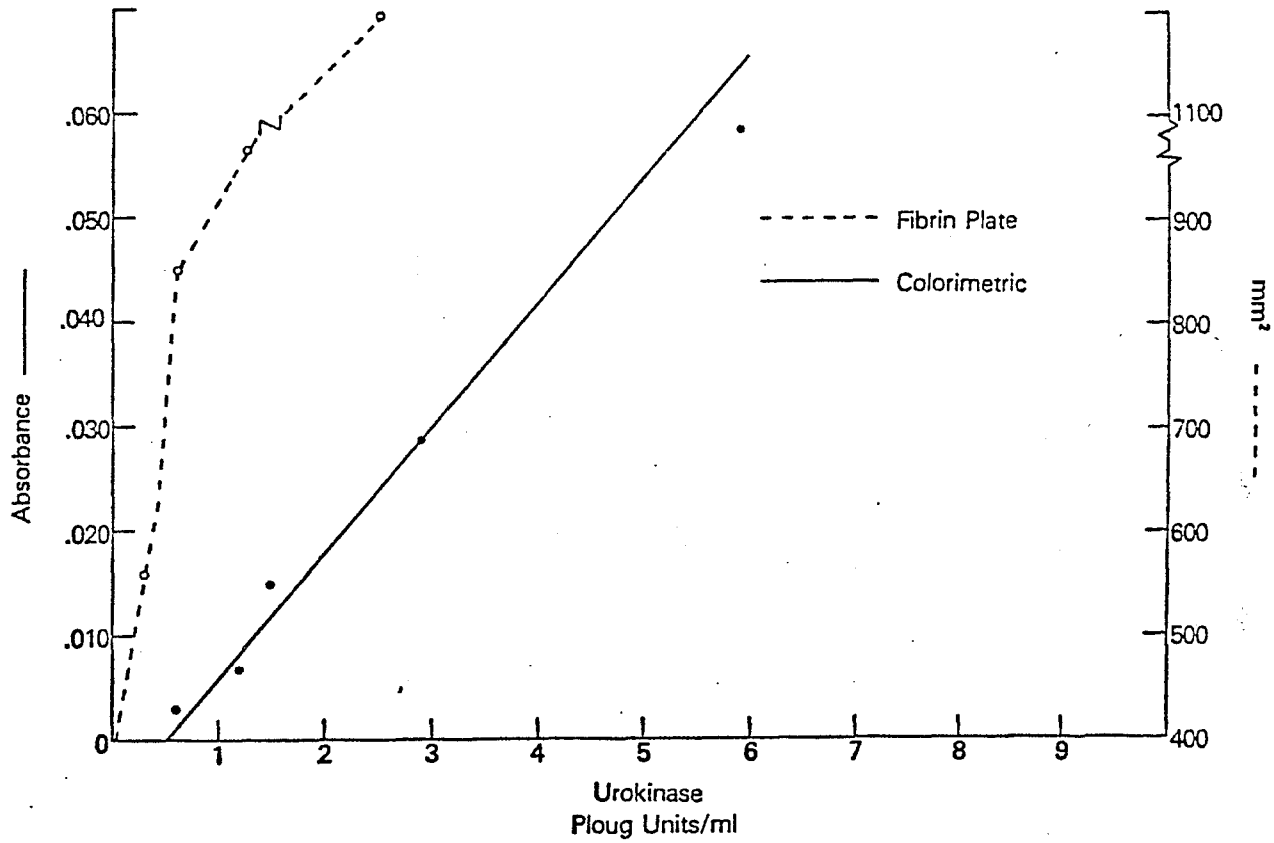


**Figure 3.** Comparison of Colorimetric and Fibrin Plate Methods for the Determination of PA Activity. Calibration curves for the direct colorimetric and fibrin plate techniques determination of PA activity were prepared using urokinase as a standard.

For the ODC determination, liver sections were rapidly thawed and homogenized in five volumes of ice cold buffer consisting of 50 mM potassium phosphate, 50 mM sodium phosphate, 0.1 mM EDTA, pH adjusted to 7.5. The following reagents were added to 50 ml buffer immediately prior to homogenate preparation: 1.0 ml of 100 mM dithiothreitol; 300 µl of 2 mM pyridoxal phosphate and 100 µl of 20 mmol phenylmethylsulfonylfluoride. The homogenates were centrifugated at 12,000 x g for 10 minutes and the resulting supernatant assayed for ODC activity. All enzyme analysis was done in duplicate and, in some cases, in triplicate. Ornithine decarboxylase activity was determined as the amount of $^{14}CO_2$ released from 0.5 µCi of L-1-$^{14}$C-ornithine (47.2 mCi/mmol New England Nuclear) in 130 µl buffer and 50 µl homogenate in 30 minutes. The assay was performed in conical centrifuge tubes fitted with stoppers containing center wells (Kontes Glass Co.). $^{14}CO_2$ was trapped on 2.3 cm filter paper (Whatman Number 3) that had been treated with 20 µl of 2N NaOH. Following injection of 0.50 ml of 1.0 M citric acid to the mixture after 30 minutes incubation, the samples were allowed to stand an additional 30 minutes at 37°C. The stoppers were removed and the filter papers were counted in 6.0 ml of toluene scintillation fluid using a Beckman liquid scintillation counter.

Protein Determination: Total protein content of the homogenates was determined utilizing a micro-technique which is a modification of the Biuret and Lowry methods (Ohnishi and Barr 1978), using bovine serum albumin as a standard.

# CHAPTER 3

# RESULTS

## Induction of Hepatic ODC and PA by TPA

In the first *in vivo* experiment, mature male Buffalo rats (350-500g) received a single intraperitoneal injection of TPA (200 µg/rat). The animals were serially sacrificed, livers excised and assayed for PA and ODC activities. As shown in Figure 4, administration of TPA led to a rapid increase in both hepatic PA and ODC. PA activity was induced to a level significantly ($p < .01$) over control at 6 hours, fell at 8 hours and appeared to climb again at 12 hours. ODC activity rapidly increased to significant levels ($p < .001$) by 2 hours, peaked at eight hours ($p < .001$) and fell to a level still significantly above control ($p < .05$) at 12 hours following TPA administration.

To further determine the biochemical relationship between hepatic PA and ODC *in vivo* and to ascertain whether the biphasic response in PA activity suggested by the rebound in enzyme activity at 12 hours in the above experiment was a physiologic event, a strain variation, or an experimental artifact, a second group of rats (Spraque-Dawley, mean weight 150 g) received 100 µg/rat TPA intraperitoneally. The animals were then serially sacrificed by decapitation over a 24

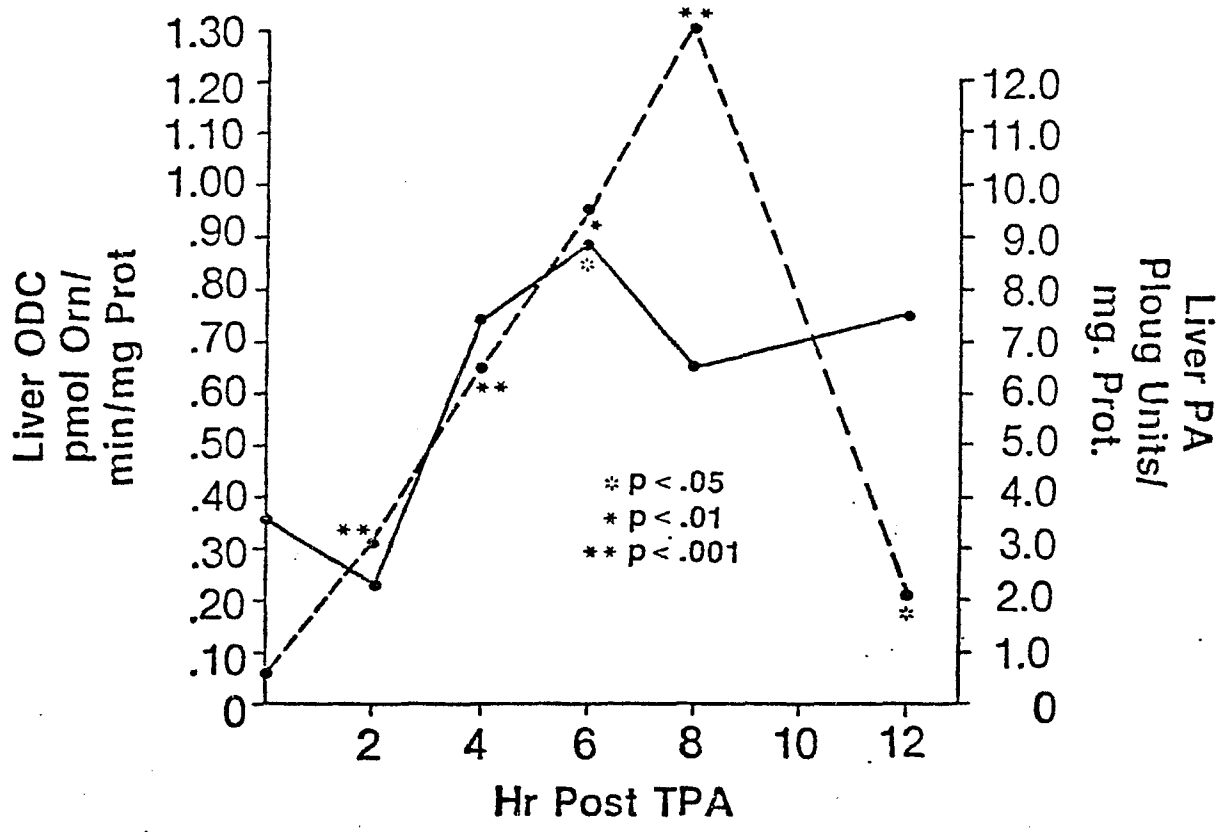


Figure 4. Temporal Relationship between ODC and PA Inductions in Buffalo Rats. Buffalo rats received 200 µg/rat i.p., TPA. The animals were serially sacrificed, livers excised and assayed for ODC and PA activities.

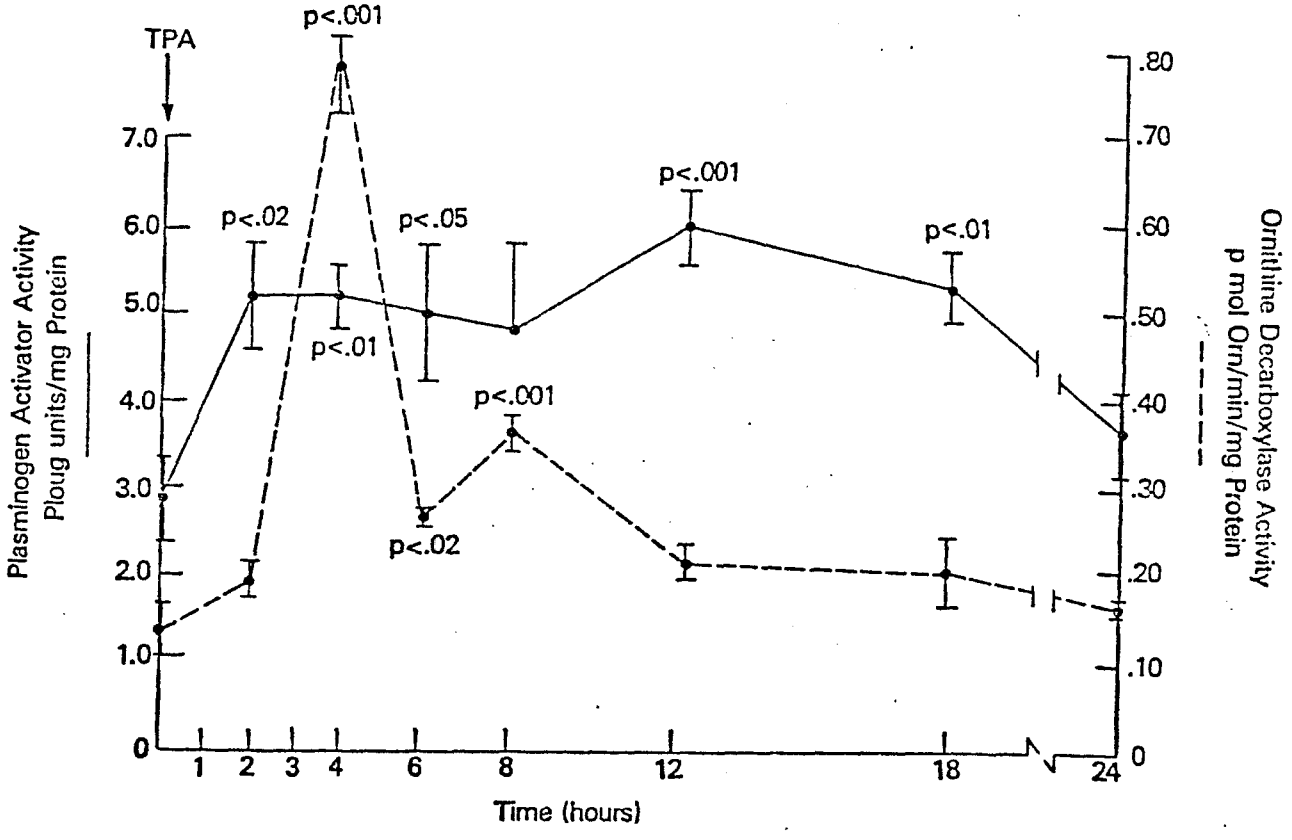


**Figure 5.** Temporal Relationship Between ODC and PA Inductions in Sprague-Dawley Rats. Rats received TPA, 100 µg/rat i.p. The animals were serially sacrificed, livers excised and assayed for ODC and PA activities.

hour period, livers excised and assayed for enzyme activities. Again, both enzymes in the liver were rapidly induced (Figure 5). PA activity exhibited a biphasic induction, reaching significantly elevated levels at two hours ($p < .02$), fell to a nadir at eight hours then climbed to a second peak at 12 hours ($p < .001$). PA activity remained elevated through 18 hours, then gradually declined to control levels by 24 hours. ODC activity was elevated at two hours ($p < .001$) and peaked at four hours ($p < .001$). ODC activity remained elevated through eight hours and then fell to control values at 12 hours where it remained.

It has been previously shown *in vitro* that induction of both PA and ODC activity elicited by TPA require *de novo* protein and RNA synthesis. To determine if this phenomena also occurs in the liver as the result of TPA exposure, another experiment was performed. Male Sprague-Dawley rats were pretreated with an intraperitoneal injection of cycloheximide (70 mg/kg) or actinomycin D (6 mg/kg), protein and RNA synthesis inhibitors, respectively, fifteen minutes prior to intraperitoneal TPA. The TPA dose was identical to that previously established to elicit PA and ODC inductions. The animals were then sacrificed and compared to TPA and vehicle-only treated controls. Induction of hepatic PA activity after a single systemic dose of TPA was obliterated by both metabolic inhibitors (Figure 6) at four hours. The peak ODC activity at four hours was also blocked by metabolic inhibitor administration (Figure 7). These data are consistent with the previously described requirement for *de novo* protein and RNA synthesis *in vitro* for both

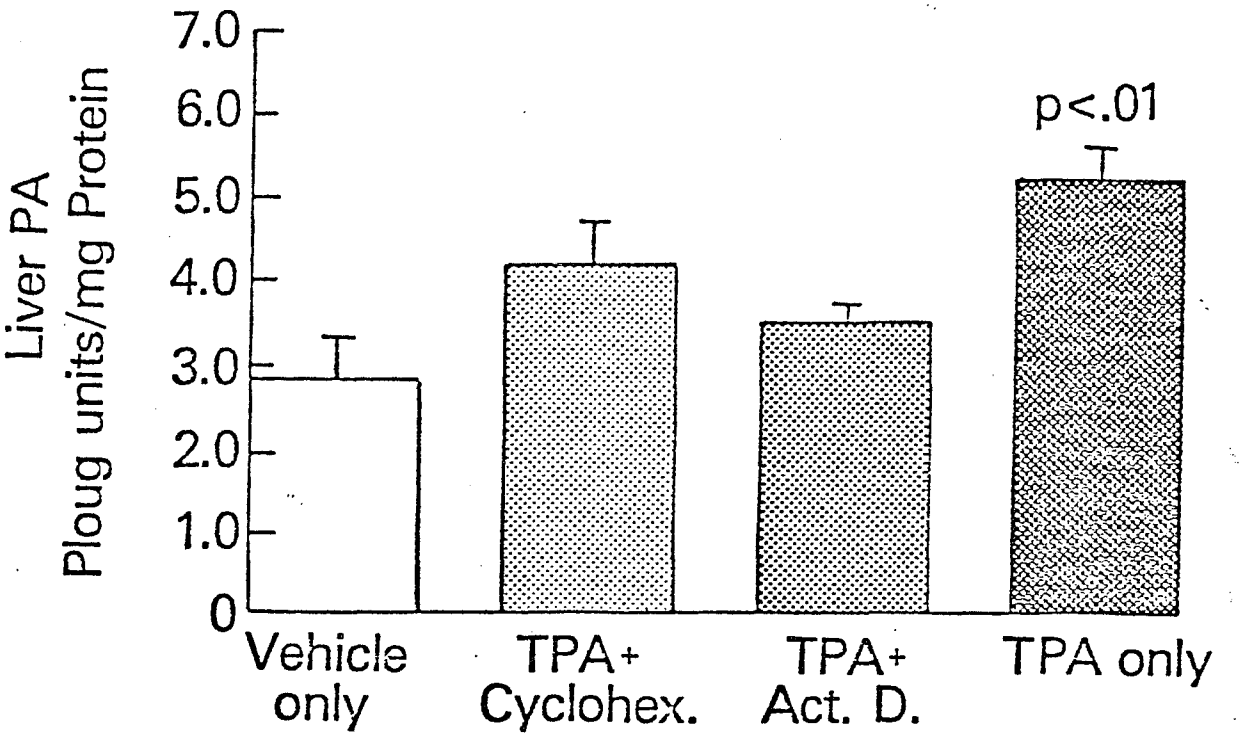


Figure 6. Effect of Metabolic Inhibitors on PA Induction by TPA. Cycloheximide (70 mg/kg) or Actinomycin D (6 mg/kg) were administered 15 minutes prior to TPA (100 µg/rat) dosing. Livers were excised and assayed for PA activity at 4 hours following TPA.

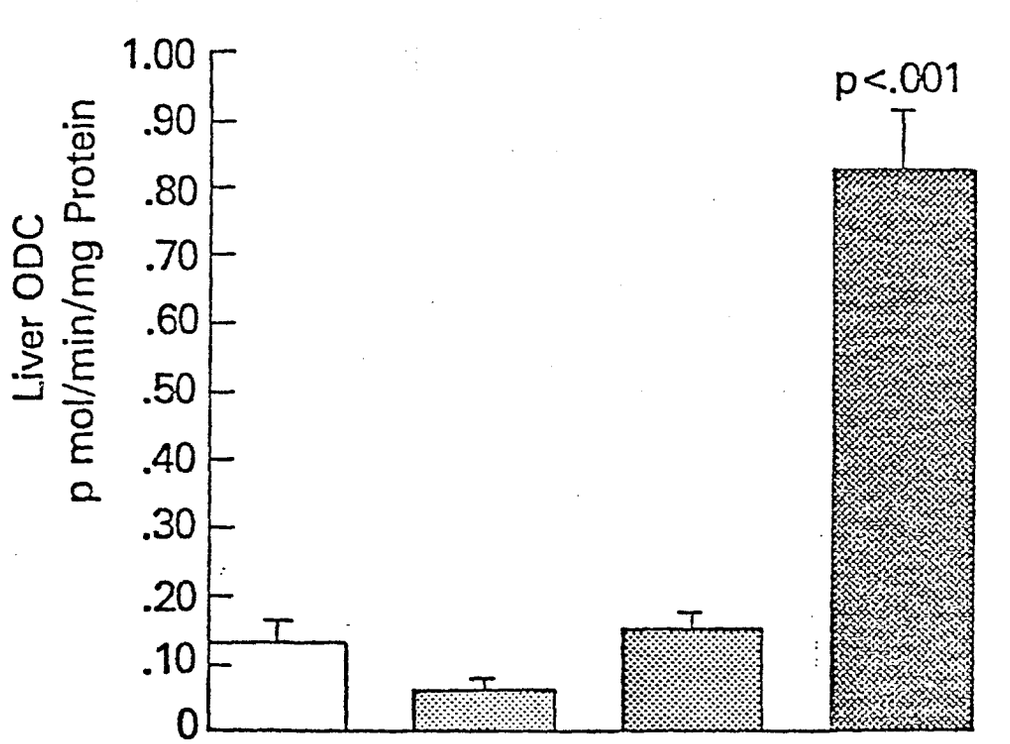


Figure 7. Effect of Metabolic Inhibitors on ODC Induction by TPA. Cycloheximide (70 mg/kg) or Actinomycin D (6 mg/kg) were administered 15 minutes prior to TPA (100 µg/rat) dosing. Livers were excised and assayed for ODC activity at 4 hours following TPA.

enzyme inductions to occur, suggesting that the requirement for de novo synthesis of these enzymes is a physiologic event following systemic exposure to TPA.

## PA-Like Activity of ODC-Primary Amine Conjugates

The literature dealing separately with the induction of the enzymes PA and ODC by tumor promoting and other agents is voluminous. There are many similarities between these two seemingly unrelated enzyme systems. As already mentioned, both enzymes are strongly influenced by physiologic concentrations of hormones and induction of PA and ODC have both been shown to be early $G_1$ events in the cell cycle. Both inductions require de novo enzyme synthesis in vitro and in vivo (Buckley et al. 1983)and a strong experimental relationship between the magnitude of PA and ODC inductions and tumor promoter dose has been shown in vitro. With these and other similarities in mind, we wondered if ODC itself or ODC-primary amine conjugates, suspected to occur physiologically, possess PA-like activity.

Putrescine and spermidine were conjugated in vitro via a γ-glutamyl linkage to partially purified ODC by the calcium dependent enzyme, transglutaminase. Once conjugated, the sample mileau was assayed for urokinase-like PA activity by the fibrin plate and colorimetric methods.

The first experiment (Table II) involved determination of PA activity of the amine-conjugated ODC by the fibrin plate method. The ODC-putrescine conjugate was found to possess about six times the PA activity of the ODC-spermidine conjugate.

After the direct spectrophotometric technique (Buckley and Putnam 1983) utilizing a synthetic tripeptide substrate was developed, further experiments were carried out to determine PA activity of other ODC-primary amine conjugates. Using this technique, the urokinase-like PA activity of ODC-putrescine and ODC-histamine conjugates was measured (Table III). Samples of nonconjugated, ODC containing, free putrescine solutions were also assayed and served as "controls". ODC-histamine was found to possess approximately the same PA activity as the ODC-non-conjugated "controls". A 50 microliter aliquot of the ODC-putrescine conjugate was again found to possess substantial urokinase-like PA activity in this new assay system.

Finally, ODC from E. coli (Sigma$^{R}$) was assayed for its inherent PA activity, by itself and in the presence of specific enzyme inhibitors. As shown in Table IV, 200µl of the bacterial ODC (0.3mg/ml) had 0.70 Ploug units of urokinase-like PA activity. When the same quantity of the bacterially generated ODC was examined in the presence of alpha-difluoromethylornithine (DFMO) there was a 54% reduction in PA activity. DFMO is a known suicide substrate that involves covalent alteration of ODC, and is therefore an irreversible inhibitor of the enzyme. When the glycoprotein, alpha$_1$-antitrypsin ($\alpha_1$AT), an endogenous physiologically active fibrinolytic inhibitor was assayed in the presence of E. coli ODC, again there was a reduction in PA activity. Human $\alpha_1$AT at the concentration of 0.1 mg/ml reduced the urokinase-like PA activity of ODC by 29%.

Table 2

*In vitro* determination of amine conjugated ODC for PA activity.

| ODC-Conjugate | Concentration | Volume Assayed | PA Activity |
|---|---|---|---|
| ODC-putrescine | 7.2 mg/ml | 10 µl | 36.9 $mm^2$ |
| ODC-spermidine | 7.2 mg/ml | 10 l | 6.0 mm |

PA activity was determined by the fibrin plate method. All values represent the mean value of assays performed in triplicate.

TABLE 3

*In vitro* determination of amine conjugated and nonconjugated ODC for PA activity.

| ODC-Conjugate | Concentration | Volume Assayed | Total PA Activity |
|---|---|---|---|
| ODC-putrescine | 1.0 nmol | 50 µl | 4.0 Ploug Units |
| ODC-histamine | 0.5 nmol | 100 µl | 2.0 Ploug Units |
| ODC-nonconjugated putrescine | 1.0 nmol | 50 µl | 2.1 Ploug Units |

PA activity was determined by the direct colorimetric method. All values represent the mean value of assays performed in triplicate.

TABLE 4

*In vitro* determination of urokinase-like PA activity of ODC in the presence of specific enzyme inhibitors.

| Inhibitor | ODC Concentration | Inhibitor Concentration | Volume Assayed | Total PA Activity |
|---|---|---|---|---|
| Tris buffer | 0.3mg/ml | 100 mM | 200 µl | 0.70 Ploug Units |
| DFMO | 0.3mg/ml | 0.002 mg/ml | 200 µl | 0.32 Ploug Units |
| alpha-1-antitrypsin | 0.3mg/ml | 0.1 mg/ml | 200 l | 0.50 Ploug Units |

PA activity was measured by the direct colorimetric technique. All values represent the mean value of assays performed in triplicate.

# CHAPTER 4

## DISCUSSION

Chemical carcinogenesis is now generally considered to be a multistage process (Figure 8). The first step, initiation, results from the interaction of an initiating agent and normal cells. Initiated cells can not be morphologically differentiated from normal cells. In the presence of a promoting agent, the second stage, they can develop into neoplastic cells that then replicate and eventually develop into gross tumors. The process of promotion first appears to be a single step but is actually a multifactorial process requiring prolonged exposure to the promoting agent.

The mechanism by which a chemical can function as an initiator usually involves an electrophilic interaction between the carcinogen and cellular macromolecular constituents. Direct alkylating and acylating carcinogens are capable of interacting directly with their target cells; most other chemical carcinogens must be metabolically "bioactivated" *in vivo* to "ultimate carcinogens" before they are capable of tumor initiation (Miller 1978). Once activated to a strongly electrophilic reactant, they are then capable of combining chemically with nucleophilic sites on cellular macromolecules in target cells, i.e., DNA, RNA and proteins (Miller 1970, Miller 1978). These electrophiles

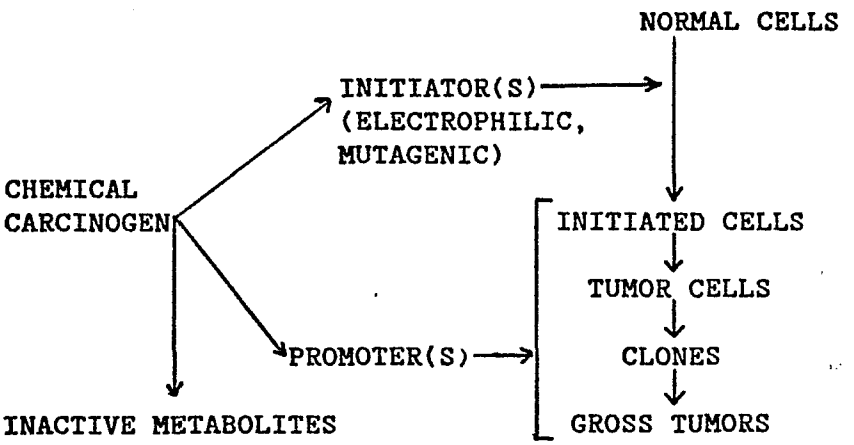


Figure 8. Simplified scheme for induction of cancer by a chemical. (Slaga, T.J., Sivak, A., Boutwell, R.K., eds. Mechanisms of Tumor Promotion and Cocarcinogenesis. New York: Raven Press, 1978.)

form non-enzymatic covalent bonds through electron sharing with the nucleophilic macromolecules. The processes concerned with tumor initiation are primarily genetic in nature, resulting in the formation of DNA-carcinogen adducts. The interaction of the carcinogen with cellular genetic molecules is irreversible and rapid. This process results in altered bases in DNA and the fixation of base-pairing errors during replication. These factors appear to be critical to the process of initiation (O'Connor et al. 1979). Although some cancer cells may arise from epigenetic mechanisms (Pierce 1970, Markert 1978, Mintz 1978), most tumors are initiated through alterations in the informational content of DNA (Miller and Miller 1981).

In contrast to initiation, the process of tumor promotion is less well understood (Miller 1978). Promotion occurs over a prolonged period of time and is reversible. TPA, for example, requires no metabolic activation to function as a potent tumor promoter (Berry et al. 1978). Also in contrast to the initiating agents, neither TPA itself nor its metabolites are electrophiles (Berry et al. 1978). Phorbol esters are non-mutagenic and do not bind covalently to DNA, RNA or protein (Hecker 1977, Soper and Evans 1977). Instead, TPA exposure results in a variety of responses both in cell culture and in epidermal cells <u>in vivo</u>. In mouse skin, application of TPA is followed by rapid increase in phospholipid synthesis, RNA synthesis and prostaglandin synthesis (Boutwell 1974, Fürstenberg and Marks 1978, Colburn 1980). In addition, TPA and other phorbol esters cause increases in certain enzymes, alterations in the degree of terminal differentiation, aberrations in growth

properties and membrane modifications (Troll 1976, Van Duuren 1976, Weinstein et al. 1979, Colburn 1980).

The primary target for TPA interaction may well be the cell plasma membrane. Horowitz and Weinstein (1982) have shown that phorbol esters manifest their biological action by first interacting with a specific "phorboid" receptor located on the cellular plasma membrane. These receptors function in a manner consistant with present knowledge of other receptor-ligand interactions; ie, they display high ligand affinity, the binding constants of ligand analogues show positive correlations with biological effects, ligand binding is reversible and exhibits a partial receptor down-regulation in the presence of continued ligand exposure (Horowitz and Weinstein 1982).

As mentioned above, one of the physiological responses elicited by phorbol ester exposure is the induction of several enzymes associated with seemingly unrelated biochemical systems. Both in vivo and in vitro, TPA has been shown to induce ornithine decarboxylase, plasminogen activator and transglutaminase. In the present study we examined the effects of a single systemic dose of TPA, on rat hepatic ODC and PA in two rat srtains. We found both hepatic enzymes rapidly induced to levels significantly elevated above controls in both rat strains following intraperitoneal TPA administration. The activities of both enzymes appeared to exhibit a biphasic response. PA activity rose to a first peak at 2 hours and a second at 12 hours before falling to control values at 24 hours. In a similiar manner, ODC climbed to a first peak of enzyme activity at 4 hours, a second at 8 hours and then

declined to baseline by 24 hours. Both enzyme inductions were completely abolished when animals were pretreated with cycloheximide or actinomycin D, indicating de novo protein and RNA biosynthesis to be a prerequisite for PA and ODC inductions to occur. This is consistent with data from cell culture systems and the mouse skin model. That these same phenomena occur when TPA is administered systemically is of particular note and may shed light on tumor promoting mechanisms in general.

The literature dealing with the biological responses elicited by tumor promoting agents and, specifically, the phorbol esters, is exhaustive. An occurance common in almost all studies reported is either inducton of ODC or PA. These two seemingly unrelated enzyme systems are capable of similiar responses to a variety of chemical agents, including physiologically occuring growth factors, hormones and exogenous carcinogenic or tumor promoting agents. There is a strong correlation in vitro between tumorigenicity, PA and ODC induction and tumor promoting dose. In light of these and other similarities, we wondered if the inductions of ODC and PA were actually the induction of a single protein that may be post-translationally modified, such that once chemically altered, the enzyme loses some or all its original substrate specificity and catalytic action and begins to function biochemically as a new and different enzyme. This modified enzyme would then function in a manner biochemically distinct from its original action. This conservation of biomolecules makes theoretical sense, suggests an alternative mechanism for enzyme modulation and is an

attractive explanation for the ubiquitous nature of PA and ODC activity-inductions following exposure to growth-promoting stimuli.

For this hypothesis to have validity a number of criteria must be met. First, the original enzyme must be capable of induction to a sufficient concentration at the appropriate time and intracellular location. Secondly, a mechanism must be present to biochemically modify the induced enzyme. Third, a technique to measure either the modifiying process or the resulting product is necessary.

Another enzyme capable of induction by TPA (Yuspa et al. 1980) and other growth-promoting stimuli (Scott et al. 1982) is transglutaminase. Transglutaminases (R-glutaminyl-peptide: amine-glutamyltransferase, EC 2.3.2.13, TGase) are a group of $Ca^{2+}$ dependent enzymes present both intracellularly and in extracellular fluids. They function biochemically by catalyzing formation of isopeptide linkages between glutamine residues contained within certain proteins and various substances containing primary amine moities. The naturally occuring polyamines, putrescine, spermidine and spermine all possess primary amine functional groups as part of their structure. That polyamines may serve as substrates for TGase <u>in vitro</u> has been well established (Clarke 1959, Williams-Ashman et al. 1977, Folk et al. 1980). That polyamines are physiological substrates for TGase has been implicated by Folk et al. (1980), Williams-Ashman et al. (1980) and Russell (1981). Russell and Haddox (1980,1981) found concomittant, increased concentrations of putrescine, spermidine and spermine conjugates and increased transglutaminase activity in rat liver nuclei following

partial hepatectomy. These conjugates required peptide bond hydolysis to release the parent compounds, indicating covalent binding of the polyamines to nuclear protein. Increases in all three polyamine derivitives were noted but when expressed as a function of nuclear protein, only the putrescine conjugate was significantly elevated. This led the authors to conclude that the increases in spermidine and spermine conjugates were a consequence of the cell doubling process, while the increases noted in the putrescine conjugate may indicate a regulatory role in the proliferative process. The temporal relationships of increased polyamine conjugates and transglutaminase, ODC induction, RNA and DNA synthesis indicated that the increases in putrescine conjugation to nuclear protein during liver regeneration is a component of the $G_1$ phase of the proliferative process.

Russell (1981) demonstrated that TGase catalyzes a post-translational conjugation of putrescine to ODC in vitro. Putrescine transamidation of purified ODC by guinea pig liver TGase resulted in a linear decrease in ODC activity. In another study, Scott and Russell (1982), examining the activation of TGase in Chinese hamster ovary cells, found the tight temporal correlation between TGase and ODC expression to be regulated by separate hormonal pathways. The authors concluded that through separate regulatory mechanisms, generation and accumulation of several polyamines may be more precisely controlled. They also speculated that the post-translational transamidation of ODC by its product, putrescine and the resulting decrease in ODC activity to be a mechanism by which ODC activity is inhibited following administration of

putrescine. This is in contrast to the ODC "antizyme" induction by putrescine hypothesized by others (Branca and Herbst 1980). Further, they suggested that this biochemically modified ODC may be the labile protein responsible for modulation of RNA polymerase I.

Yet another role for the post-translationally modified, ODC-putrescine conjugate may be one of proteolysis. As previously mentioned, exposure of mouse skin to micromolar amounts of TPA results in a 250-fold increase in ODC activity (O'Brien 1976). This vastly elevated above baseline enzyme level could provide sufficient substrate for TGase-catalyzed transamidation of ODC by primary amines. The decline in ODC activity 12 hours after systemically administered TPA is temporally co-incident with a second peak in plasminogen activator activity (Figure 5).

To examine this question, we evaluated the proteolytic activity of commercially available bacterial ODC and putrescine, spermidine and histamine transamidated ODC. E. coli ODC was found to chemically cleave the arginine-p-nitroaniline (Table IV) bond of S-2288, (Kabi, Stockholm Sweden), a substrate specific for proteolytic enzymes. When DFMO, an irreversible inhibitor of ODC, was added to the incubation mixture a decrease in the amount of proteolytic activity was noted. When $\alpha_1$-antitrypsin, a physiologically present protease inhibitor, was incubated with bacterial ODC, again a decrease in proteolytic activity resulted.

Using the semi-quantitative fibrin plate technique to measure fibrinolysis, ODC-putrescine and ODC-spermidine derivitives lysed the

formed fibrin matrix in a manner similiar to urokinase (Table II). The putrescine-ODC moiety was the most reactive with fibrin substrate. When the ODC-primary amine conjugates were assayed for proteolytic activity by the more sensitive colorimetric assay (Buckley and Putnam 1983), the ODC-putrescine compound was most active (Table III).

These data, taken together with observations from the literature, although highly correlative in nature, suggest the possibility that the plasminogen activator induction exhibited by virally transformed or tumor-promoter exposed cells both in vitro and in vivo may be the result of post-translational transamidation of ornithine decarboxylase by its reaction product, putrescine. That this modification may have a role in modulating synthesis of ribosomal RNA has been suggested by others (Scott and Russell 1982). We speculate that an additional role of proteolysis, having implications in cellular proliferation and neoplastic invasiveness, may also be served by this biochemical process.

## LIST OF REFERENCES


Astedt, B., Glifberg, I., Mattsson, W. et al, JAMA, 238, 154 (1977).

Berenblum, I., Cancer Res., 1, 44 (1941).

Berenblum, I., Prog. Exp. Tumor Res., 11, 21 (1969)

Berry, D.L., Bracken, W.M., Fisher, S.M., et al, Cancer Res., 38, 2301 (1978)

Boutwell, R.K., Crit., Rev. Toxicol., 2, 419 (1974).

Boutwell, R.K., O'Brien, T.G., Verma, A.K., et al Naturally Occurring Carcinogens - Mutagens and Modulators of Carcinogensis Baltimore, Univ. Park Press, 287 (1979).

Branca, A.A., Herbst, E.J., Biochem. J., 186, 925 (1980).

Buckley, A.R., Jr., Harrow, E.J., Putnam, C.W., Fed. Proc., 41, 923 (1982).

Buckley, A.R., Jr., Putnam, C.W., J. Colo. Wy. Acad. Sci, 8, 58 (1981).

Buckley, A.R., Putman, C.W., Russell, D.H., Toxicol. Letters 18,163 (1983).

Buckley, A.R., Putnam, C.W., Proceed. West. Pharm. Soc., 26, 73 (1983).

Clark, D.D., Mycek, M.J., Neidle, A., et al, Arch. Biochem. Biophys., 79, 338 (1959).

Cohen, R., Pacifici, M., Rubinstein, N., et al, Nature, 266, 538 (1977)

Colburn, N.H., Modifiers of Chemical Carcinogenesis, New York, Raven Press, 33 (1980).

Corasanti, J.G., Hobika, M.H., Markus, G., Science, 216, 1020 (1982).

Diamond, L., O'Brien, T.G., Rovera, G., Carcinogenesis Vol. 2 Mechanisms of Tumor Promotion and Cocarcinogenesis, New York, Raven Press, 173 (1978).

Don, S., Wiener, H., Bachrach, U., Cancer Res., 35, 194 (1975).

Folk, J.E., Park, M.H., Chung, S.I., et al, J. Biol. Chem., 255, 3695 (1980).

Furstenberg, G., Marks, F., Biochem. Biophys. Res. Commun. 84, 1103 (1978).

Goldfarb, R.H., Quigley, J.P., Cancer Res., 38, 4601 (1978)

Granelli-Piperno, A., Vassalli, J.D., Reich, E., J. Exp. Med., 146, 1693 (1977).

Griedgem, P.E., Blumberg, P.M., Cancer Res., 37, 3257 (1977).

Haddox, M.K., Russell, D.H., Fed. Proc., 39, 643 (1980).

Haddox, M.K., Russell, D.H., Proc. Natl. Acad. Sci. USA, 78, 1712 (1981).

Hecker, E., Cancer Res., 28, 2338 (1968)

Hecker, E., Mechan, Tumor Promot. and Co-carcinogen., New York, Raven Press, 11 (1977).

Hirakawa, T., Kakunaga, T., Fujiki, H., et al, Science, 216, 527 (1982).

Horowitz, A.D., Weinstein, I.B., Prostaglandins and Cancer: First International Conf., New York, Alan R. Liss, 217 (1982).

Ishii, D.N., Fibach, E., Yamosaki, H., et al, Science, 200, 556 (1978).

Johnson, R.H. and Mansfield, A. O., Acta. Haemat. 60:243 (1978).

Katz, J., Troll, W., Russo, J., et al, Endocr. Res. Commun., 1, 331 (1974).

Kinsella, A., Radman, M., Proc. Natl. Acad. Sci., 75, 6149 (1978).

Klimpel, G.R., Byus, C.V., Russell, D.H., et al, J. Immunol. 123 817 (1979).

Kluft, C., Thrombos. Haemostas., 41, 365 (1979).

Lee, L.S., Weinstein, I.B., Science, 202, 313 (1978).

Lewis, J.G., Pizzo, S.V. and Adams, D.O.: Am. J. Clin. Pathol. 76:403 (1981).

Lowry, O.H., J. Biol. Chem., 193, 265 (1951).

Markut, C.L., Cell Differentiation and Neoplasia, New York, Raven Press, 9 (1978).

Miller, E.C., Cancer Res., 38, 1479 (1978).

Miller, E.C., Miller, J.A., Cancer, 47, 1055 (1980).

Miller, J.A., Cancer Res., 30, 559 (1970).

Mintz, B.,Cell Differentiation and Neoplasia, New York, Raven Press, 27 (1978).

Mottram, J.C., J. Pathol. Bacteriol., 56, 181 (1944)

Mufson, R.A., Fisher, P.B., Weinstein, I.B., Proc, Am. Assoc. Cancer Res., 19, 183 (1978).

O'Brien, Cancer Res., 36, 2644 (1976)

O'Brien, T.G., Simsiman, R.C., Boutwell, R.K., Cancer Res., 35, 2426 (1975).

O'Brien, T.G., Simsiman, R.C., Boutwell, R.K., Cancer Res., 34, 2426 (1975).

O'Connor, P.J., Saffhill, R., Margison, G.P., Environmental Carcinogenesis: Occurrance, Risk Evaluation, and Mechanisms, Amsterdam, Elsevier/North Holland, 73 (1979).

Ohnishi, S.T., Barr, J.K., Anal. Biochem., 86, 193 (1978).

Ossowski, L., Quigley, J.R., Kellerman, T.M., et al, J. Exp. Med., 138, 1056 (1973a).

Ossowski, L., Unkeless, J.D., Tibiu, A., et al, J. Exp. Med. 137, 112 (1973b).

Pacifici, M., Holtzer, H., Am. J. Anat., 150, 207 (1977).

Pierce, G.B., Fed. Proc., 29, 1248 (1970).

Pollack, R., Risser, R., Conlon, S., et al, Proc. Nat. Acad. Sci. (WASH), 71, 4792 (1974).

Putnam, C.W., Buckley, A.R., Jr., Gastroent., 80, 1345 (1981).

Quigley, J.P., Cell, 17, 131 (1979).

Rifkin, D., Loeb, J.N., Moore, G., et al, J. Exp. Med., 139, 1317 (1974).

Rijken, D.C., Collen, D., Progress in Fibrinolysis, Malmö Sweden, Abstr. 92 (1980).

Rohrschneider, L.R., O'Brien, D.H., Boutwell, R.K., Bichem. Biophys. Acta, 280, 57 (1972).

Rous, P., Kidd, J.G., J. Exp. Med., 73, 365 (1941).

Rovera, G., O'Brien, T.G., Diamond, L., Proc. Natl. Acad. Sci., 74, 2894 (1977)

Russell, D.H., Biochem. Biophys. Res. Commun., 99, 1167 (1981).

Russell, D.H., Durie, G.G.M., Polyamines as Biochemical Markers of Normal and Malignant Growth, New York, Raven Press, (1978).

Russell, D.H., Pharmacology, 20, 117 (1980)

Russell, D.H., Snyder, S.H., Proc. Natl. Acad. Sci. USA, 60, 1420 (1968).

Scott, K.F.F., Russell, D.H., J. Cell. Phys., 111, 111 (1982).

Soper, C.J., Evans, F.J., Cancer Res., 37, 2487 (1977).

Strickland, S., Beers, W.H., J. Biol. Chem., 251, 5694 (1976).

Suss, R., Kinzel, V., Kreibich, G., Experientia, 27, 46 (1971).

Toskutoff, D.J., Paul, D., J. Cell Physiol. 97, 99 (1978).

Troll, W., Fundamentals in Cancer Prevention, Baltimore, Univ. Park Press, 41 (1976)

Troll, W., Klassen, A., Janoff, A., Science, 169, 1211 (1970).

Troll, W., Rossman, T., Katz, J., et al, Proteases and Biological Control, New York, Cold Spring Harbor Laboratory, 977 (1975).

Unkeless, J.D., Tobia, A., Ossowski, L., et al, J. Exp. Med., 137, 85 (1973).

Unkeless, J.D., Tobia, A., Ossowski, L., et al, J. Exp. Med., 137, 85 (1973).

Van Duuren, B.L., Chem. Carcinogen. ACS Monograph 173, Washington, American Chemical Society, 24 (1976).

Van Duuren, B.L., Prog. Exp. Tumor Res., 11, 31 (1969).

Vassalli, J.D., Hamilton, J., Reich, E., Cell, 11, 695 (1977).

Vassalli, J.D., Hamilton, J., Reich, E., Cell, 8, 271 (1976).

Voorhees, J.J., Colburn, N.H., Stawiski, M., et al, Control of Proliferation in Animal Cells, New York, Cold Spring Harbor Laboratory, 635 (1974).

Weiner, R.A., Byus, C.V., biochem. Biophys. Res. Comm., 97, 1575 (1980).

Weinstein, I.B., Tih-Syng, L., Fisher, P.B.., et al, Naturally Occurring Carcinogens - Mutagens and Modulators of Carcinogensis, Baltimore, Univ. Park Press, 301 (1979)

Weinstein, I.B., Wigler, M., Pietropaolo, C., Origins of Human Cancer, New York, Cold Spring Harbor Laboratory, 751 (1977).

Weinstein, I.B., Wigler, M., Fisher, P., et al, Carcinogenesis, vol. 2, Mechanisms of Tumor Promotion and Co-carcinogenesis, New York, Raven Press, 313 (1978).

Weinstein, I.B., Wigler, M., Pietropaloo, C., Action of Tumor-Promoting Agents in Cell Culture, New York, Harbor Press (1972).

Wigler, M., Weinstein, I.B., Nature, 259, 232 (1976).

Williams-Ashman, H.G., Beil, R.E., Wilson, J., et al, Adv. Enzyme Regul.,18, 239 (1980).

Williams-Ashman, H.G., Coppoc, G.L., Weber, G., Cancer Res., 32, 1924 (1972).

Williams-Ashman, H.G., Wilson, J., Beil, R.E., et al, Biochem. Biophys. Res. Commun., 79, 1192 (1977).

Yamasaki, H., Fibuch, E., Nudel, U., et al, Proc, Natl. Acad. Sci., 74, 3451 (1977).

Yuspa, S.H., Ben, T., Hennings, H., et al, Biochem. Biophys. Res. Commun., 97, 700 (1980).