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PART I. THE EFFECT OF CIGARETTE SMOKE AND 3,4-BENZO[A]PYRENE ON THE ACTIVITIES OF CERTAIN GLYCOLYTIC ENZYMES AND ON RNA AND DNA SYNTHESIS IN HUMAN DIPLOID CELLS IN CULTURE

PART II. THE EFFECT OF ZINC, CALCIUM, IRON, AND COPPER CONCENTRATIONS ON SCOPOLIN, SCOPOLETIN, AND LIGNIN IN TOBACCO TISSUE CULTURES

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PART I. THE EFFECT OF CIGARETTE SMOKE AND 3,4-BENZO[A]PYRENE ON THE ACTIVITIES OF CERTAIN GLYCOLYTIC ENZYMES AND ON RNA AND DNA SYNTHESIS IN HUMAN DIPLOID CELLS IN CULTURE

PART II. THE EFFECT OF ZINC, CALCIUM, IRON, AND COPPER CONCENTRATIONS ON SCOPOLIN, SCOPOLETIN, AND LIGNIN IN TOBACCO TISSUE CULTURES

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CHAPTER I

INTRODUCTION

Research over the past fifty years uncovered an increasing array of environmental agents that may induce neoplastic transformation, or cancer, under appropriate circumstances. Lung cancer, now the most frequent cause of death from cancer among American men, has been attributed, at least in part, to cigarette smoking. (Nelson, 1970).

Various investigations (Berenblum, 1954, 1964; Boutwell, 1964) have shown that the neoplastic transformation is not a single step process but rather a multistage one. In mouse skin carcinogenesis by chemicals, at least two steps are clearly defined: a short initial one, called "initiation", and a subsequent much longer one, called "promotion". Most of the tumor initiators found in tobacco smoke belong to the class of compounds known as polynuclear aromatic hydrocarbons (Wynder and Hoffman, 1969). The tumor promoters (cocarcinogens) are themselves not carcinogenic or only weakly so. They can, however, shorten or modify the lag period between the time of application of the carcinogen and the time of appearance of identifiable neoplastic cells. Tumor promoters

are present in several of the major fractions of tobacco smoke condensate, mainly in the acidic and phenolic fractions; however, the only group of tumor promoters in tobacco smoke which have been identified are the volatile phenols (Wynder and Hoffman, 1971).

Considerable attention has been paid to the development of a bioassay for identification of carcinogenic substances in tobacco smoke. Application of the test substance to the skin of mice has been and still is the most commonly used bioassay for carcinogenicity. These tests, in order to be meaningful, must be carried out for at least one year or, preferably, for the lifetime of the test group. Subcutaneous injection has been used as another route of administration for carcinogens in experimental animals (Van Duuren et al., 1966). A number of researchers are attempting to develop a reliable cell or organ culture system for evaluation of potential carcinogens and cocarinogens (Sivak and Van Duuren, 1970; Battista and Kensler, 1970).

Certain advantages for testing various biological effects of many agents are offered by tissue culture. The use of established cell strains, grown under constant cultural conditions, affords an opportunity to study the reactions in the cell cycle which are controlled from within the cell. A unique system for biochemical studies is provided by human diploid cell strains since these cells, in contrast to the commonly used heteroploid cell lines, retain many of the characteristics of normal cells in vivo. If normality is to be related to the condition of the cells in normal tissues in vivo, then cells multiplying rapidly in vitro are functionally very abnormal; however, cell cultures derived from normal embryonic or adult tissues have certain characteristics that are generally accepted

as criteria of normal cell growth. Some of the more important criteria are:

1. failure to grow from small inocula (Puck, 1958; Sato et al., 1960)

2. contact inhibition of movement (Abercrombie and Heaysman, 1953)

3. finite in vitro life span (Hayflick and Moorhead, 1961)

4. failure to divide when freely suspended in conventional liquid media and failure to form colonies when suspended as monodispersed suspensions in media converted to a soft gel by the inclusion of 0.3 to 0.5% agar (Macpherson and Montagnier, 1964)

5. constancy of the diploid karyotype (Hayflick and Moorhead, 1961)

6. inability to produce tumors in hosts from which the cells were originally derived (Sanford, 1958).

An important outcome of early work on prolonged treatment of cells in vitro with a number of chemical carcinogens was the observation that neoplastic rodent cells are more resistant to the toxic effects of carcinogenic hydrocarbons in vitro than their normal counterparts (Starikova and Vasiliev, 1962; Berwald and Sachs, 1965; Alfred et al., 1964). These studies were extended to include normal guinea pig and rabbit cells and normal and transformed monkey and human cells (Diamond, 1965; Diamond et al., 1967, 1968). The results of these studies showed that the carcinogenic hydrocarbons methylcholanthrene (MCA) and benzo[a]pyrene (BP) inhibited the multiplication of normal embryonic rodent cells. Concentrations of up to 10 μg/ml of the carcinogens affected neither the growth of virus-transformed or malignant rodent cells nor the multiplication of normal as well as transformed monkey and human cells (Diamond et al., 1968).

The exposure of tissue cultures to whole cigarette smoke, whole

smoke condensate or "tar," or various fractions of smoke condensate has been shown to alter patterns of cell growth and reproduction significantly. A survey of these experiments is presented in Table I-1.

During recent years, several reports have appeared on successful neoplastic transformation of cells grown in vitro with a carcinogenic polynuclear aromatic hydrocarbon found in cigarette smoke, 3,4-benzo[a]pyrene (BP). These experiments are summarized in Table I-2.

The major objective of studies on carcinogenesis is to understand the essential nature of the neoplastic cell, how it differs from its normal counterpart, and how it becomes abnormal. Much research has been devoted to the search for biochemical differences between normal and cancer cells. Following Warburg's observations that tumor tissues almost always have a higher rate of glycolysis than normal cells, one biochemical difference of real significance was thought to be found; however, the real significance of the Warburg effect remains elusive (Warburg, 1930, 1956). Consequently carbohydrate metabolism in normal and tumor cells has been studied extensively. In general, the rate of aerobic glycolysis of normal cells is lower than that found in their transformed derivatives (Broadfoot et al., 1964). Extensive study of the glycolysis pattern in a spectrum of hepatomas of different growth rates produced by chemical carcinogenesis (Morris, 1963, 1965) has been prompted by the Warburg proposal. Those glycolytic enzymes which showed an increase in activity with increasing growth rate of the hepatomas were pyruvate kinase, phosphofructokinase, and hexokinase; glycolytic enzymes exhibiting a decrease in activity with increasing growth rate were glucose-6-phosphatase, glycerol dehydrogenase, fructose diphosphatase, phosphoenolpyruvate carboxykinase, and pyruvate

TABLE I-1 Effects of cigarette smoke on cells in culture

Tissue	Treatment	Results	Reference
Human fetal lung.	Direct exposure to whole smoke for 5 - 10 seconds.	Severe changes in exposed cells, consisting of cytoplasmic vacuolization, chromosomal anomalies, and decrease in cell division.	Awa et al., 1961.
Rabbit cardio-vascular cells.	Direct exposure to whole smoke.	Toxic effects observed, i.e., increase in pinocytosis, bubbling of cytoplasmic membranes, thickening of nuclear membrane, and inhibition of growth.	Kasai and Pollak, 1964.
KB mammalian tumor cells.	Exposure to water-soluble components of whole cigarette smoke.	Inhibition of cell growth and protein synthesis.	Thayer and Kensler, 1964.
L cells.	Exposure to gas phase of smoke.	Morphological changes and a reduction in cell population.	Cooper and Goldring, 1962.
Mouse lung and kidney tissue.	Exposure to several puffs of fresh smoke from unfiltered cigarettes.	Cell damage, characterized by a succession of loss of RNA and DNA, pyknosis, and cell death.	Leuchtenberger and Leuchtenberger, 1969.
Human fetal lung.	Application of a hydro-carbon-enriched fraction of whole smoke condensate.	Cell enlargement, hyperplasia, and metaplasia.	Lasnitzki, 1968.
Chick embryo fibroblasts.	Application of tobacco smoke condensate.	Proliferation and mitotic activity inhibited at high concentrations of condensate, but both were increased at lower condensate concentrations.	Guimard, 1966.
Rodent tracheal rings.	Exposure to an aqueous extract of cigarette smoke condensate: a. 4 mg/ml b. 2.5 mg/ml.	a. Instantaneous ciliostasis b. Ciliostasis after one hour of exposure.	Donnelly, 1969.

TABLE I-2 Effects of 3,4-benzo[a]pyrene (BP) on cells in culture

Tissue	Treatment	Results	Reference
Normal human epidermis.	BP (1 µg/ml for four days).	Amount of epithelial outgrowth markedly reduced in cell BP-treated cultures; fibroblast growth unaffected; no evidence of malignant transformation.	Flaxman and Dietz, 1971.
Rat pulmonary cells.	BP	Sharp depression of the mitotic coefficient.	Guerin et al., 1970.
Mice and hamster embryo cells.	BP (5% for nine days).	Production of transformed colonies.	Berwald and Sachs, 1963, 1965.
Mouse lung bud embryonic cultures	BP	Cellular disorganization, pyknosis, nuclear size and shape irregularities.	Chan et al., 1969.
Hamster lung tissue.	BP	Increased appearance of new small chromosomes.	Borenfreund et al., 1966.
Hamster embryo cells.	BP (1-10 µg/ml).	Linear dose-response relationship for BP-induced transformation.	Huberman and Sachs, 1966.
Hamster embryo fibroblasts.	BP (10 µg/ml for 8 - 9 days).	Production of tumorigenic cell lines which were aneuploid and grew to saturation densities 10-20 times greater than controls.	DiPaolo, Donovan, and Nelson, 1969, 1971.
Hamster embryo fibroblasts.	BP	Transformed cell lines with altered karyotypes obtained which were tumorigenic in hamsters.	Sivak and Van Duuren, 1968.
C3H mouse prostate cells (fibroblasts).	BP (0.5 or 1 mg for 1-6 days).	Appearance of transformed colonies.	Heidelberger and Iype, 1967. Röller and Heidelberger, 1967.

carboxylase. Glycolytic enzymes which were not correlated with growth rate were lactic dehydrogenase, phosphohexoisomerase, 6-phosphogluconate dehydrogenase, and aldolase. The activity of glucose-6-phosphate dehydrogenase was found to be high in all hepatomas, whereas, the activity of phosphoglucomutase was low in all hepatomas (Weber et al., 1964; Shonk et al., 1965; and Weinhouse et al., 1966).

Using the Morris rat hepatomas, Lea and coworkers (Lea et al., 1966) found a progressive increase in the biosynthesis of DNA with increasing tumor growth rate. Incorporation of thymidine-2-C^{14} into DNA was increased eight-fold over that of normal liver in the slow-growing tumors and was increased 64-fold in the most rapidly-growing tumors. The DNA content of the hepatomas was also found to increase with increasing growth rate of the tumors. The incorporation of C^{14}-labelled formate into RNA correlated well with the growth rate of the hepatomas (Weber et al., 1965); however, the total RNA content in the liver tumors exhibited no correlation with growth rate.

The effects of some carcinogenic polyaromatic hydrocarbons (PAH) upon nucleic acid biosynthesis have been examined by a number of investigators (Table I-3). Most of these experiments involve the single or repeated application of the carcinogen to mouse skin. One of the carcinogenic polyaromatic hydrocarbons which has been used extensively in these studies, 7,12-dimethylbenzanthracene, has not been found in tobacco smoke (Stedman, 1968).

From the literature review presented, it is evident that extensive research has been conducted upon various metabolic activities of neoplastic cells and their normal counterparts. The main biological assay for

TABLE I - 3 Effects of carcinogens and cocarcinogens on RNA and DNA synthesis

Bioassay	Treatment	Results	Reference
Mouse skin.	BP/topical application.	Initial rapid decrease in RNA synthesis followed by a subsequent continuous increase in synthesis rising to a maximum of two times the control value at 24 hours.	Alexandrov et al., 1970.
Mouse skin.	7,12-DMBA/topical application.	Inhibition of RNA synthesis.	Sinclair and McCarter, 1964.
Mouse skin.	a. 7,12-DMBA/feeding. b. Phorbol myristate acetate (PMA).	a. Initial inhibition of RNA synthesis followed by a marked stimulation of synthesis to a maximum of 2.2 times the control value at 48 hours. b. Stimulation of RNA synthesis.	Dieter, 1969.
Mouse skin.	7,12-DMBA/topical application.	Fifty per cent inhibition of DNA synthesis within 24 hours.	McCarter and Quastler, 1962.
Mouse skin.	7,12-DMBA/topical application (100 µg)	DNA synthesis inhibited 65 per cent at six hours but returned to a normal level by 48 hours.	Hennings and Boutwell, 1969.
Mouse skin.	7,12-DMBA/topical application.	Initial inhibition of DNA synthesis with synthesis returning to normal levels within 24 hours and increasing significantly relative to the control level in two to three days.	Bates et al., 1968.
Mouse skin.	7,12-DMBA/feeding.	Initial 24 hour inhibition of DNA synthesis followed by a stimulation of synthesis reaching a maximum of two times the control value at 48 hours.	Dieter, 1969.
HeLa cells.	Tetradecanoyl-phorbol acetate (TPA).	Depression of DNA synthesis.	Freienstein et al., 1970.
Mouse skin.	Phorbol diesters from croton oil.	a. Initial inhibition of DNA synthesis followed by a large increase above control levels. b. Rapid stimulation of RNA synthesis.	Baird et al., 1971.
Mouse skin.	Croton oil (05%).	a. Initial rapid inhibition of DNA synthesis followed by a continuous increase in synthesis to a maximum of three times the control value at 18 hours. b. Immediate stimulation of RNA synthesis to a maximum rate of two times the control value at 6 hours.	Hennings and Boutwell, 1970.
3T3 mouse fibroblasts.	PMA (0.5 µg/ml)	Enhancement of RNA synthesis in stationary cultures.	Sivak and Van Duuren, 1970a.

potential carcinogens has in most cases been mouse skin application with the development of tumors being indicative of the presence of one or more carcinogens. The majority of the comprehensive studies on carbohydrate and nucleic acid metabolism in neoplastic cells have utilized tumors of rat liver induced by chemical carcinogenesis. A few studies have been made on glycolysis in transformed and untransformed cells of the same line. Several studies have beeen conducted on the variation in the rate of RNA and DNA synthesis in mouse skin treated with a variety of agents.

The goal of this research has been to investigate nucleic acid metabolism at different time periods in a human diploid cell strain treated with either a solution of cigarette smoke or 3,4-benzo[a]pyrene as compared to untreated cells of the same strain. A comparative time study of the enzyme profile of several of the enzymes involved in glucose metabolism has also been made on cultures of this cell strain that have been treated with 3,4-benzo[a]pyrene and their untreated counterparts.

CHAPTER II

MATERIALS AND METHODS

Cultivation of Cell Cultures

Stock Cultures

CF-1 cells (passage 6), derived from newborn human foreskin, were obtained from Dr. Paul F. Kruse, Jr. and Mr. Wilbur Whittle of the Samuel Roberts Noble Foundation. This strain of cells is capable of serial propagation *in vitro* while retaining a normal diploid karyotype of 46 chromosomes.

The cells were cultured in McCoy's 5a (modified) medium with 10% fetal calf serum, 100 units of penicillin per ml of medium and 100 mcg of streptomycin per ml of medium. A stock solution of one liter of 5X McCoy's 5a medium was prepared by first dissolving 60.5 g of the powdered medium in one liter of water which had been double distilled in a glass still and autoclaved at 121°C and 15 psi for 30 minutes prior to use. The solution was then filtered through a sterile stainless steel Millipore filter (500 ml) by negative pressure, poured into sterile bottles (500 ml) and refrigerated. Before this stock solution was used to make up the complete medium for the cell cultures, samples of it were cultured for a week at 37°C on blood agar plates and on modified Sabouraud's agar plates at room temperature to make certain that no contamination was present. One liter of complete medium was prepared by adding 666 ml of sterile glass distilled water (autoclaved for 30 minutes at 121°C and 15 psi),

200 ml of 5X McCoy's 5a (modified) medium, 100 ml of fetal calf serum, 20 ml of a solution containing 5000 units of penicillin per ml and 5000 mcg of streptomycin per ml, and 14 ml of a sterile solution of 7.2% (w/v) sodium bicarbonate. A sample of the complete medium was also cultured on blood agar plates at 37°C and on modified Sabouraud's agar plates at room temperature for 3-4 days prior to use. The pH of the complete medium was 6.9 - 7.0.

All stock cultures of the CF-1 line were cultivated in two liter Povitsky flasks with 150 ml of complete medium. When the cell sheet became confluent, the cultures were subcultivated in the following manner:

1. The medium was decanted and 10 ml of 0.05% (w/v) pronase (Sigma Chemical Co.) in Hank's balanced salt solution was added with the cell sheet up. The pronase was prepared by adding 50 mg of pronase to 100 ml of Hank's balanced salt solution which had been brought to pH 7.4 with 7.2% sodium bicarbonate. The pronase solution was filter sterilized by means of a 150 ml disposable Millipore filter (0.22μ) (Falcon Plastics). After allowing the pronase solution to rinse over the cells, the flask was inverted and the pronase solution was decanted. The flask was then rocked gently until the cell sheet was completely free from the surface. Next, 10 ml of complete medium was added to the flask and the cells were aspirated vigorously at least 10 times by means of a sterile 10 ml pipette plugged with cotton and a rubber bulb. The resultant cell suspension was evenly distributed between two Povitsky flasks and sufficient medium was added to bring the total volume to 150 ml per flask. Cultures were sealed with silicone stoppers (West and Company) and incubated at 37°C.

All aseptic manipulations were performed in an enclosed hood (Blickman) sterilized with ultraviolet lights and provided with rubber gloves.

Cultures for Experiments

All stock cultures comprising the cell pool used for inoculation of the flasks for experiments were grown to confluency and then starved by not changing the medium. This starvation procedure is one way of bringing about synchrony in cell cultures. When the pH of the medium dropped to about 7.0, the cells were harvested, pooled, centrifuged at 2000 rpm for 15 minutes in a Lourdes centrifuge, and resuspended in fresh medium by aspiration.

RNA and DNA Synthesis

In the experiments in which RNA and DNA synthesis were measured, the cells were grown in 30 cm^2 sterile plastic flasks (Falcon Plastics) with 5 ml of complete medium. The flasks were inoculated from a common pool of cells, the initial inoculum of each flask being $3 \times 10^5 - 5 \times 10^5$ cells per 5 ml of medium. The cultures were incubated for 14 hours at 37°C, at which time the spent medium was replaced with fresh medium and the test materials were introduced into the treated flasks. In this way, the initiation and termination of the experiments were conducted in the exponential phase of the proliferation cycle. Cultures are normally gassed with 5% CO_2 in air before they are incubated to bring down the pH of the media to 7.4 in order to reduce the lag period in the growth cycle. For human diploid cell strains the pH is especially critical since these cells will not tolerate a pH above 7.4 for any extended period. Instead of introducing another possible source of contamination by gassing, an alternate

method was used to keep the pH of the medium from rising. By using medium at a pH of 6.9 - 7.1 the cells could rapidly attach and begin proliferation before the pH of the medium rose above 7.4. Cultures in passages 15 - 23 were used in all experiments.

A stock solution of 3,4-benzo[a]pyrene at a concentration of 1 mg per ml in dimethylsulfoxide (DMSO) was filter sterilized by means of a small syringe Millipore filter and stored in a sterile dark colored bottle. After replacing the spent medium on the cultures to be used in the experiment with 5 ml of fresh medium, 0.05 ml of the 3,4-benzo[a]pyrene was introduced into the treatment flasks. The same amount (0.05 ml) of sterile DMSO was introduced into the control flasks. The flasks were then placed on wooden trays, covered with aluminum foil, and incubated at 37°C.

The cigarette smoke used in the experiments was obtained under sterile conditions (Kasai and Pollak, 1964). A cigarette was inserted into one end of a piece of glass tubing containing on the opposite end a fritted glass filter (coarse) which was submerged in the McCoy's 5a complete medium. A second piece of glass tubing, bent at a 90 degree angle, was connected to a vacuum outlet, as in Plate I. All of the equipment used to construct this smoking apparatus was sterilized by autoclaving for 50 minutes at 121°C and 15 psi and was assembled in a sterile hood. By means of this apparatus, all of the cigarette smoke could bubble into the medium. A stock solution of this cigarette "whole" smoke was made by smoking 4 cigarettes (Camel, unfiltered) to a butt length of 25 mm into 400 ml of freshly prepared McCoy's 5a medium without calf serum. This gave a solution of cigarette smoke in which 1 ml contained the smoke from 1/100 of a cigarette. This solution will be referred to as CS/100, using the

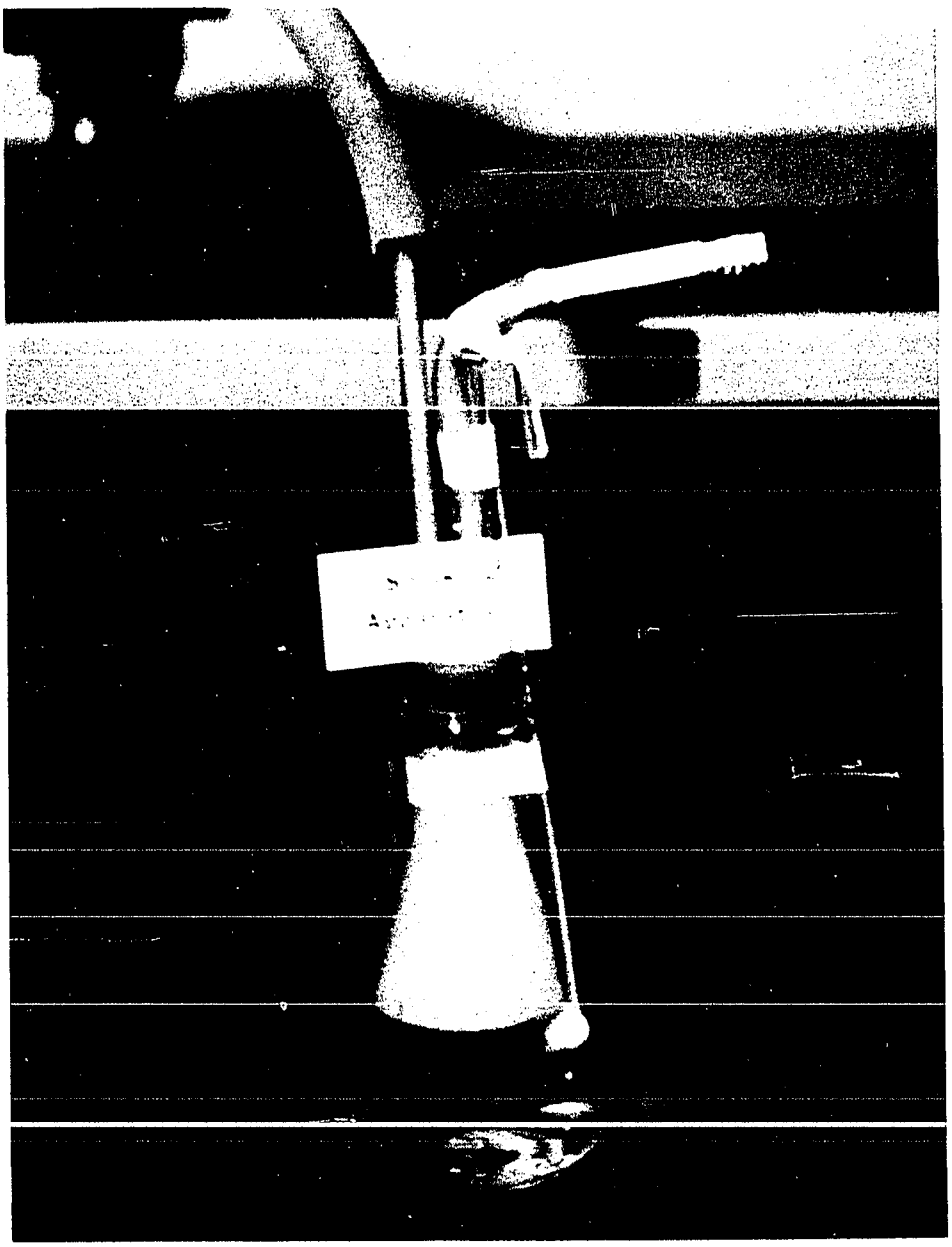

PLATE I-1. SMOKING APPARATUS.

letters "CS" to indicate the smoke from one whole cigarette. The cigarette smoke solution was then partitioned into sterile dark bottles and frozen. A sample of this solution was cultured on blood agar and on modified Sabouraud's agar and found to be sterile.

Dilutions of this stock solution of cigarette smoke were made with fresh medium and the test cultures were exposed to the various cigarette smoke concentrations by the same procedure used in the 3,4-benzo[a]pyrene experiments. An amount of fresh medium, equal to that of the cigarette smoke solution placed on the test cultures, was placed on the control cultures.

Enzyme Studies

The procedures outlined by Shonk and Boxer (1964) were adapted for the determination of glycolytic enzyme patterns in the CF-1 cell line.

For the enzyme studies the cells were grown in 2 liter Povitsky bottles, each bottle containing an initial inoculum of 1.0 - 1.5 x 10^6 cells per 150 ml of medium. After an incubation period of 14 - 16 hours, the flasks were treated with the test material as described in the preceding section.

The cells were harvested as described in the first section of this chapter, the cell pellet suspended in 5 ml of precooled extraction medium by aspiration and transferred to a precooled sonication tube (Falcon Plastics). The extraction medium, prepared in glass distilled water, contained potassium chloride, 0.15M; potassium bicarbonate, 0.05M; and disodium ethylenediamine tetraacetic acid, 0.006M. The cells were sonicated at maximum cavitation for 45 seconds at intervals of 15 seconds using a probe

that had been precooled to -20°C and immersing the sonication tube in a salt-ice-water bath between sonication periods. The sonicate was then transferred to a precooled centrifuge tube (Nalgene), immersed in a salt-ice-water bath until the temperature of the sonicate was about 0°C, and finally centrifuged at 15,000 rpm for 30 minutes at 0°C in a Sorvall RC-2B centrifuge with a SS-34 head. The supernatant was used for the enzyme assays.

Determination of RNA and DNA Synthesis

Synthesis of DNA and RNA was measured by the rate of incorporation of specific precursors which were isotopically labeled. DNA synthesis was measured by the incorporation of thymidine-methyl-H^3 (specific activity = 18.4 Ci per millimole) and RNA synthesis by the incorporation of uridine-5-H^3 (specific activity = 28.1 Ci per millimole). Both isotopes were purchased from New England Nuclear.

Cultures were pulse-labeled for 1 hour with either H^3-thymidine (0.25 µCi per ml of culture) or H^3-uridine (0.5 µCi per ml of culture) at various times after treatment with the test materials.

At the end of the labelling period, the medium was removed from the culture flasks and the cell surfaces washed three times with 2 ml of cold saline which was then discarded. After the third saline wash, 2 ml of cold 5% trichloroacetic acid (TCA) was added to each flask and the cells shaken loose by passing each flask over the probe of a sonicator several times. Each flask was washed two more times with 2 ml aliquots of cold TCA and the washings added to the first 2 ml in 12 ml centrifuge tubes (Sorvall). The combined 6 ml of material was centrifuged at 6000 rpm and

the supernatant discarded. After being washed three times with cold 5% TCA, the TCA-insoluble material was hydrolyzed in 2 ml of 5% TCA in a 95°C water bath for 45 minutes. The insoluble material remaining after hydrolysis was removed by centrifugation at 6000 rpm and the supernatant was saved for analysis.

The radioactivity in the labeled samples, expressed as counts per minute (cpm), was determined by a Beckman liquid scintillation counter at a counting error of 3% or less. Ten ml of the scintillation solution, Beckman's Cocktail-D, was mixed with 0.5 ml of each sample. Cocktail-D consists of 5 g of 2,5-diphenyloxazole (PPO)(Beckman), 100 g of naphthalene (Beckman), and enough p-dioxane (Matheson, Coleman and Bell; scintillation quality) to make one liter.

For each set of experiments, the cell numbers in a replicate set of treated and control flasks were determined by counting the cells in a hemocytometer.

Enzyme Assays

General Procedures

The buffer used in all systems was glycylglycine buffer, pH 7.6, 0.05M, containing Na_2H_2EDTA, 0.006M. All substrates, coenzymes, and assay enzymes were purchased from Sigma Chemical Company and used without further purification. All solutions for the assay procedures were prepared immediately prior to use.

Since all the enzymatic assays were systems involving the oxidation or reduction of one of the pyridine nucleotides (NAD+, NADH, NADP+, NADPH), the enzymatic measurements were made by recording rates of change in absorbance at 340 mμ on a Gilford recording spectrophotometer fitted with

an automatic sample changer. The photometric scale was set at 0-0.5 linear absorbance units full scale and the sample changer set to measure the absorbance of each cuvette for 15 second intervals. The rate of appearance of the product was equated to the rate of appearance or disappearance of the reduced form of the pyridine nucleotides. The micromoles of reduced pyridine nucleotide formed or consumed in a 3 ml assay using 1 cm cuvettes was calculated by multiplying the change in absorbance at 340 mμ by 0.482 (Horecker and Kornberg, 1948), and this was equated with the micromoles of product formed by the enzyme whose activity was being measured.

Each assay was initiated with the supernantant from the sonicated cells and was determined in duplicate at room temperature (25±1°C) with a blank being measured for each assay.

The activities of certain enzymes in the glycolytic pathway were determined using the procedures of Shonk and Boxer (1964) and Dunaway (1970).

In these experiments the number of cells at the end of each period of treatment was determined by counting the cells in a hemocytometer; therefore, the specific activity (expressed as millimicromoles of substrate utilized per minute per 10^6 cells) of each of the enzymes assayed could be calculated.

Preservation and Reconstitution of Cells

Since human diploid cell strains have a finite limit of approximately 50 subcultivations (Hayflick and Moorhead, 1961), all excess cultures of the CF-1 line were preserved by freezing in the following manner:

1. The cell sheet was removed according to the methods outlined in the first section of this chapter and the cell suspension centrifuged in

sterile plastic centrifuge tubes with caps (Nalgene) for 2 minutes at 2000 rpm on a Lourdes centrifuge. The supernatant was decanted and 5 ml of complete medium containing 10% sterile glycerine (v/v) was added to the cell button from each of the 2 liter Povitsky flasks. The pH of the glycerol medium was 6.9 - 7.0.

2. The cells were aspirated with a sterile 10 ml pipette plugged with cotton and dispensed into sterile glass tubes (6 ml) with a screw cap, 1 ml per tube.

3. The tubes were placed in a refrigerator at 4°C overnight.

4. The cells were then resuspended by agitation of the tubes, and the temperature was lowered from 4°C to -25°C at a rate of 1.5°C per minute. This was accomplished by placing the tubes in an insulated beaker containing enough acetone to cover 3/4 of the tube. This insulated beaker was then placed in a freezer (Revco, Inc. Ultralow temperature model SBZ-659) at -65°C.

5. The cells were stored at -65°C.

CF-1 cells frozen in this manner were stored up to 3 - 4 months and successfully reconstituted in the following manner:

1. The tubes to be reconstituted were removed from the -65°C freezer, immediately placed into a 37°C water bath and moved about until the solution in the tubes had warmed to 37°C.

2. The tubes were then opened, the contents poured into sterile plastic flasks (250 cm^2)(Falcon Plastics) and 20 ml of complete medium were added to each flask.

3. The medium was changed after 24 hours and the cultures were refed periodically until cell growth was confluent.

4. When the cultures were confluent, they were subcultivated as described previously.

Cleaning of Glassware

All glassware used with the cell cultures was first boiled in a 1% solution of 7X detergent (Linbro Chemical Company) in a large stainless steel pan over an open Fisher burner, scrubbed with a brush, rinsed with distilled water and next brought to a boil in a very dilute solution of glacial acetic acid. The glassware was then boiled two times in distilled water and one time in glass distilled water.

Statistical Calculations

The variance (σ^2), standard deviation (σ), and standard error (S.E.) were calculated from the raw data with a Wang Model 320K Calculator using the following relationships:

$$\sigma^2 = \frac{\Sigma x_i^2 - N(\Sigma x_i/N)^2}{N - 1}$$

$$S.E. = \sigma/\sqrt{N}$$

where x = the individual values

N = number of individual values in the series

The experimental and control means ($\overline{x}$) were compared by the Student t-test:

$$t = \frac{\overline{x}_a - \overline{x}_b}{\sqrt{(S.E._a)^2 + (S.E._b)^2}}$$

where n = number of individual values in the series; $n_a = n_b$

If the observed value of t exceeded the tabulated value at $P < 0.05$, it was stated that the experimental values differ significantly from the

controls, i.e., the differences are real. It has become customary in biological work to adopt $P < 0.05$ as a reasonable level of significance (Goldstein, 1969). In the present studies many of the differences between the control mean and the experimental mean were significant at a more decisive criterion, $P < 0.01$.

CHAPTER III

RESULTS

Effect of 3,4-Benzo[a]pyrene on the Growth of CF-1 Cells

To determine the effect of 3,4-benzo[a]pyrene (BP) on the growth of CF-1 cell cultures, cells were cultivated at 37°C in a 30 cm^2 sterile plastic flask with 5 ml of complete medium. After 14 hours the spent medium was replaced with fresh medium and BP dissolved in DMSO was added to the test cultures to give final concentrations of 5, 10, 15, 20 and 40 µg/ml. The same amount of DMSO was added to the control cultures. In a preliminary experiment, this concentration of DMSO (one per cent) had no effect on the growth of the CF-1 cells. Twelve flasks (two control and two for each concentration of BP) were harvested at selected time periods as described in Chapter II and the cells counted in a hemocytometer. The data in Table I-4 indicate that concentrations of BP greater than 15 µg/ml caused a severe inhibition of growth of the cells by 16 hours and that proliferation was inhibited to a large extent by 15 µg/ml of BP after 16 hours. Concentrations of 5 and 10 µg/ml of BP had no appreciable effect on the growth of CF-1 cells for up to 70 hours.

TABLE I-4

EFFECT OF 3,4-BENZO[A]PYRENE (BP) ON

THE GROWTH OF CF-1 CELLS

µg/ml of BP	Cell number (x 10^5)					
Hours after treatment →	2	8	16	24	48	70
0	5.58	6.30	8.32	11.7	20.3	34.6
5	5.50	6.23	8.26	11.1	19.6	33.5
10	5.52	6.20	8.20	10.8	18.7	30.2
15	5.43	6.12	7.01	8.27	12.6	18.7
20	5.41	5.90	5.02	6.45	11.4	15.2
40	5.30	5.70	4.36	4.81	8.41	12.8

Effect of Cigarette Smoke Concentrations on the Growth of CF-1 Cells

To assess the effect of various concentrations of cigarette smoke solution on the growth of CF-1 cell cultures, cells were cultivated as described in the preceding section. Various concentrations of the cigarette smoke solution were placed on the test cultures, and an equal amount of fresh medium was placed on control cultures. Cell cultures were harvested after 24 hours of treatment (Table I-5), and in a similar experiment cells were harvested and counted at selected time periods from 2 to 48 hours (Figure I-1). Smoke concentrations greater than 1/500 of a cigarette severely inhibited cell proliferation by 24 hours. Growth of the cells was inhibited to some extent with smoke concentrations of 1/600 and 1/800, with the inhibitory effect becoming more pronounced after about 36 hours of treatment. Smoke concentrations of 1/1000 and 1/5000 had only a slight inhibitory effect after 22 hours.

Effect of 3,4-Benzo[a]pyrene on the Activities of Some Glycolytic Enzymes in CF-1 Cells

The specific activities (millimicromoles of substrate utilized per minute per 10^6 cells) of several of the glycolytic enzymes and of two enzymes of the hexose monophosphate shunt were determined at selected time periods after treatment of CF-1 cell cultures with 10 μg/ml of BP as described in Chapter II. The specific activities of most of the enzymes measured in cells exposed to BP for four days (Table I-6) were lower than in control cells. The specific activity of pyruvate kinase in the treated cells was only half as great as that in the control cells

TABLE I-5

EFFECT OF VARIOUS CONCENTRATIONS OF CIGARETTE SMOKE ON THE GROWTH OF CF-1 CELLS AFTER TREATMENT FOR 24 HOURS

Experiment number →	Cigarette Smoke Concentration	Terminal cell number ($x10^6$)			% of control cells		
		1	2	3	1	2	3
	0	3.75	17.4	15.8	100	100	100
	1/30	0.16	0.77	0.69	4.03	4.42	4.37
	1/50	0.53	2.92	2.43	14.2	16.8	15.4
	1/100	1.12	5.40	5.07	29.0	31.0	32.1
	1/200	1.37	6.80	6.66	36.5	39.1	42.3
	1/400	2.10	9.05	9.16	56.0	52.0	58.0
	1/500	2.52	9.80	10.4	67.1	62.0	65.6
	1/600	2.67	12.1	11.9	71.0	73.5	75.8
	1/800	3.33	16.2	14.7	88.9	90.2	93.1
	1/1000	3.63	16.9	15.4	96.8	97.0	97.1
	1/3000	3.68	17.0	15.6	98.1	98.0	99.0
	1/5000	3.72	17.3	15.7	99.1	99.5	99.6

Cells were incubated at 37°C for 16-18 hours before treatment. Five flasks (75 cm^2) per level were counted; cell counts in the table represent the average number of cells per flask. Average number of cells per flask at beginning of treatment: 1 = 1.87×10^6; 2 = 9.44×10^6 ; 3 = 7.27×10^6.

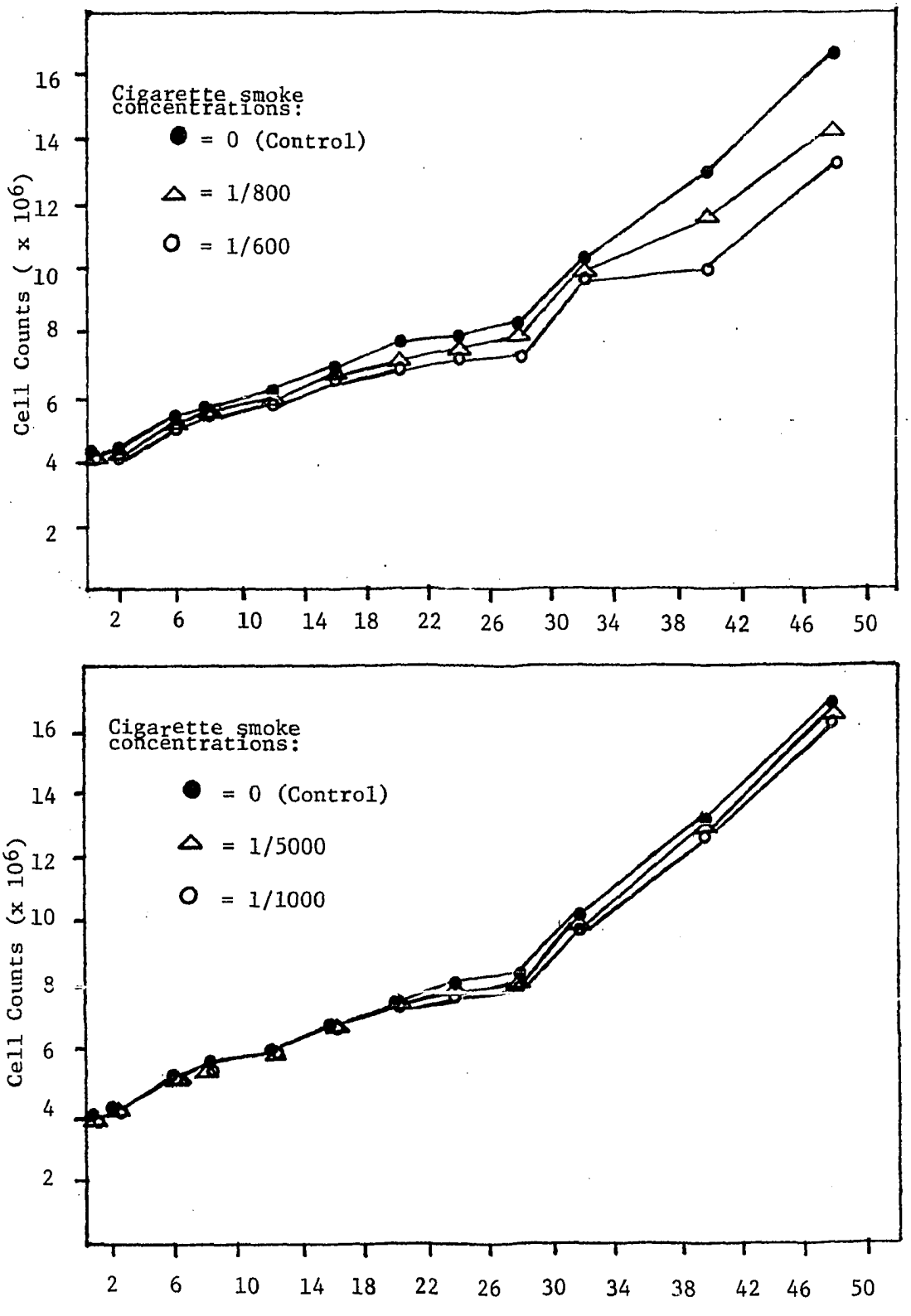

Figure I-1. Effect of Cigarette Smoke Concentrations on the Growth of CF-1 Cells.

TABLE I-6

ENZYME ACTIVITIES IN CF-1 CELLS AFTER TREATMENT

WITH 3,4-BENZO[A]PYRENE FOR FOUR DAYS

Enzyme	Activity in control cells (C_{av})		Activity in treated cells (BP_{av})		% of control $(\frac{BP_{av}}{C_{av}}) \times 100$
	$\bar{x}$	S.D.	$\bar{x}$	S.D.	
Glucose-6-phosphate Dehydrogenase	68.5	± 9.2	54.1	± 1.4	79.4
Lactate Dehydrogenase	1199	± 135	862	± 38	71.9
Hexokinase	10.6	± 1.3	6.8	± 0.5	64.1
Aldolase	14.3	± 1.9	13.4	± 1.9	93.9
Phosphoglucoisomerase	233	± 7.7	226	± 19	96.6
Pyruvate Kinase	215	± 13	105	± 5.9	48.6
6-Phosphogluconate Dehydrogenase	6.2	± 0.7	4.2	± 0.5	66.7
Phosphoglycerate Kinase	59.1	± 9.0	38.4	± 3.0	64.9

Concentration of 3,4-benzo[a]pyrene in treated flasks was 10 µg per ml. Enzyme activities expressed in millimicromoles of substrate utilized per minute per 10^6 cells. C_{av} = average ± S.D. of the activity of the enzyme of control cells from four replicate experiments. BP_{av} = average ± S.D. of the activity of the enzyme of treated cells from four replicate experiments. S.D. = Standard Deviation

($P < 0.01$). The specific activities of hexokinase, phosphoglycerate kinase, and 6-phosphogluconate dehydrogenase in the treated cells were about two-thirds of the control enzymes ($P < 0.01$), and the specific activities of lactate dehydrogenase and glucose-6-phosphate dehydrogenase in the treated cells were about three-fourths of the control enzymes ($P < 0.05$). The specific activities of aldolase and phosphoglucoisomerase were only slightly lower in the treated cells. In cells treated with BP for six days (Table I-7), the specific activities of lactate dehydrogenase, hexokinase, and pyruvate kinase remained significantly lower than the control enzymes; however, the activities of glucose-6-phosphate dehydrogenase, phosphoglucoisomerase, and phosphoglycerate kinase were about 20 per cent higher ($P < 0.05$) in the treated cells than in the control cells. The specific activities of aldolase and 6-phosphogluconate dehydrogenase were not significantly different from the controls. The specific activities of hexokinase and phosphoglucoisomerase were lower ($P < 0.01$) in cells treated with BP for eight days (Table I-8) than those in control cells. The specific activities of the other enzymes measured were not significantly different in treated and control cells. In cells exposed to BP for ten days (Table I-9), the specific activities of hexokinase and phosphoglucoisomerase were lower ($P < 0.01$) than the control enzymes, whereas the specific activities of pyruvate kinase and 6-phosphogluconate dehydrogenase were not significantly different from the control enzymes. The specific activities of glucose-6-phosphate dehydrogenase and lactate dehydrogenase were about 50 per cent higher ($P < 0.01$) in the treated cells and the specific activity of phosphoglycerate kinase about 100 per cent higher

TABLE I-7

ENZYME ACTIVITIES IN CF-1 CELLS AFTER TREATMENT

WITH 3,4-BENZO[A]PYRENE FOR SIX DAYS

Enzyme	Activity in control cells (C_{av})	Activity in treated cells (BP_{av})	% of control $(\frac{BP_{av}}{C_{av}}) \times 100$
	$\bar{x}$ S.D.	$\bar{x}$ S.D.	
Glucose-6-phosphate Dehydrogenase	64.6 ± 5.6	74.0 ± 2.2	119
Lactate Dehydrogenase	1154 ± 44	871 ± 41	75.5
Hexokinase	10.3 ± 0.7	8.4 ± 0.4	81.5
Aldolase	14.4 ± 0.6	13.5 ± 2.8	93.6
Phosphoglucoisomerase	253 ± 5.7	313 ±13	124
Pyruvate Kinase	300 ± 25	265 ±11	88.3
6-Phosphogluconate Dehydrogenase	7.3 ± 1.2	8.4 ± 1.4	115
Phosphoglycerate Kinase	66.1 ± 3.7	79.2 ± 9.6	120

Concentration of 3,4-benzo[a]pyrene in treated flasks was 10 µg/ml. Enzyme activities expressed in millimicromoles of substrate utilized per minute per 10^6 cells. C_{av} = average ± S.D. of the activity of the enzyme of control cells from four replicate experiments. BP_{av} = average ± S.D. of the activity of the enzyme of treated cells from four replicate experiments.
S.D. = Standark Deviation

TABLE I-8

ENZYME ACTIVITIES IN CF-1 CELLS AFTER TREATMENT

WITH 3,4-BENZO[A]PYRENE FOR EIGHT DAYS

Enzyme	Activity in control cells (C_{av})		Activity in treated cells (BP_{av})		% of control $\left(\frac{BP_{av}}{C_{av}}\right) \times 100$
	$\bar{x}$	S.D.	$\bar{x}$	S.D.	
Glucose-6-Phosphate Dehydrogenase	63.2	± 4.0	56.7	± 5.3	89.8
Lactate Dehydrogenase	490	± 11	508	± 4.6	104
Hexokinase	12.3	± 1.6	9.1	± 0.3	74.1
Phosphoglucoisomerase	236	± 3.7	212	± 2.9	89.8
Pyruvate Kinase	139	± 13	155	± 2.5	112
Phosphoglycerate Kinase	51.0	± 5.0	60.1	± 12.8	118

Concentration of 3,4-benzo[a]pyrene in treated flasks was 10 µg/ml. Enzyme activities expressed in millimicromoles of substrate utilized per minute per 10^6 cells. C_{av} = average ± S.D. of the activity of the enzyme of the control cells from four replicate experiments. BP_{av} = average ± S.D. of the activity of the enzyme of treated cells from four replicate experiments.
S.D. = Standard Deviation

TABLE I-9

ENZYME ACTIVITIES IN CF-1 CELLS AFTER TREATMENT

WITH 3,4-BENZO[A]PYRENE FOR TEN DAYS

Enzyme	Activity in control cells (C_{av})	Activity in treated cells (BP_{av})	% of control $(\frac{BP_{av}}{C_{av}}) \times 100$
	$\bar{x}$ S.D.	$\bar{x}$ S.D.	
Glucose-6-Phosphate Dehydrogenase	54.3 ± 4.5	79.5 ± 6.8	148
Lactate Dehydrogenase	279 ± 4.2	413 ± 9.3	148
Hexokinase	8.7 ± 0.6	5.3 ± 0.6	60.5
Phosphoglucoisomerase	140 ± 5.0	101 ± 4.9	72.0
Pyruvate Kinase	78.0 ± 5.9	79.5 ± 7.6	102
6-Phosphogluconate Dehydrogenase	6.1 ± 0.5	5.6 ± 0.3	91.0
Phosphoglycerate Kinase	24.6 ± 4.4	50.3 ± 2.2	204

Concentration of 3,4-benzo[a]pyrene in treated flasks was 10 µg/ml. Enzyme activities expressed in millimicromoles of substrate utilized per minute per 10^6 cells. C_{av} = average ± S.D. of the activity of the enzyme of control cells from four replicate experiments. BP_{av} = average ± S.D. of the activity of the enzyme of the treated cells from four replicate experiments.
S.D. = Standark Deviation

($P < 0.01$) in the treated cells. Attempts to measure the activities of phosphofructokinase and fructose-1,6-diphosphatase were unsuccessful.

The specific activities of the enzymes measured at different times after initial treatment of the cells with BP is presented graphically in Figures I-2, I-3, and I-4.

Time Studies on the Effect of 3,4-Benzo[a]pyrene on DNA and RNA Synthesis in CF-1 Cells

DNA and RNA synthesis were measured as described in Chapter II. DNA synthesis was measured by the rate of incorporation of thymidine-methyl-H^3 and total RNA synthesis measured by the rate of incorporation of uridine-5-H^3. The results presented represent the average of three separate experiments.

Cells were exposed to 10 µg/ml of BP and RNA synthesis was measured at 2 hour intervals from 1 to 38 hours in treated and control cells (Figure I-5). No significant difference between treated and control cells was observed in the rate of uridine-5-H^3 incorporation over this time span.

The effect of various concentrations of BP on DNA synthesis in CF-1 cells is represented in Figure I-6. Concentrations of 15, 20, and 40 µg/ml of BP caused a gradual decrease in DNA synthesis from 2 to 72 hours. DNA synthesis was not different from that of the controls in cells treated with 5 µg/ml of BP. A BP concentration of 10 µg/ml caused an initial increase in DNA synthesis with a maximum of 1.3 times the control value at 16 hours. DNA synthesis was measured at 2 hour intervals from 2 to 72 hours in cells treated with 10 µg/ml of BP and control cells (Figure I-7). Peaks of increase incorporation (relative to control cells) of thymidine were observed at 8 hours, 16 hours, and from 40 to 45 hours

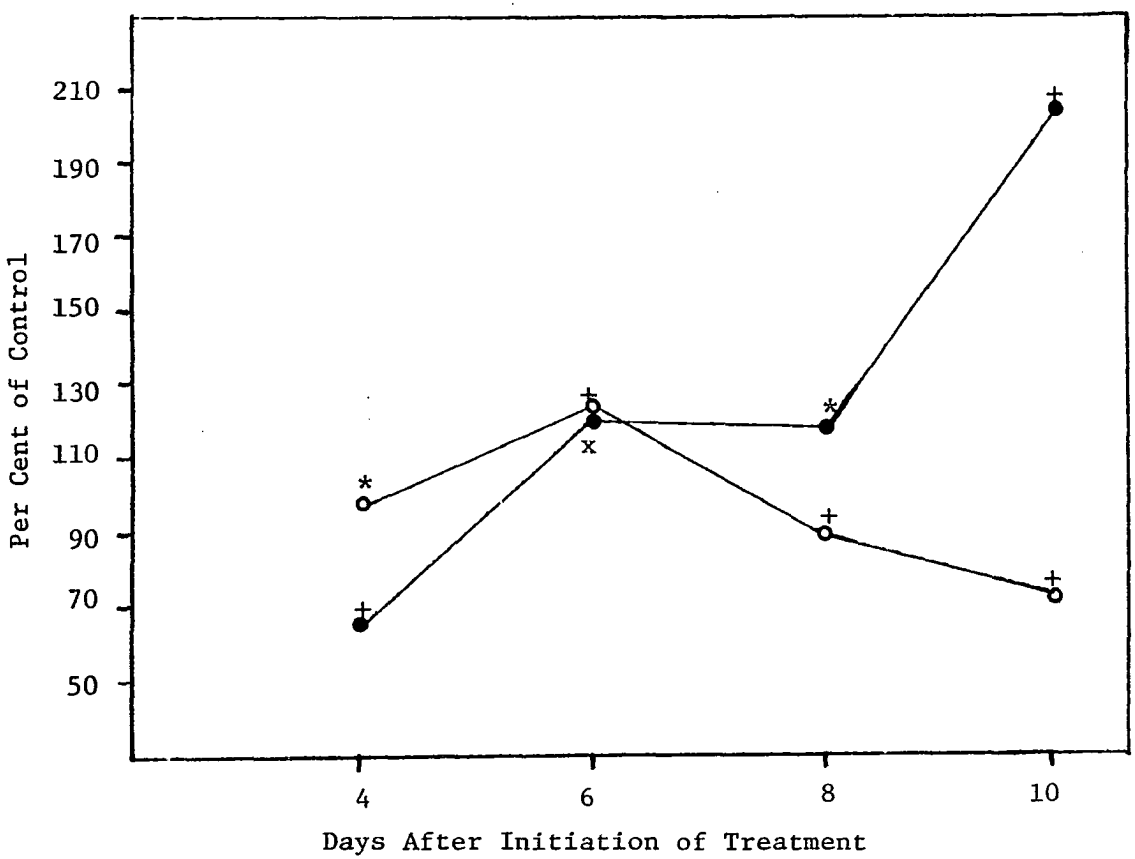

Figure I-2. Enzyme Patterns in CF-1 Cells After Treatment with 3,4-Benzo[a]pyrene (10 µg/ml). I.

○ Specific activity of phosphoglucoisomerase

● Specific activity of phosphoglycerate kinase

+ = P < 0.01; x = P < 0.05; * = not significant

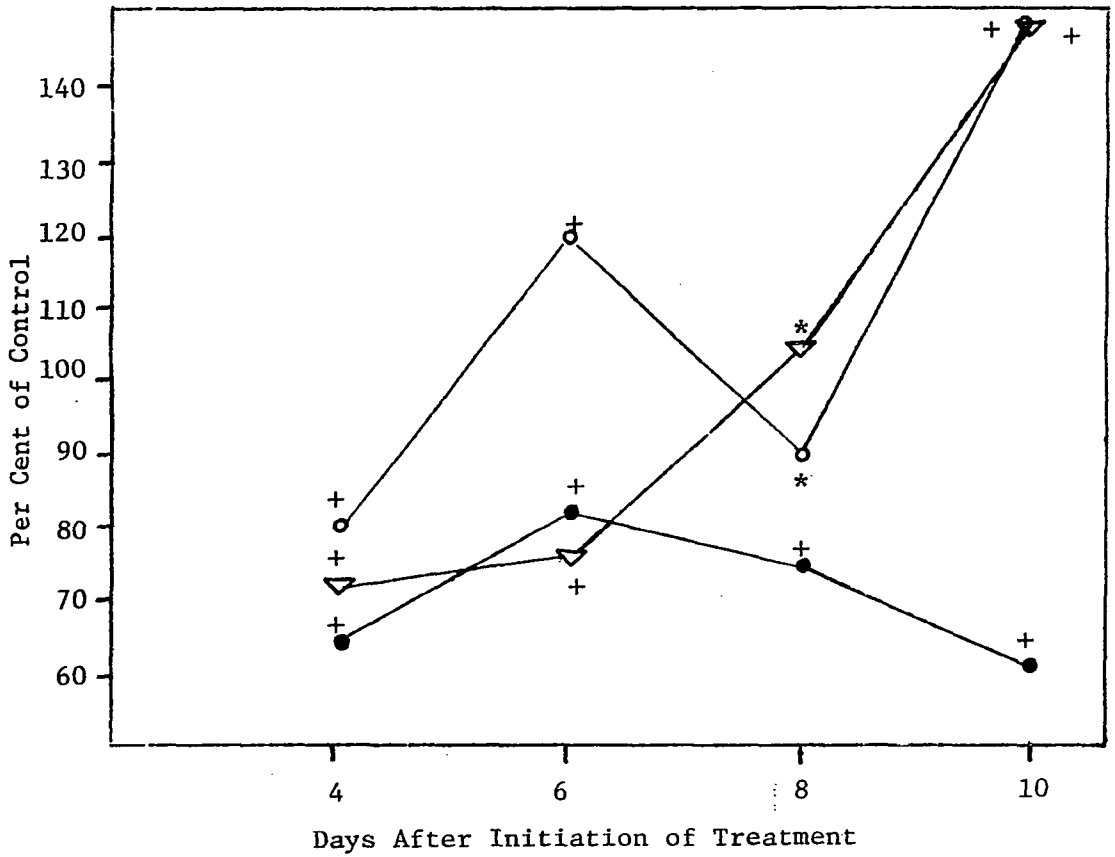

Figure I-3. Enzyme Patterns in CF-1 Cells After Treatment with 3,4-Benzo[a]pyrene (10 µg/ml). II.

○ Specific activity of glucose-6-phosphate dehydrogenase

● Specific activity of hexokinase

▽ Specific activity of lactate dehydrogenase

+ = $P < 0.01$; * = not significant

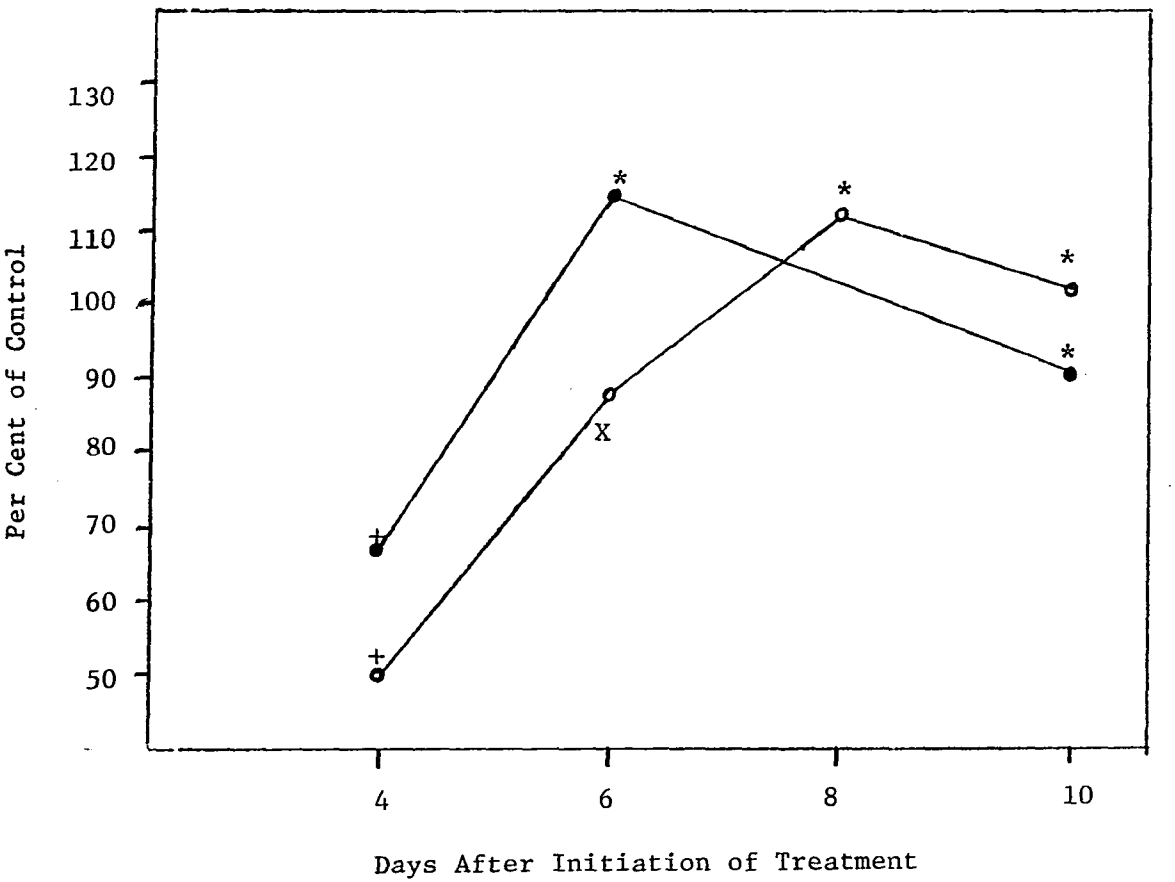

Figure I-4. Enzyme Patterns in CF-1 Cells after Treatment with 3,4-Benzo[a]pyrene (10 µg/ml). III.

○ Specific activity of pyruvate kinase

● Specific activity of 6-phosphogluconate dehydrogenase

+ = $P < 0.01$; x = $P < 0.05$; * = not significant

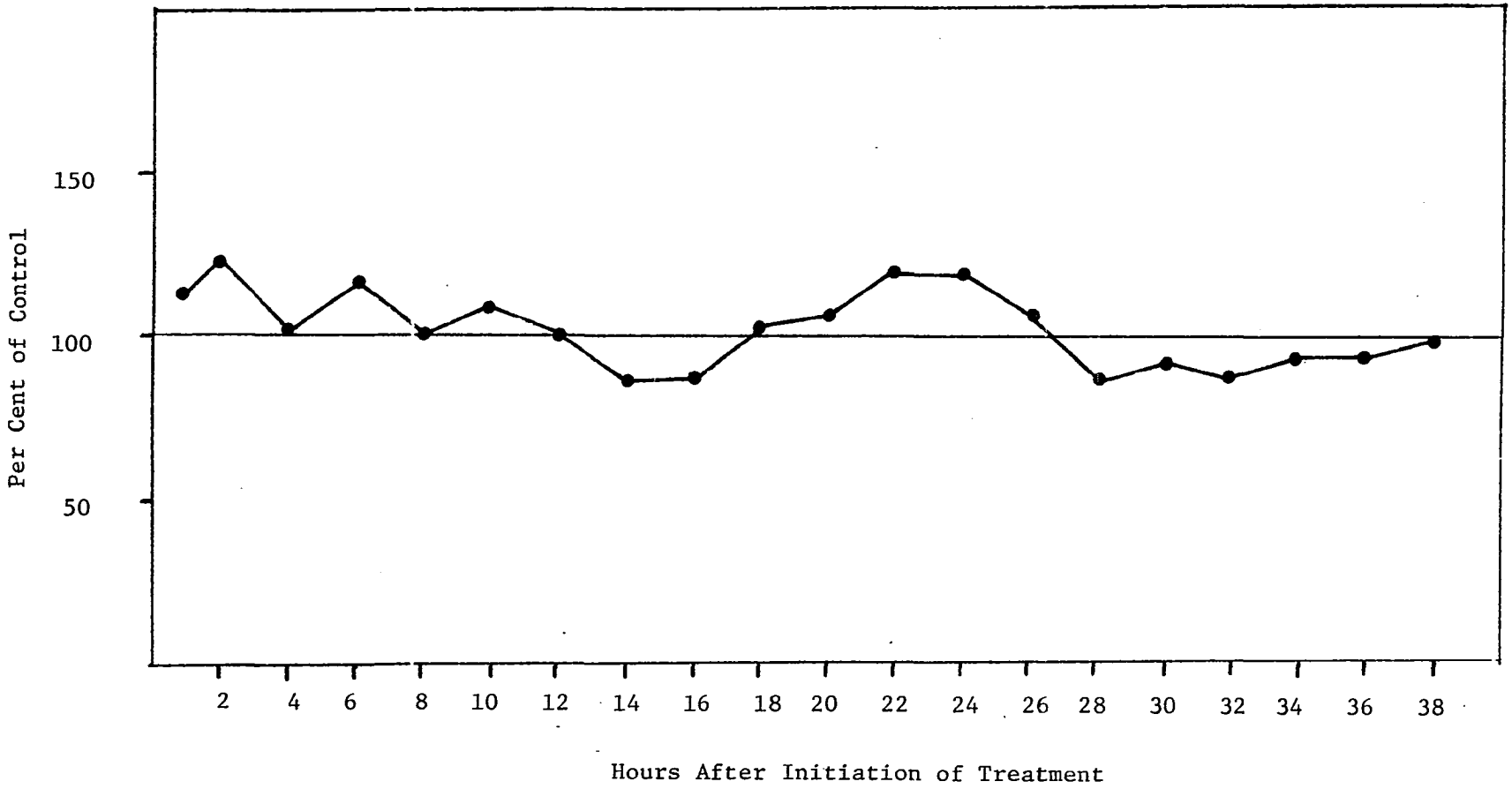

Figure I-5. Total RNA Synthesis in CF-1 Cells Treated with 3,4-Benzo[a]pyrene (10 µg/ml)

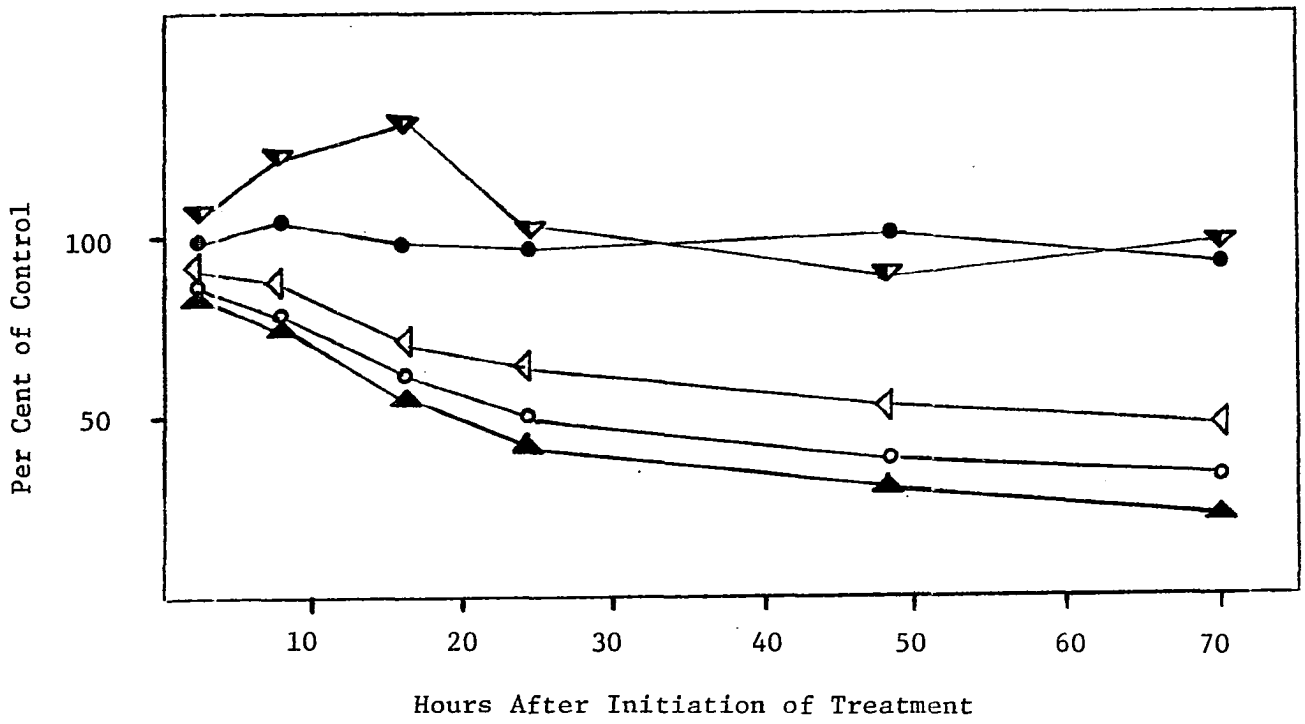

Figure I-6. Effect of Various Concentrations of 3,4-Benzo[a]pyrene on DNA Synthesis in CF-1 Cells.

● 5 µg/ml

▼ 10 µg/ml

◁ 15 µg/ml

○ 20 µg/ml

▲ 40 µg/ml

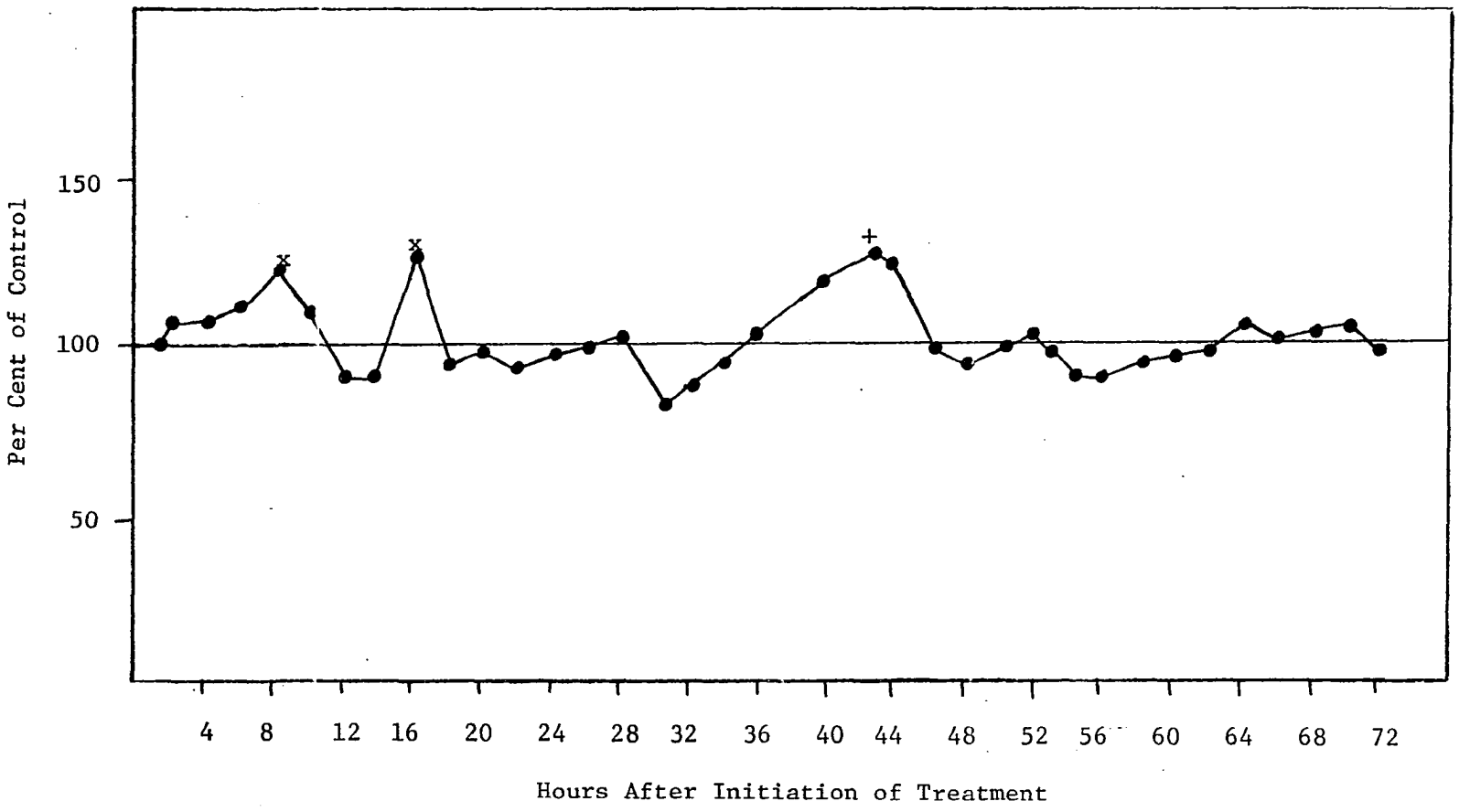

Figure I-7. DNA Synthesis in CF-1 Cells Treated with 3,4-Benzo[a]pyrene (10 µg/ml)

+ = P < 0.01

x = P < 0.05

after initial exposure. The peak at 8 hours was 123 per cent of the control ($P < 0.05$; $N = 5$) and the peak at 16 hours was 129 per cent of the control ($P < 0.05$; $N = 5$). The pooled results from hours 40, 43, and 45 showed a high level of significance ($P < 0.01$; $N = 9$) with the largest increase (1.4-fold) in DNA synthesis occurring at 43 hours. At all other time periods, treated cells were not significantly different from control cells in DNA synthesis.

Effect of Various Concentrations of Cigarette Smoke of DNA and RNA Synthesis in CF-1 Cells

The effect on DNA synthesis in CF-1 cells exposed to non-growth inhibiting concentrations of the cigarette smoke solution is represented in Figure I-8. The rate of thymidine incorporation by cells exposed to smoke concentrations of 1/1000 and 1/1200 was only 50 per cent that of the control cells after 8 hours. Cells exposed to smoke concentrations of 1/1500, 1/2000, and 1/2500 had about the same rate of thymidine incorporation as the control cells after 8 hours. Cells exposed to cigarette smoke concentrations of 1/3000 and 1/3500 had a 20 per cent greater thymidine incorporation than control cells at 8 hours, and cells exposed to smoke concentrations of 1/4000 and 1/4500 had about a 35 per cent increase over control cells in thymidine uptake. A smoke concentration of 1/5000 caused a 100 per cent increase in thymidine incorporation compared to control cells at 8 hours. After 24 hours of exposure, thymidine incorporation in all treated cells was not very different from that in control cells.

Thymidine incorporation into cells exposed to a cigarette smoke concentration of 1/1000 was measured at 2 hour intervals for 32 hours (Figure I-9). From 4 to 10 hours after initial exposure of the cells,

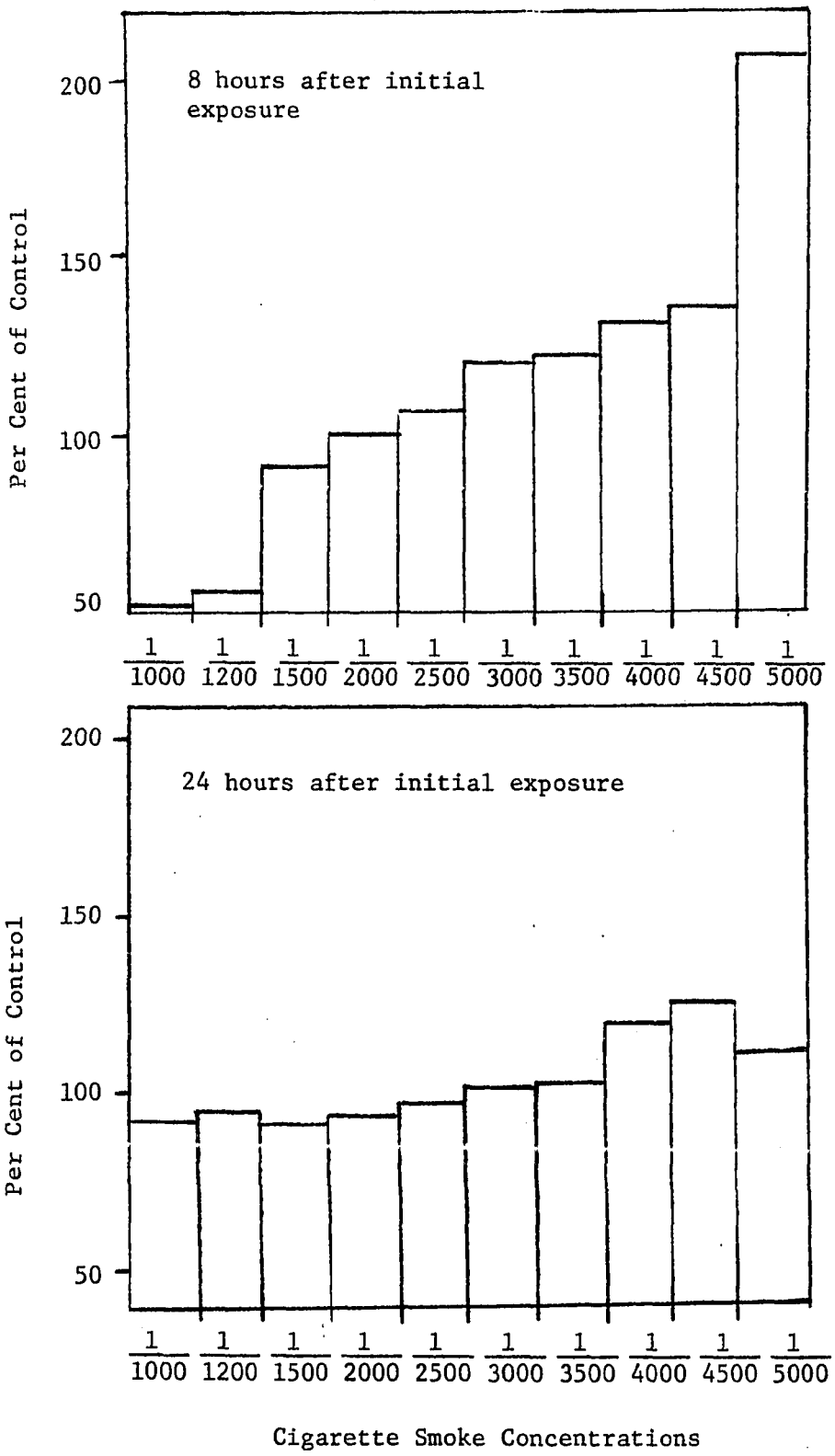

Figure I-8. DNA Synthesis in CF-1 Cells Exposed to Various Non-Growth-Inhibting Concentrations of Cigarette Smoke

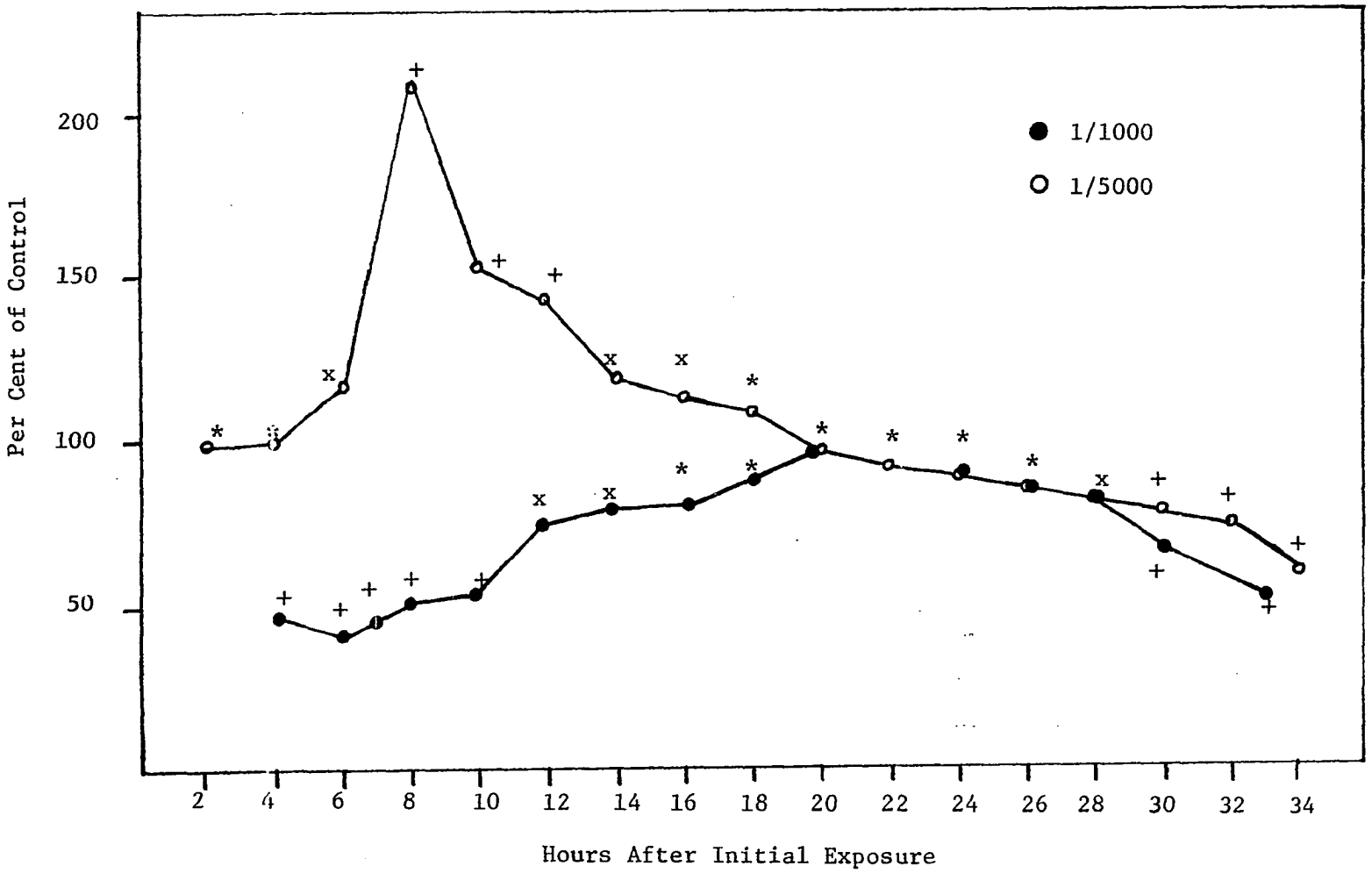

Figure I-9. DNA Synthesis in CF-1 Cells Exposed to Cigarette Smoke Concentrations of 1/1000 and 1/5000

+ = P < 0.01; x = P < 0.05; * = not significant

the rate of incorporation of thymidine by the treated cells is only 50 per cent ($P < 0.01$) that of the control cells. The rate of thymidine incorporation by the treated cells then increases during the 12 to 20 hour period, and the values from 18 to 28 hours do not differ significantly from the values in the control cells at this time period. The thymidine incorporation value in the treated cells begins to decrease from the control value after about 26 hours. A similar pattern of thymidine incorporation was observed in cells exposed to a smoke concentration of 1/1200 (Figure I-10). The thymidine incorporation pattern in cells exposed to a smoke concentration of 1/5000 (Figure I-9) is quite different from that in cells exposed to the smoke concentrations of 1/1000 and 1/1200. A large peak of increased thymidine incorporation ($P < 0.0001$) with a maximum of about two times the control value at 8 hours was observed from 6 to 16 hours after initial exposure of the cells to this smoke concentration. Table I-10 represents the data obtained from additional experiments performed with cells treated with smoke concentrations of 1/1000 and 1/5000.

Hours after treatment	DNA synthesis (CPM thymidine-methyl-H^3)						
	Control		$\frac{CS}{1000}$		$\frac{CS}{5000}$		N
	$\bar{x}$	S.E.	$\bar{x}$	S.E.	$\bar{x}$	S.E.	
8	2860	87	1418	74[1]	5875	81[1]	8
24	6163	431	5693	208[2]	5547	379[2]	6

Table I-10. Effect of cigarette smoke concentrations of 1/1000 and 1/5000 on DNA synthesis in CF-1 cells. $\bar{x}$, mean; S.E., standard error of the mean; N, number of determinations; P, probability (Student's t-test). [1]Significantly different ($P < 0.0001$) from the controls by Student's t-test. [2]Not significantly different from the control.

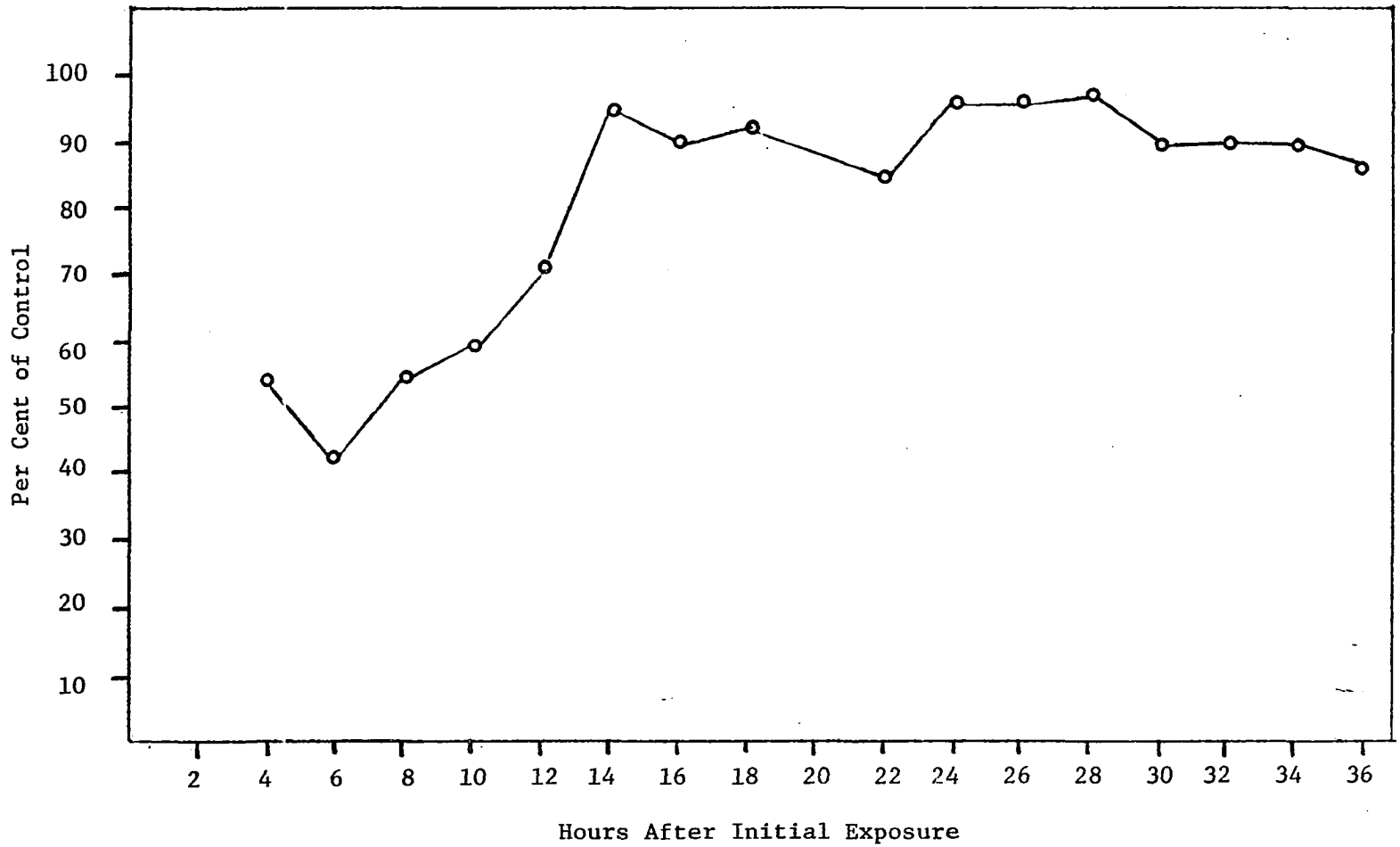

Figure I-10. DNA Synthesis in CF-1 Cells Exposed to a Cigarette Smoke Concentration of 1/1200.

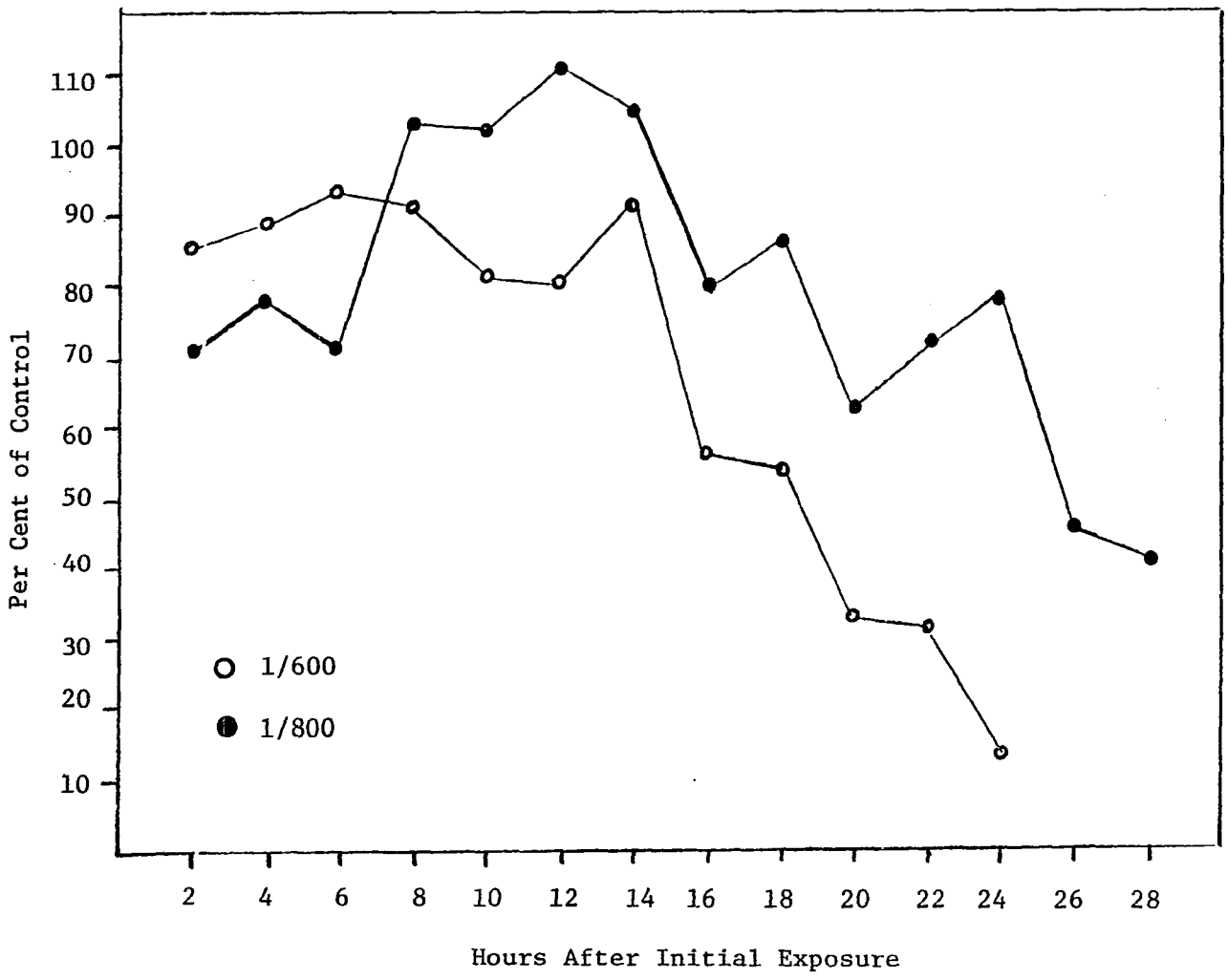

Figure I-11. Total RNA Synthesis in CF-1 Cells Exposed to Cigarette Smoke Concentrations of 1/600 and 1/800.

The time course of uridine incorporation in cells exposed to a cigarette smoke concentration of 1/600 is represented in Figure I-11. The rate of incorporation of uridine by the treated cells is about 80-90 per cent that of the control cells until 16 hours after initial exposure when the incorporation value of the treated cells decreases to 60 per cent that of the control cells. The uridine incorporation values in the treated cells show only progressive decreases from the control cells at all time periods measured after 16 hours. Uridine incorporation into cells treated with a smoke concentration of 1/800 (Figure I-11) was lower (70-85 per cent) than that of the control cells for 24 hours after initial exposure except between 8 and 14 hours ,when the rate of incorporation in treated and control cells was essentially the same. At the time periods measured after 24 hours, the uridine incorporation values of the treated cells were progressively lower than those of the control cells. A decrease ($P < 0.01$) in the uridine incorporation values was observed between 14 and 20 hours in cells exposed to a cigarette smoke concentration of 1/1000 (Figure I-12). The uridine incorporation in the treated cells did not differ significantly from that of the control cells at all other time periods. The uridine incorporation values in cells treated with a smoke concentration of 1/5000 (Figure I-12) was lower ($P < 0.05$) than that in control cells at 2, 6, between 10 and 14, 20 to 22, 32, and 36 hours but did not differ significantly from the controls at all other times. As can be seen from Figure I-12 , the pattern of uridine incorporation in cells exposed to a smoke concentration of 1/5000 is almost the mirror image of the pattern observed in cells exposed to a smoke concentration of 1/1000.

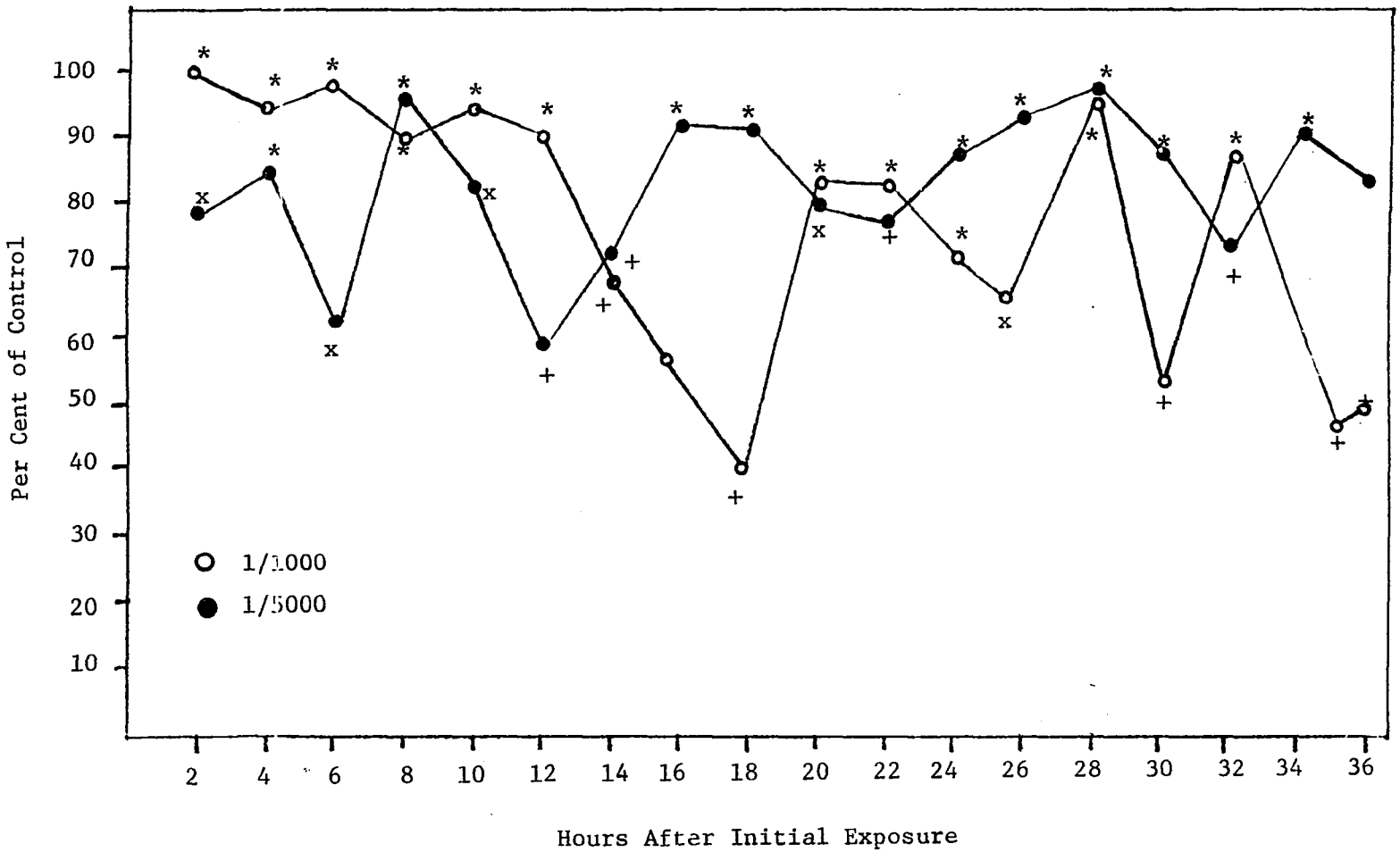

Figure I-12. Total RNA Synthesis in CP-1 Cells Exposed to Cigarette Smoke Concentrations of 1/1000 and 1/5000.

+ = P < 0.01; x = P < 0.05; * = not significant

CHAPTER IV

DISCUSSION

Concentrations of 5 or 10 µg/ml of benzo[a]pyrene (BP) had only a very slight inhibitory effect on the proliferation of the human fibroblast strain CF-1. At higher concentrations of BP (15-40 µg/ml), cell multiplication was inhibited to a greater extent (Table I-4). Diamond (1965) reported that multiplication of a diploid strain of human fibroblasts (WI-38) was not inhibited by exposure to BP at a concentration of 10 µg/ml for 47 days or by treatment with 20 µg/ml of BP for 17 days. Exposure of outgrowth cultures of normal human epidermis to BP at a concentration of 1 µg/ml for 4 days did not affect fibroblast growth (Flaxman and Dietz, 1971).

Toxicity, as demonstrated by inhibition of cell proliferation, was evident in all cigarette smoke concentrations greater than 1/800 (Table I-5). A very slight inhibitory effect upon cell growth was observed even with the lowest smoke concentration of 1/5000. Smith (1968) observed a similar pattern of inhibition of proliferation of both HeLa and WI-38 cell cultures exposed to various concentrations of the water-soluble portion of cigarette smoke tar. Water-soluble components of whole cigarette smoke inhibited growth in the KB line of human cells (Thayer and Kensler, 1964). Nakanishi and his coworkers (Nakanishi et

al., 1961) reported that fibroblast-like cells from human lung tissue showed cellular damage when exposed to cigarette smoke condensate in dilutions of 1:400, 1:800, and 1:1600. At 1:3200 and 1:10,000 dilutions, however, the cells were not damaged for at least 7 days. Experiments conducted by Kasai and Pollak (1964) showed that proliferation of rabbit's aortic and myocardial cells in vitro was immediately inhibited upon addition of cigarette smoke concentrations of 1/50 and 1/100, moderately inhibited at a smoke concentration of 1/200 and only slightly inhibited at a smoke concentration of 1/400. Guimard (1966) found that proliferation and mitotic activity in fibroblast tissues from chick embryos in vitro were inhibited at high concentrations of smoke condensate (0.1 % tobacco tar) but that, after a 24 hour period of inhibition, both were increased above the controls at lower tar concentrations (0.05 % tobacco tar).

The pattern of RNA synthesis as measured by uridine-H^3 incorporation into CF-1 cell cultures exposed to 10 µg/ml of BP for 38 hours (Figure I-5) was essentially the same as that of the control cultures. Most of the reported studies on the effects of carcinogens or of tumor-promoting substances of RNA synthesis involve the mouse skin application bioassay (Table I-3). As can be observed from these studies, the results obtained depend upon many variables; the investigator(s), the treatment material, and the biological system being a few of the most apparent of these variables. These experiments demonstrate that, in general, application of a carcinogen to mouse skin causes an initial decrease in RNA synthesis followed by a stimulation of RNA synthesis at some later time. In contrast, application of a cocarcinogen, or tumor-promoter, to mouse

skin causes an immediate stimulation of RNA synthesis. Enhancement of RNA synthesis was reported in a cell culture system (3T3 mouse fibroblasts) exposed to a tumor-promoter (Sivak and Van Duuren, 1970).

CF-1 cells exposed to 10 μg/ml of BP for 72 hours had increased peaks (relative to control cells) of DNA synthesis at 8 hours (1.2-fold), 16 hours (1.3-fold), and from 40 to 45 hours (1.4-fold). As with RNA synthesis, other reported studies on the effects of carcinogens or cocarcinogens on DNA synthesis involve the mouse skin application bioassay (Table I-3). Results of these studies show that application of either a carcinogen or a cocarcinogen to mouse skin causes an initial depression of DNA synthesis which, in some cases, was followed by a subsequent stimulation of DNA synthesis.

It is now known that different types of carcinogens interact with several components of cells both *in vivo* and *in vitro* including proteins, RNA, and DNA (Abell and Heidelberger, 1962; Alfred and DiPaolo, 1968; Brookes and Lawley, 1964; Diamond *et al.*, 1967; Duncan and Brookes, 1970; Duncan *et al.*, 1969; Kuroki and Heidelberger, 1971). In most cases, BP was found to be bound to proteins to a greater extent than to nucleic acids, whereas 7,12-DMBA was bound mostly to nucleic acids. Kuroki and Heidelberger (1971) found that when cultured embryonic cells (fibroblasts) from C3H mice were treated with labeled hydrocarbons, BP was bound to RNA with a specific activity about one-half that of 7,12-DMBA and bound to DNA to about one-fourth the extent of 7,12-DMBA.

Duncan *et al.* (1969) reported that the "binding index" of a polycyclic hydrocarbon (amount bound divided by amount metabolized) is more closely related to its carcinogenic activity than is binding itself.

The "binding index" of BP to mouse embryo cells was of the same magnitude for DNA, RNA, and protein and was about ten-fold less than the value for 7,12-DMBA "binding index" to RNA and DNA.

It is difficult to correlate the results obtained in the in vitro studies done with CF-1 cells exposed to BP with similar studies that have been reported, most of which involve an in vivo situation. Not only do the two bioassay systems differ greatly, but also the cell types differ in their response to polycyclic hydrocarbons. Diamond (1965) observed that normal rodent cells are sensitive to polycyclic aromatic hydrocarbon (PAH)-induced cytotoxicity; transformed or malignant rodent cells are resistant to PAH-induced cytotoxicity; and normal and transformed or malignant primate cells are resistant to PAH-induced cytotoxicity. This difference in response between sensitive and resistant cells in response to carcinogen-induced cytotoxicity was related to the amount of PAH that was found firmly bound to the cells. By fluorescence microscopy it was shown that BP concentrated in the cytoplasm of both sensitive and resistant cells; however, normal rodent cells bound ten to fifty times more hydrocarbon than did normal or transformed primate cells (Diamond, 1969).

Binding of carcinogens may require metabolism to a reactive form and resistant cells may be unable to metabolize the hydrocarbon or may metabolize it to a chemically unreactive form (Diamond, 1970). Cells which are relatively resistant to hydrocarbon toxicity have a much reduced capacity (relative to sensitive cells) to metabolize the hydrocarbons to water-soluble derivatives. Conversion of BP to water-soluble products was very inefficient in human diploid cells (Diamond

et al., 1968). One route of conversion of polycyclic hydrocarbons to water-soluble products in tissue culture is presumably by way of the microsomal aryl hydroxylase (benzpyrene hydroxylase) enzyme complex first described in rat liver by Conney et al., (1957). In cell cultures that are sensitive to the cytotoxic effect this enzyme has been found to be present and inducible by certain polycyclic aromatic hydrocarbons, while little or no activity has been found in cultures that are resistant to cytotoxicity (Alfred and Gelboin, 1967; Nebert and Gelboin, 1968). Thus, the difference in the magnitude of the response between the CF-1 cells and the mouse skin cells to exposure to polycyclic hydrocarbons may be due in part to a relatively small amount of binding of BP by the macromolecules of the CF-1 cells relative to that found in rodent cells. Also, most of the other studies involve effects seen when the biological system is exposed to 7,12-DMBA which binds to DNA and RNA to a much greater extent than does BP (Kuroki and Heidelberger, 1971).

Uridine-H^3 incorporation into CF-1 cells was inhibited at cigarette smoke concentrations of 1/600, 1/800 (Figure I-11), 1/1000, and 1/5000 (Figure I-12). The inhibition was gradually increased after 14 hours in the cells exposed to smoke concentrations of 1/600 and 1/800. A sharp drop in uridine-H^3 incorporation was observed between 14 and 20 hours in cells exposed to a cigarette smoke concentration of 1/1000 with incorporation returning to slightly below normal values by 28 hours. Uridine-H^3 incorporation into cells exposed to a smoke concentration of 1/5000 was very erratic, ranging between 60 to 95 per cent of the control from 2 to 32 hours. Leuchtenberger and Leuchtenberger

(1969, 1970) observed that one exposure of primary mouse kidney tissues in culture, covered with media, to ten puffs from the first half or to four puffs from the second half of an unfiltered cigarette resulted in a marked loss of RNA from the cytoplasm and nucleoli within 6 hours, followed by subsequent cell degeneration.

A 50 per cent decrease in incorporation of thymidine-H^3 into DNA was observed for up to 10 hours after exposure of CF-1 cells to cigarette smoke concentrations of 1/1000 (Figure I-9) and 1/1200 (Figure I-10). Thymidine incorporation returned to the level of the control by 18 hours in cells exposed to the 1/1000 concentration and was at control levels by 14 hours in cells exposed to a smoke concentration of 1/1200. After 20 hours there was a gradual decrease in thymidine incorporation in cells exposed to the 1/1000 smoke concentration, but thymidine incorporation remained only slightly below control levels in cells treated with a smoke concentration of 1/1200. Cells exposed to a cigarette smoke concentration of 1/5000 (Figure I-9) exhibited a marked stimulation of thymidine incorporation between 8 and 16 hours after initial exposure. A maximum rate of incorporation of two times the control value was observed at 8 hours with the level of synthesis returning to the level of the control by 20 hours. A gradual decrease in thymidine incorporation was observed after 20 hours. A similar response for thymidine-H^3 incorporation as measured by autoradiography was found *in vivo* in lungs taken from hamsters which had been exposed to cigarette smoke. Thymidine incorporation was enhanced in alveolar cells 8 hours after the beginning of the exposure with the labeling counts returning to control levels by 32 hours (Boren, 1970).

A low concentration of cigarette smoke (1/5000) caused a stimulation of DNA synthesis at 8 hours, whereas a higher smoke concentration (1/1000) exhibited an initial inhibitory effect on DNA synthesis (Table I-9). A stimulation of DNA synthesis at 8 hours was observed also in CF-1 cells treated with 10 μg/ml of BP (Figure I-6). The increase in DNA synthesis at 8 hours was much more pronounced with the low concentration of cigarette smoke (1/5000) than with BP. Of course, BP is only one representative of a large group of polyaromatic hydrocarbons which are the main suspected carcinogenic agents in cigarettes. Exposure of a biological test system to whole tobacco smoke involves simultaneous exposure to a variety of compounds (over 4000), and, while it is possible to ascribe a certain biological activity to some of the individual chemical components of tobacco smoke, i.e., carcinogenic, cocarcinogenic, and tumor-inhibitory agents, the complex nature of whole tobacco smoke and the possibility that a single chemical can have multiple biologic effects make it extremely difficult to determine the resultant effects of one type of agent upon another (Van Duuren et al., 1971).

Other studies indicate that carcinogens and cocarcinogens alter DNA synthesis in vivo (Table I-3). Several of these investigations have shown that stimulatory effects on macromolecular synthesis, especially DNA synthesis, precede hyperplasia (Hennings and Boutwell, 1970; Baird et al., 1971). A postulated scheme of the relationship between alteration of macromolecular synthesis by carcinogens and hyperplasia and neoplasia is given in Figure I-13.

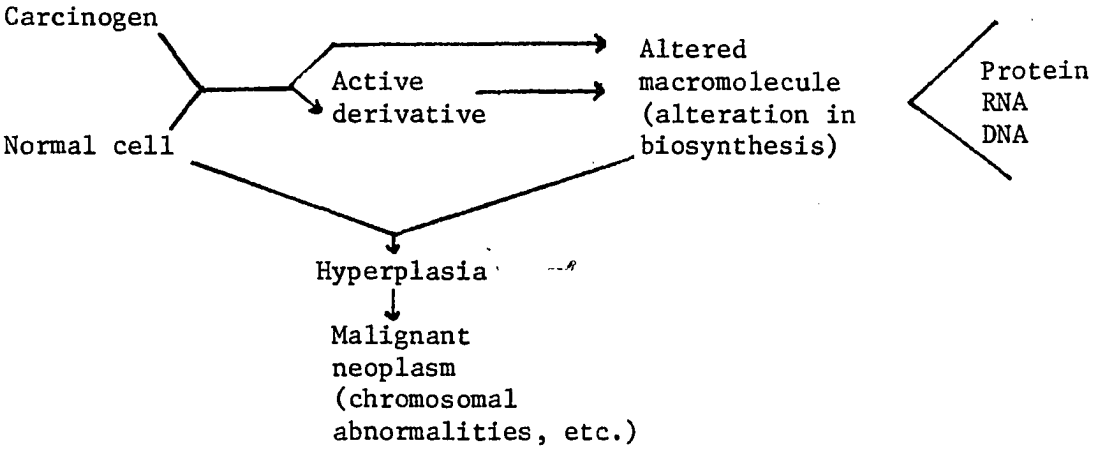

Figure I-13. Diagrammatic Representation of Some of the Interactions Between Cells and Carcinogens (from Farber, 1968).

One of the most remarkable features of this investigation was the observation that, although DNA synthesis at 8 hours differed by 400 per cent in cells exposed to cigarette smoke concentrations of 1/1000 and 1/5000; in each case the cell number did not differ significantly from the control cells at this time. Even at 24 hours there was little difference between the two groups in the number of cells. These results may reflect a change in the DNA content of the cells. Leuchtenberger and Leuchtenberger (1969) observed that primary mouse kidney tissue and embryonic mouse lung organ cultures showed a loss of DNA after exposure to several puffs of whole cigarette smoke. These investigators also measured total DNA by cytochemical methods in a 3T3 fibroblast cell line (Swiss mouse strain) that had been exposed to cigarette smoke from 2 days to 3 weeks and found that the exposed cultures had abnormally large nuclei with variable and sometimes high DNA content

(Leuchtenberger and Leuchtenberger, 1970).

A few cases have also been reported in which there is an increase in the synthesis of DNA without a corresponding increase in cell division rate. Doemer and Oehlert (1964) demonstrated that, following a single topical application of 20-MCA, a stimulation of DNA biosynthesis was induced in mouse skin, while the mitotic index did not differ significantly from the controls. Thus, in a biological system exposed to carcinogenic substances there is an indication that DNA may accumulate without a corresponding increase in cell division rate. This accumulation of DNA may reflect chromosome anomalies that have been found in several instances. DiPaolo et al., (1971) reported that primary cell cultures derived from primary tumors induced by subcutaneous injection of BP into hamsters showed an abrupt decrease of cell multiplication after the first few days in culture with the subsequent appearance of giant multinucleated cells. Borenfreund et al. (1966) found that transformed colonies derived from a Chinese hamster lung tissue line treated with 0.5 µg/ml of BP appeared to have chromosomal changes which did not occur in the untreated cell line. A large proportion of the carcinogen-treated cells appeared to have two additional chromosomes.

The increase in thymidine incorporation observed in CF-1 cells treated with the 1/5000 smoke concentration and after the initial inhibition of incorporation in cells exposed to smoke concentrations of 1/1200 and 1/1000 could also reflect DNA repair. Lieberman et al. (1971) have shown that some carcinogens as well as other alkylating agents were found to stimulate thymidine incorporation into DNA in nondividing human lymphocytes in the presence of hydroxyurea which

suppressed background incorporation of thymidine. Eighty to eighty-five per cent of the label appeared to be internal.

The specific activities of several of the glycolytic enzymes in CF-1 cells treated with 10 µg/ml of BP differed significantly from those of the control cells measured at the same time period. The specific activity of hexokinase was significantly lower in CF-1 cells exposed to 10 µg/ml of BP for 4, 6, 8, and 10 days (Tables I-6, I-7, I-8, and I-9). The specific activities of lactate dehydrogenase and phosphoglycerate kinase exhibited a gradual increase from below control levels after 4 days of BP treatment to a 1.5- and 2.0-fold increase respectively (relative to controls) after 10 days of treatment (Figures I-2 and I-3). Pyruvate kinase specific activity increased from 50 per cent of the control value after 4 days of exposure to BP to a level equal to that found in the control cells after 10 days of BP treatment (Figure I-4). The specific activity of phosphoglucoisomerase was slightly higher than the control value after 6 days of treatment with BP but decreased to 72 per cent of the value observed in control cells after 10 days of treatment (Figure I-2). After 4 days of exposure to BP the specific activity of glucose-6-phosphate dehydrogenase was 80 per cent of the control but was 1.5-fold higher than the control after 10 days of treatment (Figure I-3). The specific activity of 6-phosphogluconate dehydrogenase increased from a low of 67 per cent of the control after 4 days of the BP treatment to a level that was not significantly different from that of the control after 6 days (Figure I-4).

No experiments similar to these enzymatic rate studies have been reported. Several extensive studies have been reported which compare

the glycolysis pattern in a spectrum of hepatomas of different growth rates that were produced by feeding rats low doses of the liver carcinogen N-2-fluorenylphthalamic acid (FPA) (Morris, 1963, 1965). Two of the glycolytic enzymes which showed an increase in activity with increasing growth rate of the hepatomas were pyruvate kinase and hexokinase. The activity of glucose-6-phosphate dehydrogenase was found to be high in all hepatomas. All of the other enzymes involved in glucose metabolism that were measured in the CF-1 cell cultures were reported to be not correlated with growth rate of the hepatomas. In the slowest growing hepatoma the specific activities of hexokinase, pyruvate kinase, phosphoglucoisomerase, aldolase, and lactate dehydrogenase were 83, 157, 70, 41, and 65 per cent respectively of the values found in the liver of normal rats (Weber et al., 1964; Shonk et al., 1965; Weinhouse et al., 1966).

Dunaway and Smith (1971) found lower specific activities of glucose-6-phosphate dehydrogenase (48.4 per cent of the control), 6-phosphogluconate dehydrogenase (62.3 per cent of the control), and hexokinase (42.3 per cent of the control) in an SV-40-transformed cell line of human embryonic lung tissue (WI-38VA13A) than in its normal counterpart (WI-38). The activity of phosphoglucoisomerase appeared to be slightly higher in WI-38VA13A (1.3-fold) than in WI-38.

Since the biological systems and various carcinogenic agents are different, it is difficult to correlate directly the results of the enzymatic rate studies of the CF-1 cells exposed to BP for different lengths of time with either the enzyme patterns reported by Dunaway and Smith (1971) for WI-38 or WI-38VA13A or those patterns observed in

the spectrum of liver tumors (Weber et al., 1964; Shonk et al., 1965; Weinhouse et al., 1966).

A correlation that is readily apparent among the reported studies and this investigation is the observation of a low specific activity of hexokinase relative to the controls. From the data in Table I-6 it is evident that the specific activities of most of the enzymes assayed is significantly lower in the cells exposed to BP for 4 days than in the control cells. After treatment with BP for 6 days (Table I-7) the specific activities of most of the enzymes are closer to the values for these enzymes in the control cells with four of the enzymes having slightly higher specific activities than those in the control cells. After 10 days of treatment (Table I-9) the activities of glucose-6-phosphate dehydrogenase, lactate dehydrogenase, and phosphoglycerate kinase are 1.5-, 1.5-, and 2.0-fold higher, respectively, in the treated cells, whereas hexokinase and phosphoglucoisomerase are 1.6- and 1.4-fold higher in activity in the control cells. The activities of pyruvate kinase and 6-phosphogluconate dehydrogenase are nearly the same in both the treated and control cells.

Since BP has been shown to interact with DNA, RNA, and protein both in vivo and in vitro, the above results may be related to an initial block in the synthesis of the enzymes followed by an increase in synthesis as the BP is either metabolized to inactive products and the block is released or is diluted by partitioning and loss due to cell division. Binding of BP to protein may bring about a decrease in the activity of some of the enzymes which may be alleviated by metabolism, partitioning, and loss of the active carcinogen.

The pattern of activities of enzymes involved in glucose metabolism measured for the CF-1 cells and their BP-treated counterparts appears to be similar to that observed in WI-38 and WI-38VA13A cultures (Dunaway and Smith, 1971), i.e., 6-phosphogluconate dehydrogenase, hexokinase, and aldolase are in the low activity group in all three cell lines; phosphoglycerate kinase and glucose-6-phosphate dehydrogenase are in the intermediate activity group (about six times more active than the low activity group) in CF-1 cells, whereas only glucose-6-phosphate dehydrogenase is in this group in WI-38 and WI-38VA13A; the rest of the enzymes measured are in the high activity group in all three cell lines. Thus, treatment of CF-1 cells with 10 μg/ml of BP does not change the relative relationship of the various glycolytic enzymes based upon their specific activities.

Despite the fact that the two human fibroblast cultures originate from different tissues, the specific activities of most of the enzymes measured in the CF-1 cultures are of the same magnitude as those for WI-38 cultures (Tables I-6 - I-9; Dunaway and Smith, 1971). The activity of phosphoglycerate kinase appears to be about 5-fold greater in the WI-38 cells than in the CF-1 cells, whereas the activity of lactate dehydrogenase is about 3-fold higher in CF-1 cells grown for 4 to 6 days. The CF-1 cell cultures that were grown for 10 days had a lactate dehydrogenase activity similar to that measured in the WI-38 cultures. The data indicate that the activities of the high-activity enzymes in CF-1 cells decrease significantly as the cells are grown for longer than 6 days. The activities of the same enzymes also decrease in the cells exposed to BP; however, the activities of

lactate dehydrogenase and pyruvate kinase decrease only half as much in the treated cells as in the untreated cells. This decrease in the activities of these enzymes could possibly be a result of the contact inhibition of growth normally exhibited by human diploid cells in culture (Hayflick, 1965) since cessation of mitotic activity in confluent cultures of normal cells is associated with contact inhibition (Macpherson, 1970). Levine et al. (1965) have also found that depression of DNA and RNA synthesis and a reduction in free cytoplasmic polyribosomes occurs in association with the onset of contact inhibition in strains of human diploid fibroblasts. Both the control CF-1 cells and the treated cultures had reached confluency by the eighth day of growth. That the activities of lactate dehydrogenase and pyruvate kinase are not decreased by the same factor in the cells exposed to BP, despite the fact that the cell numbers are not significantly different in the two cultures after 10 days (BP = 9.10×10^5; control = 1.02×10^6), could reflect a change in the normal response to cell-cell contact by the BP treatment.

CHAPTER V

SUMMARY

The effect of cigarette smoke and one of its carcinogenic components, 3,4-benzo[a]pyrene (BP),upon DNA and RNA synthesis was studied in a human diploid strain of cells, CF-1. The effect of BP on the specific activities of several enzymes involved in glucose metabolism was also investigated. Cell proliferation was severely inhibited at smoke concentrations greater than 1/800 of a cigarette (CS/800). Concentrations of BP greater than 10 µg/ml inhibited cell multiplication to some extent. Total RNA synthesis was unaffected in cells treated with 10 µg/ml of BP for 38 hours, whereas increased peaks (relative to control cells) of DNA synthesis were observed at 8 hours (1.2-fold), 16 hours (1.3-fold), and from 40 to 45 hours (1.4-fold) after initial exposure. RNA synthesis was inhibited in cells exposed to cigarette smoke concentrations of CS/600, CS/800, CS/1000, and CS/5000 for up to 36 hours with the inhibition pattern differing depending upon the smoke concentration. A 50 per cent decrease in DNA synthesis was observed for up to 10 hours after exposure of the cells to smoke concentrations of CS/1000 and CS/1200; however, exposure of the cells to a smoke concentration of CS/5000 resulted in a marked stimulation of DNA synthesis between 8 and 16 hours. Although DNA synthesis at 8 hours differed

by 400 per cent in cells exposed to smoke concentrations CS/1000 and CS/5000, the cell number in each case was almost the same as from the control cells up to 48 hours, which may reflect a change in the DNA content of the cells. Thus, cigarette smoke and BP, to a lesser extent, appear to have a concentration and time-dependent effect on nucleic acid biosynthesis in CF-1 cells. Exposure of the cells to 10 μg/ml of BP for up to 10 days resulted in significant differences in the specific activities of several of the glycolytic enzymes. The specific activity of hexokinase was lower in treated cells measured at 4, 6, 8, and 10 days. The specific activities of glucose-6-phosphate dehydrogenase, lactate dehydrogenase, and phosphoglycerate kinase were 1.5-, 1.5-, and 2.0-fold greater, respectively, in the treated cells than in the control cells after 10 days. Therefore, the effect of BP on each of the enzymes measured varies with the length of exposure.

II. THE EFFECT OF ZINC, CALCIUM, IRON, AND COPPER CONCENTRATIONS ON SCOPOLIN, SCOPOLETIN, AND LIGNIN IN TOBACCO TISSUE CULTURES

CHAPTER I

INTRODUCTION

Zinc was recognized as an essential component of biological systems in 1869 when Raulin showed that zinc was indispensable for the growth of Aspergillus niger. In 1926 zinc was shown to be essential for the growth of a number of green plants in culture solution. (Sommer and Lipman, 1926).

Evidence of deranged metabolism in plants deficient in zinc is observable by chlorosis and blanching of the leaves; thickening, curling, and underdevelopment of leaves; and by stunting and rosetting due to failure of internodes to develop their normal length. Leaves of zinc deficient plants also frequently contain abnormal quantities of phenolic substances (Chesters and Rolinson, 1950).

Zinc ions are known to be required for a variety of metabolic processes in plants. The activities of a number of enzymes, i.e., alcohol dehydrogenase of Neurospora (Nason, Kaplan, and Colowick, 1951), carbonic anhydrase of oat and tomato plants (Wood and Sibly, 1952), nitrate reductase of wheat seedlings (Harper and Paulson, 1969), aldolase of higher plants (Quinlan-Watson, 1951), and D-lactate dehydrogenase of Euglena

gracilis have been reported to be related to the amount of zinc available.

A marked deficiency of auxin is evident in plants deficient in zinc. Skoog (1940) showed that when tomato plants were grown in a zinc deficient solution, the plants failed to elongate and their auxin content was much lower than that of the control. Addition of zinc to the culture solution caused an increase in auxin in the plant within twenty-four hours, with elongation of the stem soon taking place. Recent studies have shown that free tryptophan and tryptamine accumulate in leaves of zinc deficient maize seedlings (Takaki and Kushizaki, 1970).

The presence of an excess amount of zinc also results in a deranged metabolism in plants. Studies done by Lee, Craddock and Hammer (1969) showed that the presence of large amounts of soluble zinc in the soil is a cause of abnormal plant growth with symptoms resembling zinc-induced iron deficiency. These investigators studied the influence of iron on the growth of fiber flax at various levels of zinc sulfate and found that plant growth in nutrient solutions containing high levels of zinc sulfate was increased by the addition of iron both as ferric chloride and as sodium ferric ethylenediamine di-[0-hydroxyphenylacetate]. Zinc appeared to interfere with iron uptake, whereas iron did not interfere with zinc uptake. In a study of the effect of zinc on the translocation of iron in soybean plants by Ambler, Brown, and Gauch (1970), zinc was observed to interfere with translocation of iron from roots to above ground parts of the soybean plants. Addition of iron as sodium ferric ethylenediamine di-[0-hydroxyphenylacetate] to the growth medium overcame the interference of zinc.

Results of studies by Carles et al. (1969) on various types of green plants grown in the field indicate that the role of zinc in plants appears to be related to the abundance of certain other elements such as calcium. According to observations by these researchers, calcium appears to facilitate the utilization of zinc and to diminish its toxicity to such an extent that certain plants become indifferent to an overabundance of zinc.

Magnesium salts appear to be more effective than iron in counteracting the growth supression in Aspergillus niger brought about by high concentrations of zinc.

Reed (1938) found that the leaves of plants suffering from a deficiency in zinc frequently show an accumulation of phenolic materials. The coumarin scopoletin and its 7-glucoside scopolin have been reported to occur in many plants. Wender and associates have shown that environmental stress conditions such as low daytime temperature, excess amounts of growth regulators, and relatively high intensity ultraviolet and x-ray irradiation cause the concentrations of scopoletin and scopolin to increase above normal levels (Wender, 1970). Leaves of boron deficient tobacco plants have been reported with a 20-fold increase of scopolin over leaves of control plants (Watanabe, et al., 1961). Loche and Chouteau (1963) reported that calcium, magnesium, and phosphorus deficient conditions cause an accumulation of certain phenolic compounds in tobacco. Loche reported that deficiencies in nitrogen and also in sulfur are accompanied by an accumulation of chlorogenic acids and rutin. Studies done by Armstrong (1968) at the University of Oklahoma on various mineral deficiencies on tobacco plants showed that increases in scopolin relative

to controls were evident after one week in nitrogen deficient plants, after three weeks in magnesium deficient plants, and after five weeks in potassium deficient plants.

Lignin is a complex polymer formed from the polymerization of free radicals derived from certain phenolic compounds (Brown, 1966). The relationship between lignin, scopoletin, and scopolin remains obscure. Some stress conditions that cause an increase in scopolin cause a decrease in lignification (Wender, 1970); however, other stress conditions have been shown to cause an increase of both phenolic compounds and lignin (Cheng and Marsh, 1968; Smith and Attridge, 1970). Recently it has been demonstrated that neither scopolin nor scopoletin is a lignin precursor but that scopoletin can be polymerized to a lignin-like compound (Lowenberg, 1970; Innerarity, 1970). Lipetz (1962) found that high concentrations of calcium result in a reduction of lignification in sunflower gall tissue in culture. Wheat plants grown with low calcium and low phosphate contained a higher percentage of lignin than normal plants (Parish and Miller, 1969). The data obtained in a preliminary experiment by Schafer and Wender (1970) with tobacco tissue cultures indicate that the degree of lignification is increased in tissues grown on media with low concentrations of calcium.

Preliminary experiments were designed to determine if zinc concentration would affect the growth of W-38 tobacco tissues in culture and if an effect on the concentration of scopolin and scopoletin could be observed in these tissues with varying zinc concentrations. Based on the results of various zinc studies mentioned previously, experiments were designed to determine the effects of varying zinc and calcium concentrations upon the growth, concentration of scopolin and scopoletin, and the

degree of lignification in W-38 tobacco tissue culture. Additional experiments were designed for the study of the effects of iron and copper on these same parameters in W-38 tissues grown on media containing high concentrations of zinc.

CHAPTER II

METHODS AND MATERIALS

Growth of Tobacco Tissue Cultures

Stock Cultures

Callus tissue from tobacco, Nicotiana tabacum L. variety Wisconsin #38 (W-38), obtained from Dr. Folke Skoog of the University of Wisconsin, was used throughout these studies. The tissues were grown on the revised medium (RM-1964) of Linsmaier and Skoog (1965) with 2 mg/l of indoleacetic acid, 0.2 mg/l of kinetin, and 1.0 mg/l of thiamine hydrochloride. The complete medium was prepared according to a procedure used by Smith (1968).

The flasks were inoculated with small pieces of firm, white callus tissue from four to five week old stock cultures. Each flask was inoculated with three disks obtained by cutting a cylinder of callus tissue with a #3 sterile cork borer, extruding the cylinder from the borer with a sterile glass rod, and cutting transverse slices of approximately 2 mm thickness. The callus disks were placed on the surface of the agar with sterile forceps. All cultures were prepared in a clean laminar flow hood (Agnew-Higgins, Inc.) which was swabbed with 70% ethanol just prior to use. The flasks were placed on a table in diffuse light at room temperature until harvested five weeks later.

Experimental Cultures

For the experimental studies the callus tissues were grown in test tubes made of Kimble #N-51A "noncorrosible" glass (Bellco Glass Co., Vineland, N.J.) containing 25 ml of the revised medium (RM-1964) without agar. The tissues were supported on a filter pad constructed by folding a 5.5 cm disk of filter paper so as to form a cylinder with one open end which was then inserted, open end first, into the tube and the pad so formed pushed down until its surface was just level with that of the medium (Heller, 1965). Each tube was inoculated with one disk of white callus tissue approximately 8 mm in diameter and 2 mm thick, prepared in the same manner as the disks for the stock cultures, from five week old stock cultures. Either five or seven tubes were inoculated for each of the levels of the cations being studied in a particular experiment. The tubes were then placed upright in racks and allowed to grow for 35 days at room temperature in diffuse light.

All glassware used in the experiments was cleaned in 0.1M Na_2EDTA for 30 minutes, rinsed with distilled water, soaked in 10% (v/v) HNO_3 for 30 minutes, and rinsed thoroughly in double distilled water (Harper and Paulsen, 1969).

Fixation and Extraction

At the end of the growth period (35 days) the tissues from all of the tubes at each level were pooled, weighed, and fixed by boiling in isopropyl alcohol for five minutes.

The fixed samples were then ground in a blender at high speed for about one minute, transferred into paper extraction thimbles which were contained in a funnel, constructed in the shape of a thimble, and then ex-

tracted by the procedure used by Innerarity (1970).

The residue was brought to a known volume (50 ml) with isopropanol: benzene:methanol:water (2:1:1:1, v/v/v/v) (IBMW) in the following manner: the round bottom flask containing the residue was rinsed with 2 ml of benzene and 2 ml of water, followed by four ml of benzene and 4 ml of water (two times), then by 10 ml of methanol, and brought to volume with isoproanol.

The plant material is thus divided into two main fractions by this extraction procedure. The combined extracts represent the IBMW-soluble fractions and the residue remaining in the thimble respresents the IBMW-insoluble fraction.

Determination of Total Extractable Residue, Non-extractable Residue, and Dry Weight

From each sample, an aliquot (10 ml) of the IBMW-soluble material was pipetted into a preweighed bottle. The solvent was removed under reduced pressure in a vacuum drying oven and the residue weighed to a constant weight. The total extractable residue (ER) for each sample was then determined using this weight.

The residue remaining in the Soxhlet thimble following the extraction (IBMW-insoluble fraction) was transferred to a preweighed sintered glass filter of coarse porosity and dried to a constant weight. The weight of the material in the coarse sintered glass filter is designated as the non-extractable residue (NER).

The dry weight (DW) of each sample is equal to the total extractable residue weight plus the non-extractable residue weight of the sample.

Lignin Determination

Lignin determinations were made on each sample using the procedure of Bergman (Bergman, 1964). A portion (≤150 mg) of the non-extractable residue (NER) was weighed and placed in a preweighed 100 ml round bottom flask. The sample was then hydrolyzed for 24 hours by shaking in 72% sulfuric acid at room temperature. The samples were then diluted with distilled water to 3% sulfuric acid and further hydrolyzed by autoclaving for one hour at 121°C and 15 psi. The samples were then cooled, filtered through preweighed sintered glass filters of medium porosity, washed free of sulfate with distilled water and weighed to a constant weight. The residue is called Klason lignin or 72% sulfuric acid lignin.

Scopoletin and Scopolin Determinations

Quantitation of scopoletin and scopolin was performed using one-dimensional paper chromatography (Koeppe, 1968; Einhellig, 1969). The papers were then developed in a descending chromatographic chamber with one of two ternary solvent systems - (1) methylisobutyl ketone: formic acid: water (14:3:2, v/v/v) (KFW) for scopolin and (2) isopropanol: formic acid: water (5:0.1:95, v/v/v) (IFW) for scopoletin. After development for the appropriate amount of time (20 hours for scopolin and 6 hours for scopoletin), the papers were dried and the fluorescent band of either scopolin or scopoletin could be observed under an ultraviolet light. The fluorescent bands were circled with a pencil, cut out, and eluted with 5% methanol, and the fluorescence of each sample was measured on a Turner Model 110 Fluorimeter. The instrument was zeroed using a blank which had been run through procedures identical with those of the particular set of samples

for which fluorescence was being measured.

Standard reference curves were prepared for scopolin and scopoletin by running known quantities of the authentic compounds through the entire procedure. The concentration of scopolin and scopoletin was determined either by using the standard reference curve or from standards developed at the same time as the samples.

CHAPTER III

RESULTS

Tobacco callus tissues were grown in tubes containing liquid medium as described in the experimental section. At each concentration of zinc, of calcium and zinc, or of zinc, iron, and copper either five or seven tubes were grown. The tissues were harvested after a five week growth period, weighed, extracted, and analyzed. The sets of data in calcium and zinc experiments (Tables II-1, II-2, II-4, II-5, II-7, and II-8; Figures II-1, II-2, II-3, and II-4) were obtained four months apart. The data on the zinc, iron, and copper experiments (Tables II-3, II-6, and II-9; Figure II-5) were obtained four months after the last set of calcium and zinc data. Each experiment was run in duplicate and the data presented represent the average of the two experiments with the exception of the values for lignin. The lignin was derived from the pooled non-extractable residue of the duplicate experiments.

Effect of Calcium and Zinc Concentrations on the Growth of W-38 Tobacco Tissue

As the calcium concentration in the medium was reduced, the tissues became smaller and darker brown. No significant variation in the color or the size of the tissues within a particular level of calcium was observed when the concentrations of zinc were varied from 0.0 mg per liter to 86 mg per liter (10X); however, tissues grown in medium contain-

ing higher concentrations of zinc, i.e., 430 mg per liter (50X) or 860 mg per liter (100X), were small, very dark brown, and dry. Tissues grown at a calcium concentration of 1452 mg per liter (3.3X) were similar in color and size to tissues grown at the normal calcium concentration.

The data in Tables II-1 and II-2 show that, in general, the fresh weight of tissues grown at lower calcium levels was less than that of tissues at the 1X calcium level. Tissues grown at the highest zinc level (100X) showed the greatest reduction in fresh weight. The dry weights of the tissues paralleled the fresh weights, i.e., there was no significant change in the per cent water in the tissues grown at all of the various calcium and zinc levels except for those tissues at the highest concentration of zinc, 860 mg per liter (100X). The amount of water in this group was significantly less ($P<0.01$) than in the tissues that had been grown at the other concentrations of zinc.

The amounts of non-extractable residue (NER) and extractable residue (ER) in the tissues were affected by both the calcium and zinc concentration. The extractable residue was reduced as the calcium concentration was either decreased or increased from the 1X calcium level. Tissues grown at the 100X zinc level showed the greatest reduction in extractable residue. The amount of non-extractable residue showed a decrease at concentrations of calcium both lower and higher than the normal (1X) calcium concentration; however, as is reflected in the ratio of the total extractable residue to the non-extractable residue (ER/NER), the amount of NER increases relative to the ER in tissues grown at either increased or decreased calcium concentrations (compared to the 1X level), and this is particularly evident for tissues grown at the 100X level of zinc.

TABLE II-1

THE EFFECT OF CALCIUM AND ZINC CONCENTRATION IN THE MEDIUM

ON THE GROWTH OF W-38 TOBACCO CALLUS TISSUE I

Amount of $CaCl_2 \cdot 2H_2O$ x = 440 mg/l of medium	Amount of $ZnSO_4 \cdot 4H_2O$ x = 8.6 mg/l of medium	Fresh Weight g	ER g	NER g	$\frac{ER}{NER}$	Dry Weight (DW)	$\%H_2O$
1/100 x	0	7.86	0.193	0.122	1.58	0.315	96.0
1/100 x	1x	7.87	0.191	0.113	1.69	0.304	96.1
1/100 x	10x	6.17	0.200	0.075	2.67	0.275	95.5
1/100 x	100x	4.63	0.145	0.103	1.41	0.248	95.0
1/10 x	0	9.05	0.234	0.116	2.02	0.350	96.1
1/10 x	1x	9.08	0.260	0.111	2.34	0.371	96.0
1/10 x	10x	11.14	0.294	0.126	2.34	0.420	96.5
1/10 x	100x	4.65	0.141	0.088	1.60	0.229	95.0
1 x	0	9.87	0.257	0.111	2.32	0.368	96.1
1 x	1x	12.47	0.459	0.147	3.12	0.606	96.8
1 x	10x	9.99	0.273	0.112	2.44	0.385	96.1
1 x	100x	3.95	0.163	0.069	2.36	0.232	94.2
3.3 x	0	7.69	0.299	0.126	2.38	0.425	94.6
3.3 x	1 x	11.52	0.335	0.101	3.32	0.436	96.1
3.3 x	10 x	8.60	0.245	0.102	2.40	0.347	96.0
3.3 x	100 x	4.55	0.151	0.089	1.69	0.240	94.7

ER : Total extractable residue
NER : Non-extractable residue
Each level contained ten pieces of tissue.
Data represent the average of duplicate experiments.

TABLE II-2

THE EFFECT OF CALCIUM AND ZINC CONCENTRATION IN THE MEDIUM

ON THE GROWTH OF W-38 TOBACCO CALLUS TISSUE II

Amount of $CaCl_2 \cdot 2H_2O$ x= 440 mg/l of medium	Amount of $ZnSO_4 \cdot 4H_2O$ x = 8.6 mg/l of medium	Fresh Weight g	ER g	NER g	ER / NER	Dry Weight (DW)	$\%H_2O$
1/250 x	0	6.72	0.211	0.157	1.34	0.368	94.5
1/250 x	1 x	9.02	0.264	0.162	1.66	0.429	95.1
1/250 x	50 x	8.80	0.261	0.188	1.39	0.449	94.9
1/100 x	0	7.41	0.343	0.145	2.46	0.488	93.4
1/100 x	1 x	10.16	0.292	0.179	1.63	0.471	94.9
1/100 x	50 x	8.41	0.253	0.170	1.49	0.423	95.0
1/50 x	0	6.42	0.198	0.144	1.38	0.342	94.6
1/50 x	1 x	8.86	0.289	0.162	1.78	0.451	95.0
1/50 x	50 x	7.94	0.264	0.169	1.56	0.433	94.6
1 x	0	8.05	0.338	0.124	2.73	0.462	94.2
1 x	1 x	9.58	0.344	0.169	2.04	0.513	94.7
1 x	50 x	7.97	0.327	0.138	2.37	0.465	94.3

ER : Total extractable residue
NER : Non-extractable residue
Each level contained ten pieces of tissue.
Data represent the average of duplicate experiments.

Effect of Zinc, Iron, and Copper Concentrations on the Growth of W-38 Tobacco Tissue

The data in Table II-3 show that the tissues grew optimally in a medium having 8.6 mg of $ZnSO_4 \cdot 7H_2O$ per liter (1X), 27.8 mg of $FeSO_4 \cdot 7H_2O$ per liter (1X), and 0.025 mg of $CuSO_4 \cdot 5H_2O$ per liter (1X). The growth of the tissues at the normal zinc (1X) level was inhibited by increased concentrations of both copper and iron. The weight of the tissues grown in medium with a $FeSO_4 \cdot 7H_2O$ concentration of 139 mg per liter (5X) and normal levels (1X) of zinc and copper was 44 per cent of the normal tissues, and at a concentration of iron of 278 mg per liter (10X) the tissue weight decreased to 32 per cent of the normal tissue. When the concentrations of iron and zinc were at normal levels (1X), increased concentrations of copper did not have as detrimental an effect on the growth of the tissues; i.e., the weight of the tissues grown in medium with a $CuSO_4 \cdot 5H_2O$ concentration of 0.125 mg per liter (5X) was 93 per cent of the control, and at a copper concentration of 0.25 mg per liter (10X) the weight of the tissues was 67 per cent of the control. When the level of both iron and copper was 5X, the weight of the tissues was 49 per cent of the control.

The tissues grown in medium having a $ZnSO_4 \cdot 4H_2O$ concentration of 645 mg per liter (75X) and normal levels of iron and copper had a fresh weight that was only 38 per cent of the normal tissues. Increased levels of copper (5X and 10X) and/or iron (5X) decreased the inhibition in growth caused by the high zinc concentration; i.e., when the $CuSO_4 \cdot 5H_2O$ concentration was increased to 0.125 mg per liter (5X), the tissue weight

TABLE II-3

THE EFFECT OF ZINC, IRON, AND COPPER CONCENTRATION IN THE MEDIUM

ON THE GROWTH OF W-38 TOBACCO CALLUS TISSUE

Amount of $ZnSO_4 \cdot 4H_2O$ x = 8.6 mg/l of medium	Amount of $FeSO_4 \cdot 7H_2O$ x= 27.8 mg/l of medium	Amount of $CuSO_4 \cdot 5H_2O$ x=0.025 mg/l of medium	Fresh Weight g	ER g	NER g	$\frac{ER}{NER}$	Dry Weight (DW)	$\%H_2O$
1 x	1 x	1 x	23.93	0.555	0.367	1.51	0.922	96.4
1 x	5 x	1 x	10.61	0.250	0.241	1.04	0.491	95.4
1 x	1 x	5 x	22.34	0.554	0.340	1.63	0.894	95.1
1 x	5 x	5 x	11.76	0.296	0.285	1.04	0.581	95.0
1 x	10 x	1 x	7.74	0.287	0.208	1.38	0.495	93.8
1 x	1 x	10 x	16.12	0.410	0.307	1.33	0.717	95.6
75 x	1 x	1 x	9.09	0.286	0.261	1.09	0.547	94.0
75 x	5 x	1 x	10.91	0.327	0.224	1.46	0.551	95.1
75 x	1 x	5 x	14.59	0.411	0.358	1.15	0.769	94.6
75 x	5 x	5 x	12.29	0.369	0.277	1.29	0.646	94.4
75 x	10 x	1 x	8.46	0.276	0.236	1.17	0.512	94.0
75 x	1 x	10 x	13.37	0.411	0.299	1.37	0.710	94.8

ER : Total extractable residue
NER : Non-extractable residue
Each level contained fourteen pieces of tissue.
Data represent the average of duplicate experiments.

was 61 per cent of the control (1X), the weight of the tissues at the 10X level of copper was 56 per cent of the control; the weight of the tissues at the 5X level of iron was 46 per cent of the control but only 35 per cent of the control at the 10X iron level; and the weight of the tissues and the 5X iron, 5X copper level was 52 per cent of the control.

The per cent water in the tissues grown at the 75X zinc concentration and at the various levels of iron and copper was less ($P<0.05$) than the tissues grown at the various iron and copper levels at the 1X zinc concentration.

The amount of extractable material (ER) in the tissues at the normal zinc level was greatly decreased by increased concentrations of iron and slightly decreased by increased concentrations of copper. The largest reduction in the ER was observed in tissues grown at the 75X zinc level and normal levels of iron and copper. The amount of non-extractable material (NER) exhibited the same trend as the ER. Increasing the levels of copper (5X and 10X) and/or iron (5X) helped to overcome the reduction in NER and ER in tissues grown at the 75X zinc level. The ratio of ER to NER appeared not to be affected to any great extent by increased levels of copper, iron, or zinc.

Effect of Calcium and Zinc Concentrations on Lignification

The degree of lignification occurring in W-38 tobacco tissue was affected by the concentrations of calcium and zinc in the medium (Tables II-4 and II-5). The amount of lignin, as per cent of the non-extractable residue and as per cent of the dry weight, increased at concentrations of

TABLE II-4

THE EFFECT OF CALCIUM AND ZINC CONCENTRATION IN THE MEDIUM

ON NON-EXTRACTABLE RESIDUE AND LIGNIN I

Amount of $CaCl_2 \cdot 2H_2O$ X = 440 mg/l of medium	Amount of $ZnSO_4 \cdot 4H_2O$ X = 8.6 mg/l of medium	Dry Weight (DW) g	NER g	Lignin mg	Lignin as % NER	Lignin as % DW
1/100 X	0	0.315	0.122	13.6	5.3	5.7
1/100 X	1 X	0.304	0.113	12.8	11.3	4.2
1/100 X	10 X	0.275	0.075	5.6	7.5	2.0
1/100 X	100 X	0.248	0.103	21.8	21.2	8.8
1/10 X	0	0.350	0.116	8.6	7.4	2.5
1/10 X	1 X	0.371	0.111	6.9	6.2	1.9
1/10 X	10 X	0.420	0.126	4.9	3.9	1.2
1/10 X	100 X	0.229	0.088	12.2	13.9	5.4
1 X	0	0.368	0.111	2.7	2.4	0.7
1 X	1 X	0.606	0.147	3.8	2.6	0.6
1 X	10 X	0.385	0.112	7.4	6.6	1.9
1 X	100 X	0.232	0.069	6.7	9.7	2.9
3.3 X	0	0.425	0.126	6.2	4.9	1.5
3.3 X	1 X	0.436	0.101	9.1	9.0	2.1
3.3 X	10 X	0.347	0.102	6.0	5.9	1.7
3.3 X	100 X	0.240	0.089	11.5	12.9	4.8

NER : Non-extractable residue
Each level contained ten pieces of tissue.

TABLE II-5

THE EFFECT OF CALCIUM AND ZINC CONCENTRATION IN THE MEDIUM

ON NON-EXTRACTABLE RESIDUE AND LIGNIN II

Amount of $CaCl_2 \cdot 2H_2O$ X = 440 mg/l of medium	Amount of $ZnSO_4 \cdot 4H_2O$ X = 8.6 mg/l of medium	Dry Weight (DW) g	NER g	Lignin mg	Lignin as % NER	Lignin as % DW
1/250 X	0	0.368	0.157	33.8	21.6	9.2
1/250 X	1 X	0.429	0.162	25.0	15.5	5.8
1/250 X	50 X	0.449	0.188	42.5	22.6	9.5
1/100 X	0	0.488	0.145	26.7	18.6	5.1
1/100 X	1 X	0.471	0.179	27.8	15.6	5.9
1/100 X	50 X	0.423	0.170	38.8	22.8	9.1
1/50 X	0	0.342	0.144	32.4	22.5	9.5
1/50 X	1 X	0.451	0.162	22.8	14.1	5.1
1/50 X	50 X	0.433	0.169	37.2	21.9	8.6
1 X	0	0.462	0.124	4.9	3.9	1.1
1 X	1 X	0.513	0.169	14.3	8.4	2.8
1 X	50 X	0.465	0.138	6.4	4.7	1.4

NER : Non-extractable residue
Each level contained ten pieces of tissue.

calcium both lower and higher than the normal calcium concentration. Different concentrations of zinc within a particular calcium level also affected lignification. Tissues grown at the higher levels of zinc (50X and 100X) had an amount of lignin that was two to eight times that of the control.

The data are presented graphically in Figures II-1, II-2, II-3, and II-4.

Effect of Zinc, Iron, and Copper Concentrations on Lignification

The data in Table II-6 (Figure II-5) show that the degree of lignification was increased relative to the control by high concentrations of iron and decreased by high concentrations of copper in the tissues grown in medium with the normal zinc concentrations. The amount of lignin, as per cent of the non-extractable residue and as per cent of the dry weight, was increased relative to the control in the tissues grown in the high zinc concentration (75X) with normal concentrations of copper and iron. Increasing concentrations of copper and of iron decreased the amount of lignin as per cent NER and as per cent DW relative to that at the 75X zinc, 1X iron, 1X copper level.

Effect of Calcium and Zinc Concentrations on Scopolin and Scopoletin

The data in Tables II-7 and II-8 (Figures II-1, II-2, II-3, and II-4) show that the concentrations of both scopolin and scopoletin increased as the concentration of calcium in the medium decreased relative to the normal calcium concentration. The concentration of scopolin at

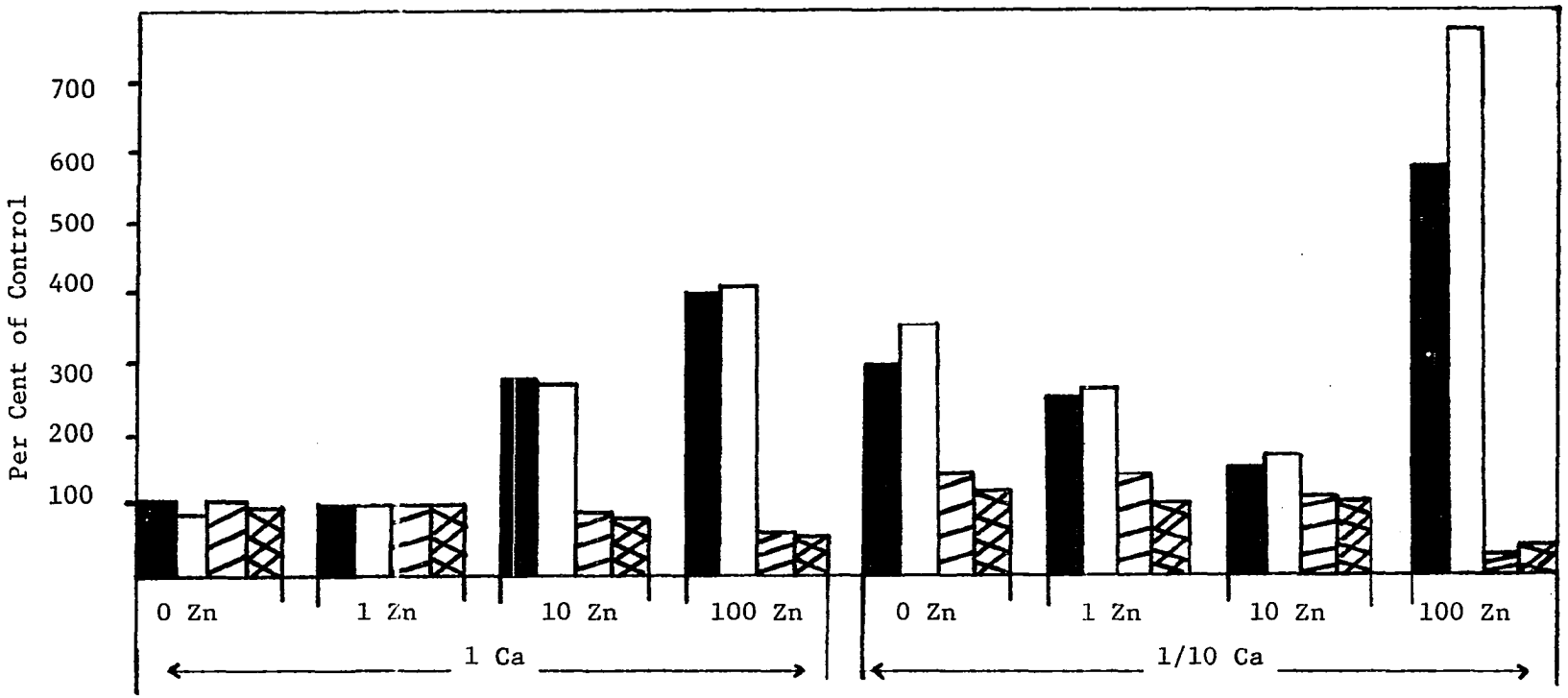

Figure II-1. The Effect of Calcium and Zinc Concentration on the Degree of Lignification and on the Concentrations of Scopolin and Scopoletin Ia.

- ■ Lignin as per cent of non-extractable residue
- □ Lignin as per cent dry weight
- ▨ Concentration of scopolin (µg/ml/g fresh weight)
- ▩ Concentration of scopoletin (µg/ml/g fresh weight)

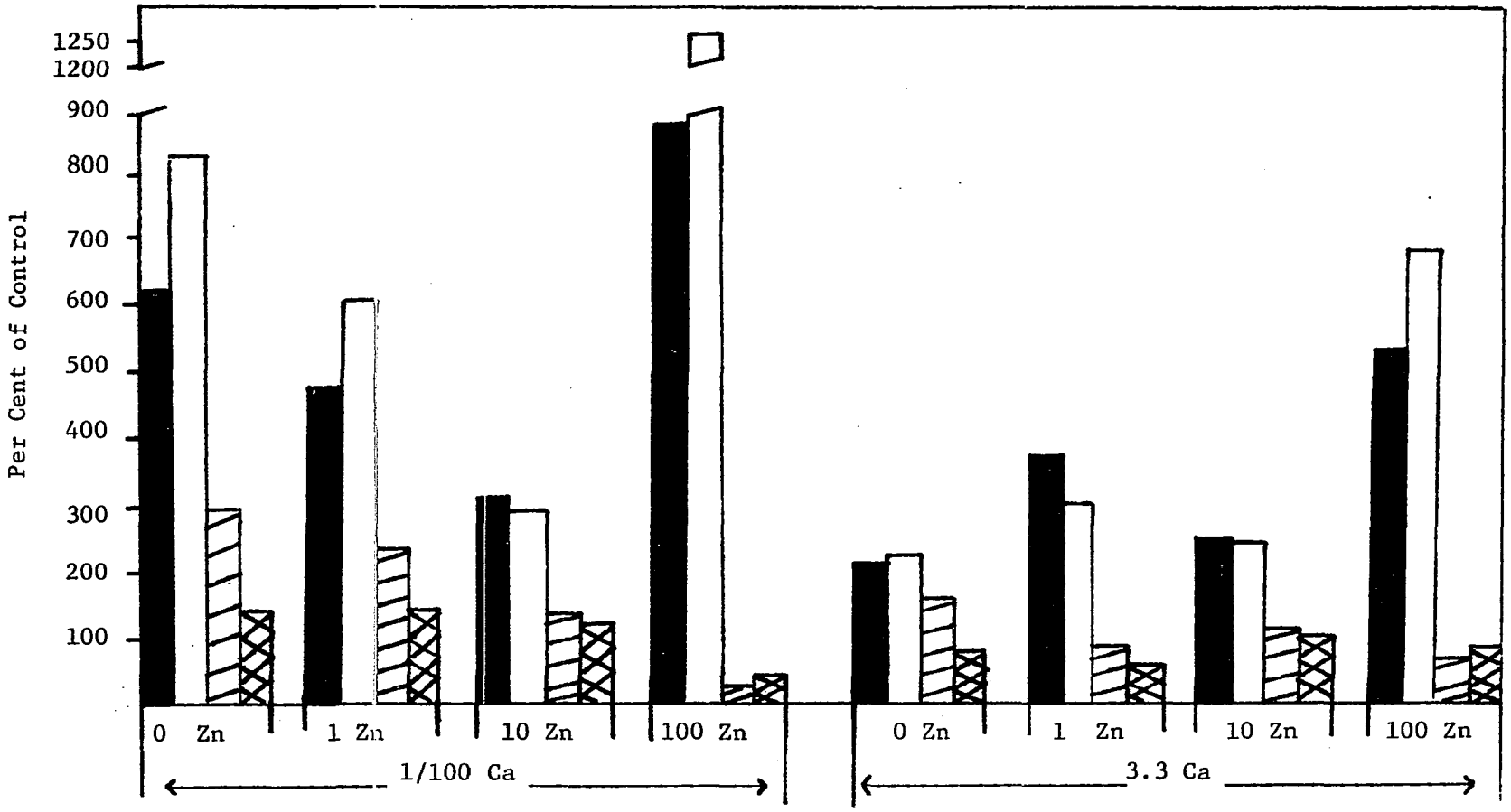

Figure II-2. The Effect of Calcium and Zinc Concentration on the Degree of Lignification and on the Concentrations of Scopolin and Scopoletin Ib.

- Lignin as per cent non-extractable residue
- Lignin as per cent dry weight
- Concentration of scopolin (µg/ml/g fresh weight)
- Concentration of scopoletin (µg/ml/g fresh weight)

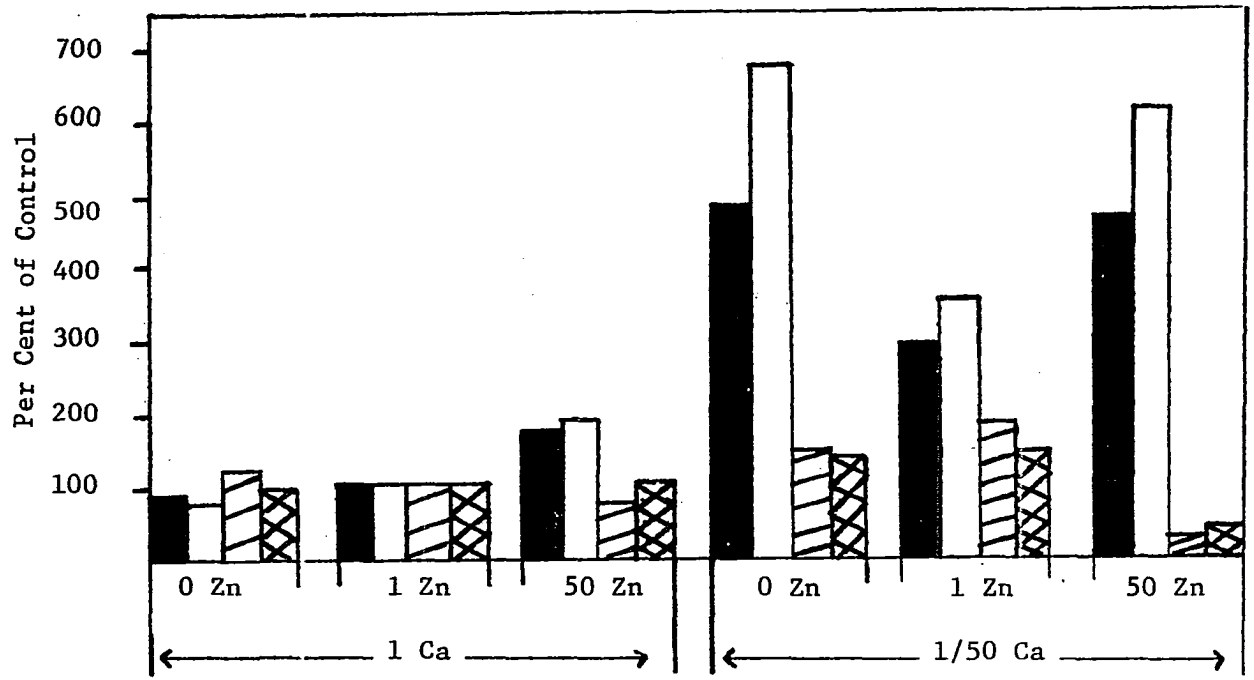

Figure II-3. The Effect of Calcium and Zinc Concentration on the Degree of Lignification and on the Concentrations of Scopolin and Scopoletin IIa.

■ Lignin as per cent non-extractable residue

□ Lignin as per cent dry weight

▨ Concentration of scopolin (µg/ml/g fresh weight)

▩ Concentration of scopoletin (µg/ml/g fresh weight)

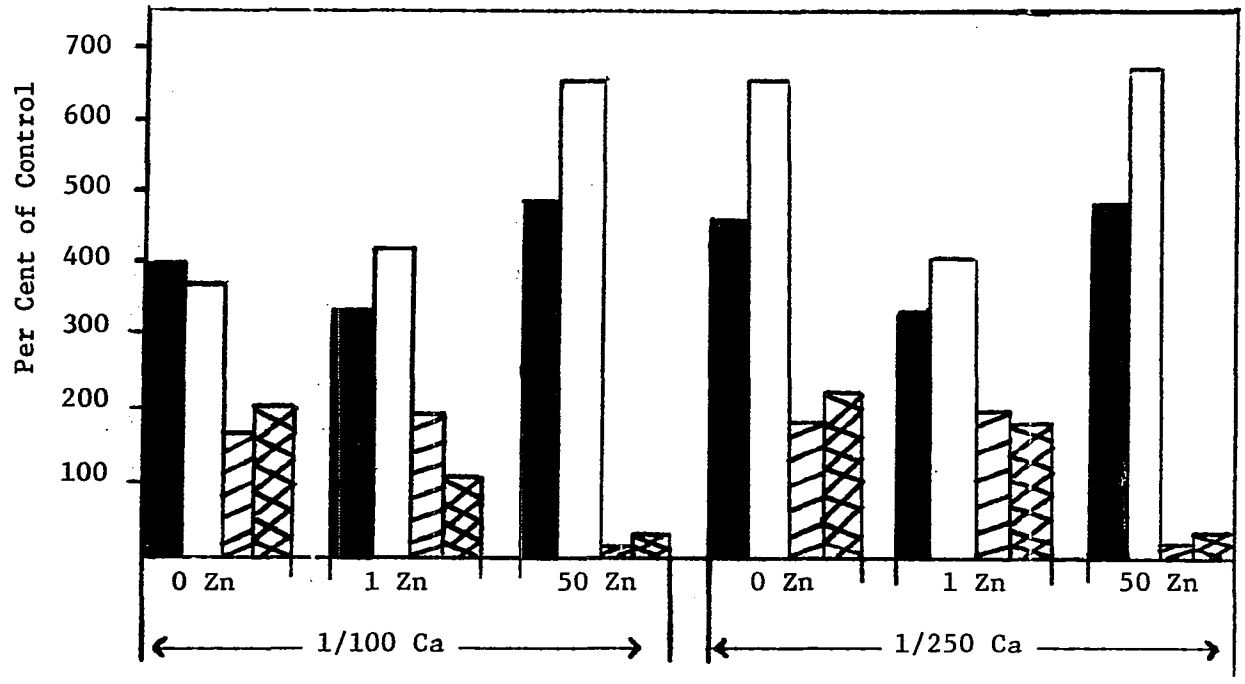

Figure II-4. The Effect of Calcium and Zinc Concentration on the Degree of Lignification and on the Concentrations of Scopolin and Scopoletin IIb.

- Lignin as per cent non-extractable residue
- Lignin as per cent dry weight
- Concentration of scopolin (µg/ml/g fresh weight)
- Concentration of scopoletin (µg/ml/g fresh weight)

TABLE II-6

THE EFFECT OF ZINC, IRON, AND COPPER CONCENTRATION IN THE MEDIUM

ON NON-EXTRACTABLE RESIDUE AND LIGNIN

Amount of $ZnSO_4.4H_2O$ X = 8.6 mg/l of medium	Amount of $FeSO_4.7H_2O$ X = 27.8 mg/l of medium	Amount of $CuSO_4.5H_2O$ X = 0.025 mg/l of medium	Dry Weight (DW) g	NER g	Lignin mg	Lignin as % NER	Lignin as % DW
1 X	1 X	1 X	0.922	0.367	25.2	6.8	2.7
1 X	5 X	1 X	0.491	0.241	29.8	12.4	6.1
1 X	1 X	5 X	0.894	0.340	16.4	4.8	1.8
1 X	5 X	5 X	0.581	0.285	22.0	7.7	3.8
1 X	10 X	1 X	0.495	0.208	25.2	12.1	5.1
1 X	1 X	10 X	0.717	0.307	14.3	4.7	2.0
75 X	1 X	1 X	0.547	0.261	30.7	11.8	5.6
75 X	5 X	1 X	0.551	0.224	18.0	8.0	3.3
75 X	1 X	5 X	0.769	0.358	35.0	9.8	4.6
75 X	5 X	5 X	0.646	0.277	26.4	9.5	4.1
75 X	10 X	1 X	0.512	0.236	21.6	9.2	4.2
75 X	1 X	10 X	0.710	0.299	18.8	6.3	2.7

NER : Non-extractable residue
Each level contained fourteen pieces of tissue.

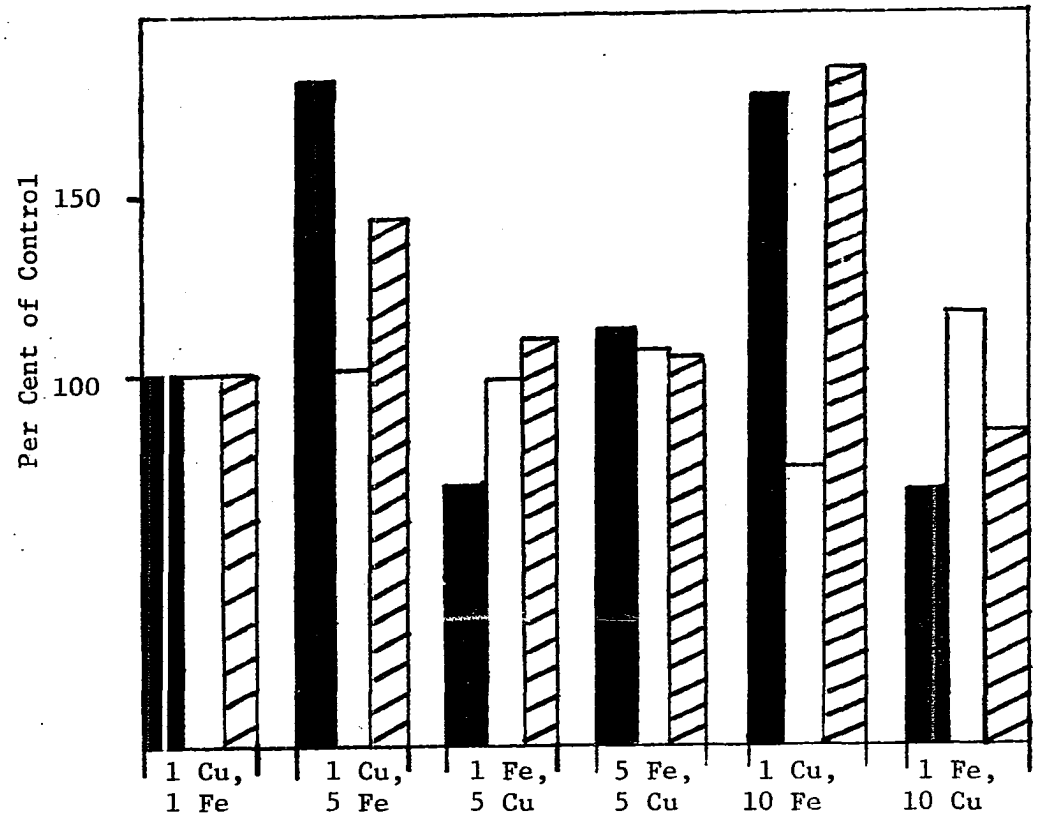

Figure II-5. The Effect of Zinc, Iron, and Copper Concentration on the Degree of Lignification and on the Concentrations of Scopolin and Scopoletin at a Zinc Concentration of 1X

■ Lignin as per cent non-extractable residue

□ Concentration of scopolin (µg/ml/g fresh weight)

▨ Concentration of scopoletin (µg/ml/g fresh weight)

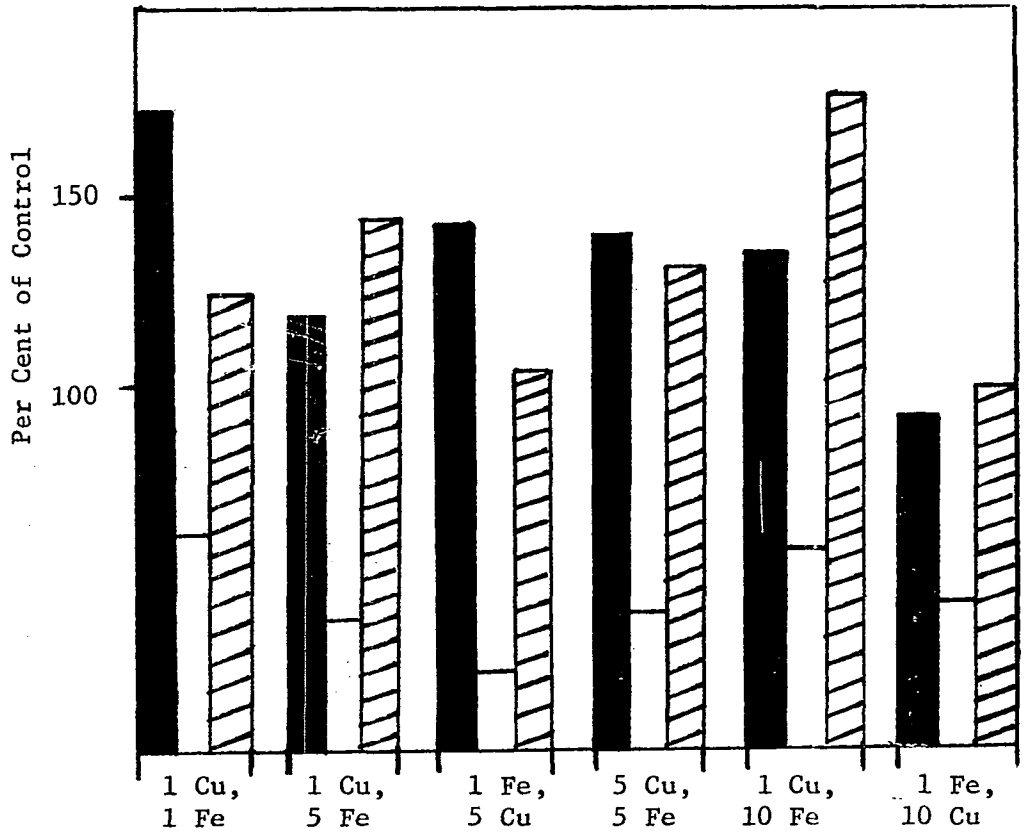

Figure II-6. The Effect of Zinc, Iron, and Copper Concentration on the Degree of Lignification and on the Concentrations of Scopolin and Scopoletin at a Zinc Concentration of 75X

■ Lignin as per cent non-extractable residue

□ Concentration of scopolin (µg/ml/g fresh weight)

▨ Concentration of scopoletin (µg/ml/g fresh weight)

TABLE II-7

THE EFFECT OF CALCIUM AND ZINC CONCENTRATION IN THE MEDIUM

ON THE CONCENTRATION OF SCOPOLIN, SCOPOLETIN, AND

ON THE SCOPOLIN-SCOPOLETIN RATIO I

Amount of $CaCl_2 \cdot 2H_2O$ X = 440 mg/l of medium	Amount of $ZnSO_4 \cdot 4H_2O$ X = 8.6 mg/l of medium	µg of scopolin per g - fresh weight	µg of scopoletin per g - fresh weight	Scopolin / Scopoletin
1/100 X	0	226	4.78	47.3
1/100 X	1 X	178	4.93	36.2
1/100 X	10 X	101	4.26	23.7
1/100 X	100 X	27	1.35	20.0
1/10 X	0	114	4.30	26.6
1/10 X	1 X	115	3.89	29.6
1/10 X	10 X	93	4.04	23.0
1/10 X	100 X	22	1.34	16.4
1 X	0	84	3.54	23.8
1 X	1 X	78	3.62	21.6
1 X	10 X	68	2.91	23.4
1 X	100 X	49	2.22	22.1
3.3 X	0	104	2.28	45.6
3.3 X	1 X	65	2.38	27.4
3.3 X	10 X	85	3.55	24.0
3.3 X	100 X	55	3.16	17.4

TABLE II-8

THE EFFECT OF CALCIUM AND ZINC CONCENTRATION IN THE MEDIUM ON THE CONCENTRATION OF SCOPOLIN, SCOPOLETIN, AND ON THE SCOPOLIN-SCOPOLETIN RATIO II

Amount of $CaCl_2 \cdot 2H_2O$ X = 440 mg/l of medium	Amount of $ZnSO_4 \cdot 4H_2O$ X = 8.6 mg/l of medium	µg of scopolin per g - fresh weight	µg of scopoletin per g - fresh weight	Scopolin / Scopoletin
1/250 X	0	182	8.36	21.8
1/250 X	1 X	204	6.90	29.6
1/250 X	50 X	15.8	1.16	15.1
1/100 X	0	162	7.60	21.3
1/100 X	1 X	196	5.95	33.0
1/100 X	50 X	16.4	1.17	14.1
1/50 X	0	149	5.26	28.3
1/50 X	1 X	187	5.51	33.9
1/50 X	50 X	24.4	1.53	16.0
1 X	0	121	3.57	33.9
1 X	1 X	78.4	4.17	18.8
1 X	50 X	101	3.76	26.8

the lower levels of calcium increased to a greater extent than the concentration of scopoletin. Different concentrations of zinc within a particular calcium level also affected the concentrations of scopoletin and scopolin. The tissues grown at the higher levels of zinc (50X and 100X) had a large decrease in the concentrations of both scopolin and scopoletin. The per cent decrease in the concentration of scopolin at the higher concentrations of zinc was more than the per cent decrease in scopoletin. These changes in the relative concentrations of scopolin and scopoletin are reflected in the value of the ratio of scopolin to scopoletin. The concentration of both scopolin and scopoletin in the 3.3X level of calcium did not differ to any great extent from the values obtained at the normal calcium level.

Effect of Zinc, Iron, and Copper Concentrations on Scopolin and Scopoletin

The most striking effects of zinc, iron, and copper concentrations on scopolin and scopoletin are those of the high zinc (75X) on scopolin concentration and of the high iron levels (5X and 10X) on the concentration of scopoletin (Table II-9; Figure II-5). Tissues grown at the zinc concentration of 75X had an average scopolin concentration that was only 41 per cent that of tissues at the 1X zinc level. Tissues at the high iron levels (5X and 10X), excluding the 5X iron, 5X copper levels, had an average scopoletin concentration that was 1.6-fold greater than the average scopoletin concentration of tissues at all levels containing a 1X iron concentration. When the 5X iron, 5X copper levels were included in the high iron levels, the average scopoletin concentration was 1.4-fold

TABLE II-9

THE EFFECT OF ZINC, IRON, AND COPPER CONCENTRATION IN THE MEDIUM ON THE CONCENTRATION OF SCOPOLIN, SCOPOLETIN, AND ON THE SCOPOLIN-SCOPOLETIN RATIO

Amount of $ZnSO_4 \cdot 4H_2O$ X = 8.6 mg/l of medium	Amount of $FeSO_4 \cdot H_2O$ X = 27.8 mg/l of medium	Amount of $CuSO_4 \cdot 5H_2O$ X = 0.025 mg/l of medium	µg of scopolin per g - fresh weight	µg of scopoletin per g - fresh weight	Scopolin / Scopoletin
1 X	1 X	1 X	139	3.05	45.6
1 X	5 X	1 X	141	4.34	32.5
1 X	1 X	5 X	137	3.37	40.6
1 X	5 X	5 X	147	3.24	45.4
1 X	10 X	1 X	104	5.61	18.5
1 X	1 X	10 X	163	2.55	63.9
75 X	1 X	1 X	80.3	3.75	21.4
75 X	5 X	1 X	46.7	4.37	10.7
75 X	1 X	5 X	39.7	3.10	12.8
75 X	5 X	5 X	48.8	3.97	12.3
75 X	10 X	1 X	69.9	5.37	13.0
75 X	1 X	10 X	53.0	2.99	17.7

greater than the average found in tissues grown at all levels of 1X iron. All tissues grown at the high zinc concentration showed a large decrease in the scopolin to scopoletin ratio at all various levels of copper and iron.

Effect of Zinc Concentration on Growth and on the Concentration of Scopolin

The data in Table II-10 show that the growth of the W-38 tissues was decreased in all levels of zinc relative to the normal zinc concentration (1X).

The concentration of scopolin was slightly increased when no zinc was present in the medium and was decreased at the highest zinc concentration (100X).

TABLE II-10

THE EFFECT OF ZINC CONCENTRATION IN THE MEDIUM

ON THE CONCENTRATION OF SCOPOLIN

Amount of $ZnSO_4.4H_2O$ X = 8.6 mg/l of medium	Fresh Weight g	µg of scopolin per g - fresh weight
0	16.56	79.0
1 X	19.01	66.5
10 X	13.79	65.4
100 X	11.54	41.7

Each level contained twelve pieces of tissue.
Data represent the average of three replicate experiments.

CHAPTER IV

DISCUSSION

The present investigation clearly indicates an interrelationship between calcium and zinc and between zinc, iron, and copper in relation to their effects on growth, concentration of scopolin and scopoletin, and degree of lignification. The relationship between growth, lignin, scopoletin and scopolin, however, has not yet been clearly defined. As can be seen in the abbreviated biosynthetic scheme shown in Figure II-7, ferulic acid is a common intermediate for lignin, scopolin, and scopoletin. Scopolin is metabolized very slowly, but scopoletin can be polymerized to cell wall material similar to lignin (Lowenberg, 1970; Innerarity, 1970).

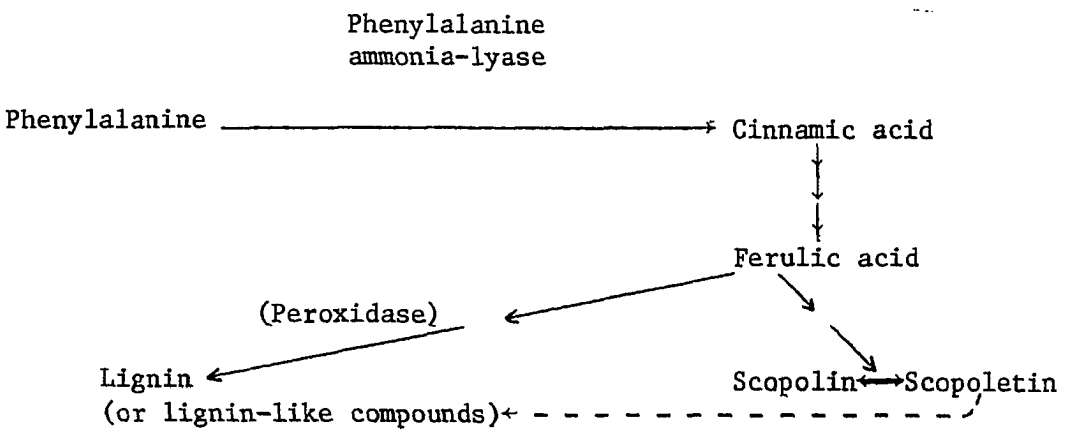

Figure II-7. A Proposed Abbreviated Biosynthetic Route to Scopolin and Lignin from Phenylalanine. (from Gamborg, 1967; Hanson et al., 1967; Steck, 1967a and 1967b; Wender, 1970; Schafer and Wender, 1970; Brown, 1966; Lowenberg, 1970; Innerarity, 1970.)

In all experiments in this investigation, an inverse correlation between growth (fresh weight) and amount of lignin as per cent of non-extractable residue (NER) or as per cent dry weight (DW) was evident. Therefore, an accumulation of lignin under these conditions may be related to the growth potential of the tissues. The most outstanding example of this effect is seen in the tissues grown at low calcium levels (1/250X to 1/10X) and high zinc levels (50X and 100X). The fresh weight of the tissues at the high zinc levels is greatly decreased relative to normal tissues and to tissues in the other treatments (Tables II-1 and II-2), and the amount of lignin in these tissues is from two to eight times that of the control tissues (1X). Studies by Lavee and Galston (1968) demonstrated that the growth potential in vitro of explants of tobacco pith taken at various distances from the apex of a mature stem show an inverse correlation with peroxidase activity. The fresh weight of the tissues in vitro after thirty days was inversely proportional to the total peroxidase activity in the original pith. Thus, the tissues at the high zinc levels could possibly contain more of the particular isoperoxidase responsible for lignification, a result of which may lead to the large increase in lignification observed in these tissues.

Wender (1970) suggested that an accumulation of scopolin in stressed plants might result from a block in the pathway to lignin (see biosynthetic pathway shown in Figure II-7). Some conditions that cause an increase in scopolin do cause a decrease in lignification. Watanabe et al. (1961) found an accumulation of scopolin in the leaves of boron-deficient tobacco plants, and, under the same condition (boron deficiency), Dutta and McIlrath (1964) observed significantly lower levels of lignin

and peroxidase activity in sunflower tissues. Since peroxidase is required for lignification (Brown, 1961; Freudenberg and Neish, 1968), the block in the pathway to lignin postulated by Wender (1970) may actually be due to a lower activity of peroxidase.

The results of the present investigation show that, under certain conditions, the inverse relationship may also be true, i.e., an increase in the degree of lignification with a concurrent decrease in the concentrations of scopolin and scopoletin. Such is the case in the tissues grown at high levels of zinc (50X and 100X) and low levels of calcium (1/250X - 1/10X) (Tables II-4, II-5, II-7, II-8). These results suggest that the intermediates common to lignin and to scopolin and scopoletin may have been shunted to lignin or that scopoletin itself may have been polymerized to lignin or lignin-like compounds by an increased activity of peroxidase.

The data in Tables II-4 and II-5 confirm the finding of Schafer and Wender (1970) that there is an increase in the degree of lignification in W-38 tissues grown at concentrations of calcium lower than the concentration optimal for growth (1X). Other studies also indicate that the degree of lignification in certain plant tissues may be affected by the calcium. Lignification was reported to be inhibited in sunflower gall tissue grown in medium containing a high concentration of calcium (Lipetz, 1962), which is believed to act by releasing peroxidase bound to the cell wall (Lipetz and Garro, 1965). Wheat plants grown at low calcium levels contained a higher percentage of lignin than normal plants and also had a significantly higher percentage of total peroxidase activity loosely bound to the internode cell wall (Parish and Miller, 1969). Thus, the

increase in the degree of lignification in the W-38 tissues grown at low calcium concentrations could possibly result from an increase in the cell wall-bound peroxidase.

Results of studies by Carles et al. (1969) indicate that the role of zinc in plants appears to be related to the concentration of certain other elements such as calcium. In tissues at the low calcium levels there was an increase in the degree of lignification when zinc was excluded from the medium and when the medium contained high levels of zinc. Tissues grown at the 100X level of zinc show an increase in the degree of lignification even at the normal and high levels of calcium. The large increase in lignification caused by high zinc concentrations may have been due to a direct influence of zinc on a particular peroxidase, by an alteration in the effects of calcium on cell wall-bound peroxidase, or by some as yet unknown mechanism.

Increased lignification has been shown to be correlated not only with increased peroxidase activity but also with increased phenylalanine ammonia-lyase activity (Cheng and Marsh, 1968; Rubery and Northcote, 1964, 1965). As can be seen from the biosynthetic scheme in Figure II-7, an increase in phenylalanine ammonia-lyase might result in an increase in scopolin, scopoletin, and lignin as was observed in the W-38 tissues grown at low concentrations of calcium and zinc. The increase in the concentrations of scopolin and, to a lesser extent, of scopoletin at lower levels of calcium is in agreement with experiments conducted by Schafer (Schafer and Wender, 1970) during the same time period as the present investigation.

However, results of subsequent experiments by Schafer (Schafer, 1971) were quite variable, and therefore, were not able to be used to confirm the results of his preliminary experiments and the results of the experiments in this study.

Some relationship between the amount of browning in the tissues and the degree of lignification was also apparent. Conditions which caused the tissues to become darker, i.e., decreasing calcium concentrations or high concentrations of zinc (50X and 100X), also caused a parallel increase in the degree of lignification. Since the brown color was not removed upon extraction of the tissues, the increased browning may be associated with the degree of lignification. Similar results were observed by Schafer (1971) in tobacco callus tissues grown on low calcium levels. Another study was reported (Lipetz, 1962) which demonstrated that a reddish-brown pigment found in sunflower gall tissue grown in culture on low calcium was deposited on the cell wall and appeared to be lignin.

The amount of water in tissues grown at the 100X zinc level was less ($P < 0.01$) than in tissues grown at all other zinc and calcium levels which is another indication of increased cell wall material in these tissues.

The data in Table II-3 indicate that the inhibition of growth resulting from a high level of zinc (75X) in the medium is decreased by the additional amounts of iron and/or copper. Craddock and Hammer (1969) found that the growth of fiber flax in nutrient solutions containing high levels of zinc sulfate was increased by the addition of iron. Zinc appeared to interfere with iron uptake, whereas iron did not interfere with zinc uptake. Ambler et al. (1970) reported that zinc interfered with

translocation of iron from roots to above ground parts of soybean plants. Addition of iron overcame the interference of zinc. Since the present studies were conducted in tissue culture instead of in whole plants, it is more probable that excess zinc would either interfere with iron uptake from the medium or would interfere with iron metabolism in the plants instead of being involved in the translocation mechanism. Excess zinc has also been shown to interfere with copper metabolism. Lee and Matrone (1969) reported that rats on high zinc diets exhibited effects of a zinc-induced copper deficiency which was alleviated by the administration of copper or of both iron and copper.

The degree of lignification was increased relative to the control in tissues grown at the high zinc level with normal concentrations of iron and copper. Addition of more copper, iron, or both copper and iron reduced lignification in these tissues (Table II-6). The concentration of scopolin observed in tissues grown with the high level of zinc and normal levels of copper and iron was less than that in the 1X zinc level, and increasing the concentration of copper or iron appeared only to enhance the decrease. The concentration of scopoletin showed an increase in tissues grown at high levels of iron (5X and 10X).

These experiments demonstrate that zinc, iron, and copper may be interrelated in the nutrition of W-38 tissues. Zinc toxicity in these tissues may be alleviated to some extent by the addition of extra amounts of iron and/or copper.

CHAPTER V

SUMMARY

The interrelationship between calcium and zinc and among zinc, iron and copper was studied in relation to effects on growth, concentration of scopolin, and scopoletin, and the degree of lignification in W-38 tobacco callus tissues. Tissues grown at calcium levels lower than the normal level (1X) exhibited reduced fresh weight and dry weight yields, an increase in the amount of lignin, and an increase in the concentrations of scopolin and scopoletin relative to the control level. High zinc concentrations (50X, 75X, and 100X) caused the greatest reduction in fresh weight, dry weight, and the concentration of scopolin in the tissues with a concomitant large increase in the degree of lignification. At low calcium levels and especially at the 100X zinc levels, the dry weight of the tissues was composed of a larger proportion of insoluble material. Increased levels of copper (5X and 10X) and/or iron alleviated to some extent the inhibition in growth and the increase in the amount of lignin in the tissues caused by a high zinc concentration (75X). The cellular parameters studied are affected by different combinations of zinc and calcium and of zinc, iron, and copper. Thus, these studies suggest an interrelationship among these minerals in the growth and metabolism of W-38 tissues.

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