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EFFECTS OF ENVIRONMENTAL POLLUTANTS ON GROWTH AND PROLINE CONTENT OF PLANT AND ANIMAL CELL CULTURES

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Cell and tissue culture methods offer certain advantages for testing the effects of environmental pollutants which more complex biological systems do not have (49). These are:

1. They allow more precise control over experimental conditions such as temperature, pH, time and the chemical environment.

2. They provide uniform living material from which many replicate samples can be taken.

3. They demand less complex experimental equipment and methods.

4. They allow accurate observation at the cellular level.

5. They permit the recording of results in the dynamic relationship of time-change.

6. They are usually more sensitive to changes in the chemical environment.

7. They usually offer greater economy in time and expense.

8. They offer the possibility of working with human material under precisely controlled conditions.

On the other hand, cell culture methods have certain inherent

disadvantages such as:

1. There is little indication of the effect that a change in the chemical environment may have on the functional activity of the living organism as a whole.

2. Observations on the effects of a change are limited to modifications of cellular activity and growth.

3. Cells in vitro are extremely sensitive to non-specific stimuli.

4. If the material being tested is altered in any way by the intact organism, cell culture may not show the effect of this.

A number of investigators have used both plant and animal tissue cultures to investigate the effects of environmental pollutants. Ordin and Skoe (62) studied the effects of ozone on plant cell walls. They pretreated cells of Avena coleoptile for 6 hours with 130 ppm of ozone and then incubated the cells for 4 hours with glucose-U-C^{14}. They found respiration increased by this treatment. In the isolated cell walls they found a decrease in cellulose. The treatment with 130 ppm ozone gave definite cell wall damage and inhibition of growth.

Boney and Corner (11) have reported that low concentrations of polycyclic aromatic carcinogens produced an increase in cell production in sporelings of marine red algae whereas non-carcinogenic polycyclic aromatics showed inhibition of cell population. Moutschen-Dahmen and Moutschen-Dahmen (58) found that the mutagens, aluminum chloride, maleic hydrazide and nitrogen mustard oil, were toxic to tobacco cultures and caused breaking and fusing of chromosomes. The loci of attack on the chromosomes were not random but were specific for each mutagen.

In carrot cultures, Menoret and Morel (55) found 2,4-dichloro-

phenoxyacetic acid (2,4-D) to give a response similar to that of indole-acetic acid. Free amino nitrogen doubled in 12 hours at the expense of the protein nitrogen of the medium. Alanine accounted for 60 per cent of the increase.

The investigation of the effects of pollutants on animal cell cultures has been much more extensive than for plant cell cultures. Mammalian cells in culture have been exposed to the gases nitrogen dioxide and sulfur dioxide (63, 70, 84). Thompson and Pace (84) found sulfur dioxide to be inhibitory to cell growth, especially when the animal cells were directly exposed. Media containing serum exhibited a protective influence. Nitrogen dioxide exhibited similar results. In addition, there was multinucleation of cells, changes in the shape of nuclei and changes in the ultrastructure of mitochondria during nitrogen dioxide treatment.

The effects of a variety of solid particulates (dusts) have been examined in tissue culture. Metal colloids (32, 39), including colloids of silicon, tin, zirconium, titanium, iron, silver, gold, platinum, lead, mercury, antimony, bismuth and selenium, stimulate cell growth in low concentrations but inhibit growth at high concentrations. Metallic salts (39) behave in the same manner except that silver and lead salts are inhibitory at all concentrations. Perelygin (67) found native blast dust to be toxic to cells in vitro. Minerals and alkaloid components were more toxic while fibrous constituents were less toxic. Marks, Mason and Nagel-Schmidt (54) observed that quartz, tridymite, cristobalite, feld-

spar, mica, kaolin, coal, ferric phosphate, calcium fluoride, aluminum phosphate and alumina were toxic to leucocytes and toxicity correlated with in vivo fibrogenic activity. Karnovsky and Wallach (37) have shown that phagocytozing cells exhibit specific metabolic changes when exposed to solid particles. There were large increases in respiration, glycolysis, conversion of glucose to carbon dioxide and oxygen uptake. It was assumed that these changes provided energy for phagocytosis although non-phagocytozing cells (Ehrlich carcinoma) exhibited the same changes in metabolism when exposed to solid particles.

Silicic acid has shown no toxic properties nor has it induced any morphological changes in cells exposed in concentrations to 65 µg per ml of media (40, 41, 42).

Alkyl benzene sulfonate, used in detergents, has been found to be toxic to human cells in vitro at all concentrations tested (7). There was a decrease in cell growth, decreased protein synthesis, decreased DNA synthesis and cytopathological changes.

Lewis and Richards (52) reported that dichlorodiphenyltrichloroethane (DDT) in saturation concentrations (approximately 1 ppm) was without observable effect on chick embryo cells. Even a 1 per cent emulsion of DDT showed no toxicity although 0.5 ml is a lethal dose for a mouse.

Recently, a method for testing the toxicity of plastics in cell culture has been reported (69). A total of 112 plastics was tested. Most of the plastics showing toxicity could be made non-toxic by extracting the plastic with refluxing 95 per cent ethanol for 24 hours. This testing method was found to be more sensitive, more rapid and less expensive than the standard intramuscular implantation technique.

A number of investigators has examined the effects of carcinogens on mammalian cells. Andrianov et al. (4) have shown that both normal and neoplastic fibroblasts absorb 3,4-benzpyrene from the medium and Alfred (1) reported that neoplastic cells absorbed ten-fold greater concentrations of tritiated 3,4-benzpyrene as compared to normal cells. Approximately 80 per cent of the radioactivity was bound to proteins in the soluble fraction. Alfred et al. (2) reported that normal mouse and hamster cells showed high susceptibility to the toxic effects of 3,4-benzpyrene and 3-methylcholanthrene, whereas neoplastic cells showed little if any toxic response. The non-carcinogen, chrysene, exhibited no cytotoxicity in normal or neoplastic cells. Diamond (19) has reported that, in rodent cells, 3,4-benzpyrene and 3-methylcholanthrene inhibited proliferation in normal cultures but not in neoplastic or virus transformed cultures. In contrast, these carcinogens showed no inhibition of proliferation in either normal or malignant human cell cultures. Berwald and Sachs (8, 9) found normal hamster cells to exhibit a high rate of neoplastic transformation when exposed to 3,4-benzpyrene, 3-methylcholanthrene, 7,12-dimethylbenzanthracene or 10-methylbenzanthracene whereas no transformation occurred with the non-carcinogens, 8-methylbenzanthracene, chrysene or pyrene. In normal cells, others (3, 50, 57, 59) have reported that, although relatively high concentrations of carcinogenic hydrocarbons in the medium inhibit proliferation, low concentrations cause hyperplasia. Ultraviolet irradiation of the medium containing low concentrations of carcinogens reversed the proliferative effect. Hyperplasia was accompanied by abnormal cell morphology and mitosis. The carcinogens were concentrated in the lysosomes and the

co-carcinogens, croton oil and Tween 60, increased the permeability of lysosomal membranes.

The effects of smoke on cells in culture have been examined by a number of workers. Kasai and Pollak (38) found that whole cigaret smoke caused toxic manifestations when bubbled through media in which rabbit cardiovascular cells were growing. These effects included an increase in pinocytosis, budding of the cytoplasmic membrane, thickening of the nuclear membrane, inhibition of proliferation and cell death at high concentrations. Awa, Ohnuki and Pomerat (5) have reported that human fetal lung cells in direct contact with whole smoke for 5-10 seconds exhibit vacuolization, "blebbing" and pyknosis of nuclei, a decrease in cell division, an increase in abnormal mitotic figures and chromosomal anomalies. The smoke from cigaret paper was more toxic than the smoke from tobacco alone or from cigarets.

Cooper and Goldring (16) report that smoke gases cause granularity, pyknosis, cell enlargement, vacuolization, surface "blebbing" and a reduction in the cell population. Smoke gases from onion skin or cigaret papers were more toxic than cigaret smoke gases or cigaret tobacco smoke gases (17). Thayer and Kensler (82) found that the water soluble components of cigaret smoke gases inhibited KB cell proliferation and protein synthesis.

Cigaret smoke condensate has been reported (27, 43, 45, 82) to reduce cell "take" upon inoculation, inhibit cell proliferation and to enhance cytoplasmic granularity and nuclear pyknosis with ultimate cytolysis. Thayer and Kensler (82) found protein synthesis to be inhibited which would be expected when proliferation is inhibited. Guimard

(27) found that proliferation and mitotic activity were inhibited at higher concentrations of smoke tar but that both were increased at lower tar concentrations. Lasnitzki (51) reported that certain fractions, especially hydrocarbon fractions, of smoke condensate, induced hyperplasia of human fetal lung cells in culture. The effects observed were much like those obtained with 3-methylcholanthrene and 3,4-benzpyrene. The toxic effects of nicotine upon cells in culture have been reported (43, 44, 45) to be similar to those of smoke condensate but having one-thirtieth the potency.

Few studies have been reported concerning the effects of rubber upon cells in culture although it has long been known that most rubber formulations show some toxicity to cells in culture while some are violently toxic (64). This toxicity has been assumed by some tissue culturists to be due to the sulfur components in the rubber and Parker (64) gives an effective means for removing the so-called sulfur "bloom" from rubber stoppers to be used as closures in tissue culture. Parker, Morgan and Morton (65) reported that all types of rubber stoppers which they examined were toxic to chick embryo cell cultures. Toxicity could not be eliminated by extraction with organic solvents, hot basic solutions, or hot acidic solutions. Vasington et al. (89) reported that a number of the rubber closures being used in the pharmaceutical industry showed toxicity to cells in culture.

A number of polycyclic aromatic hydrocarbons has been identified in rubber stoppers and vehicular tire rubber. Falk et al. (26) reported the existence of pyrene, 3,4-benzpyrene, chrysene, 1,12-benzperylene and cyclopentenophenanthrene. Tentatively identified were alkylpyrene,

1,2-benzpyrene and alkyl 1,2-benzanthracene. Benzene extracts of rubber stoppers were found to be carcinogenic when painted upon the skin of mice. Smith (76) detected coronene, 3,4-benzpyrene, fluoranthene, 1,2-benzpyrene and pyrene as components of vehicle tire rubber. A number of these compounds has carcinogenic activity and possibly contribute to the toxicity which most rubber formulations exhibit in tissue culture.

Thompson, Nau and Lawrence (86, 87) have shown vehicle tire rubber to be present in the roadway dust of a parking garage and the air of the Holland and Lincoln tunnels of New York City. Recently, before a meeting of the Oklahoma City Chamber of Commerce, Dr. René Dubos (83) stated:

> "Pulverized rubber has shown up as the largest single component in an analysis of New York air. . . . When the returns are all in I wouldn't be surprised to see the finger put on pulverized rubber as the arch villian in air pollution."

One of the major components of vehicle tire rubber is carbon black. Tire tread rubber contains 30 to 50 parts by weight of carbon black per 100 parts by weight of rubber and more than 90 per cent of the carbon black produced in the United States is used by the rubber industry (77). Nau, Neal and Stembridge (60) have pointed out that carbon black is incorporated into rubber goods used in food processing (tubing, conveyor belts, etc.), into inks (newsprint, etc.) and in certain foods as a coloring agent (jelly beans, licorice, gum drops, etc.). Carbon black is also used extensively in paints and plastics. Although a number of hydrocarbons, including carcinogens, have been extracted from carbon blacks (25), no publication was found concerning the effects of carbon black on cells in culture.

It is apparent that relatively few metabolic studies have been reported in which cells in culture were exposed to environmental pollutants. It was noticed during the course of this research at the University of Oklahoma that tobacco callus *in vitro* accumulates large quantities of free proline when exposed to rubber dust. Thus, studies on amino acid metabolism became of interest.

There has been a number of previous reports of quantitative changes in free proline in biological systems subjected to stress. Different investigators have variously attributed this effect to be due to an altered nitrogen metabolism in the organism, an alteration in the capacity of the cell membrane to absorb certain nitrogen-containing compounds, proteolysis of cellular protein, reduction of growth with a concomitant delay of incorporation of proline into protein, increase in growth with a concomitant increase in production of free amino acids, or a combination of these factors. The reported free proline changes are presented in Table 1.

At times, the change in free proline concentration with stress is phenomenal. For example, the free proline increases by a factor of 70 in tobacco stem crown gall tumor tissue as compared to the normal stem tissue (75). In hyperprolinemia of humans, a genetic defect involving proline oxidase, the rise in free proline is more moderate. A case history is reported (31) in which the free proline in the plasma was 4 times the normal value and terminated fatally. Three female siblings had hyperprolinemia with varying degrees of damage to bodily organs. It is of interest that a decrease of proline in DDT-poisoned cockroaches was progressive with the symptoms of paralysis, and restoration of proline to

normal levels relieved the symptoms in paralyzed insects (18).

TABLE 1

REPORTED CHANGES IN FREE PROLINE IN BIOLOGICAL SYSTEMS UNDER VARIED CONDITIONS

BIOLOGICAL SYSTEM	CONDITION	CHANGE	REFERENCE
Bermuda grass leaf	Water deficit	Increase	6
Barley leaf	Water deficit	Increase	74
Wheat leaf	Water deficit	Increase	48
Wheat leaf	Water deficit	Increase	81
Wheat leaf	Water deficit	Increase	68
Corn plant	Electrified soil	Increase	46
Ladino clover leaf	Water deficit	Increase	71
Potato leaf	Potato rolled virus	Increase	66
Potato tuber	Maleic hydrazide or 2,4-D	Increase	29
Tomato stem	Crown gall tumor	Increase	75
Pumpkin plant	Water deficit	Increase	47
Cabbage plant	Low temperature	Increase	56
Sugar beet plant	Beet yellows virus	Increase	56
Jerusalem artichoke tuber (*in vitro*)	Kinetin deficit	Increase	56
Tobacco leaf	Potato virus Y	Increase	12
Tobacco stem	Crown gall tumor	Increase	75
Tobacco plant	Ca or B deficit	Increase	88
Tobacco plant	Maleic hydrazide	Increase	93
Creosote bush leaf	Water deficit	Increase	73
Almond bud	Low temperature	Increase	36
Cockroach	DDT poisoning	Decrease	18
Rat foot pad	Injection of formaldehyde, dextran or serotonin	Increase	35
Rat tissue	Starvation	Decrease	85
Rat plasma	Chilled or exercised	Decrease	92
Human	Genetic	Increase	31

It seems likely that there is much yet to be discovered relating to proline function and metabolism in living organisms. Proline is a constituent of every protein that has been studied (91). Proline facil-

itates the assumption of an α-helical conformation by proteins, hence its frequent occurence in collagen (28). Proline also serves as a precursor for glutamic acid, arginine, ornithine and hydroxyproline.

Hydroxyproline does not occur free in any quantity in plant or animal tissue except in certain exceptional situations (78, 80). It is found in quantity only in collagen or, in plants, in collagen-like proteins (80, 91). Steward and Pollard (80) found such proteins to be essential for growth in plants. They reported that exogenous hydroxyproline in concentrations as low as 10 ppm, inhibited the incorporation of proline into protein and inhibited growth. Added proline could reverse this inhibition and growth resumed. Labeled proline was incorporated and hydroxylated yielding a protein that, like collagen, was metabolically inert. Twenty-four hours after introducing labeled proline into plants, the label was almost equally distributed between proline and hydroxyproline. They concluded that direct incorporation of proline into protein and its hydroxylation are necessary parts of the growth induction response.

In recent years there has been much interest in the so-called collagen diseases. These are disorders in which there are proliferative and degenerative changes in connective tissue. In collagen synthesis, proline is incorporated into protein and hydroxylated only after its release from the ribosome (10). Autoradiographic studies have shown hydroxylation to be intracellular , resulting in extracellular collagen (34). Ferric ion and molecular oxygen are essential for hydroxylation (14, 15). Ascorbate is thought to be the hydrogen donor *in vivo* but *in vitro*, 2-amino-4-hydroxydimethylpteridine has also been reported to

be effective (14). Cross-linkage occurs, probably by the esterification of free aspartic and glutamic acid carboxyls with glucose hydroxyls (61). Various collagens contain 30-34 per cent glycine, 10-13 per cent L-proline, 5-10 per cent 4-hydroxy-L-proline and 0.5-1 per cent 5-hydroxy-L-lysine (28). From 1-4 per cent of the hydroxyproline in collagen occurs as 3-hydroxy-L-proline (14).

CHAPTER II

PURPOSE AND SCOPE

Very few studies have been performed to investigate the effects of environmental pollutants upon the metabolism of cells. No publication was found in which cells in culture have been exposed to commercial carbon blacks. The few reported investigations of the effects of rubber formulations on cells have been confined to toxicity studies, that is, the degree of inhibition of cell proliferation in culture. The only rubber formulations examined have been those used for closures. Although the effects of cigaret smoke on cells in culture have been investigated extensively, studies have been primarily confined to cellular toxicity, morphological changes and mitotic activity.

Since little is known in this area, it was of interest to determine what basic changes, if any, occur in metabolism when cells are exposed to these materials now so common in our environment. Viewed from the health aspect and the widespread exposure of the general population, cigaret smoke assumes great importance as an atmospheric pollutant. The fact that more than 150 million vehicular rubber tires are sold each year coupled with the fact that approximately one-third of each tire is carbon black, indicates that more attention should be given to those materials as potential contributors to atmospheric pollution.

This study was undertaken to observe selected metabolic changes that occur in plant or animal cells exposed to rubber dust, carbon blacks or cigaret smoke components. The metabolic change to be monitored is the change in free proline content in tobacco callus cells and in human diploid and neoplastic cells when exposed in vitro to rubber dust, thermal carbon black, furnace carbon black and cigaret smoke tar.

CHAPTER III

EXPERIMENTAL MATERIALS AND METHODS

Plant Tissue Culture Experiments

The materials that were essential to the experiments, with their source and purity, are as follows:

Callus tissue from tobacco, Nicotiana tabacum variety Wisconsin number 38, was obtained from Dr. Folke Skoog of the University of Wisconcin.

Carbon blacks, produced by the thermal and the furnace processes, were obtained from the Institute of Environmental Health, 800 Northeast 13th Street, Oklahoma City, Oklahoma.

Rubber dust was obtained from the buffing wheel of a local tire recapping shop and was screened through a number 40 mesh sieve (0.417 mm). This dust represented both passenger and truck tire rubber.

Cigaret smoke particulate materials were obtained from a popular brand of 100 mm filter-tip cigarets smoked under the supervision of Philip Schafer in a smoking machine designed by the Research Laboratory of the American Tobacco Company, Inc. and manufactured by Phipps and Bird, Inc. Eight puffs were taken from each cigaret. The Cambridge filter discs upon which the tar from main-stream smoke was deposited were extracted for 48 hours with methanol in a Soxhlet apparatus. The solvent was

removed in a rotary evaporator and the residue was stored in a methanol-benzene solution (1:9 v/v) in the refrigerator for 2 weeks before use. The solution contained 33.0 mg tar per ml and each milliliter represented the tar from two cigarets.

All chemicals and solvents were analyzed reagent grade and all water used in culture was redistilled in an all glass still.

The medium (Table 2) that was used in the plant tissue culture experiment was that of Linsmaier and Skoog (53) except that the thiamine concentration was increased. Linsmaier and Skoog (53) found that thiamine degrades on autocalving and an increased amount provides a safety factor.

A stock solution of the last four mineral salts as listed in Table 2, and which are present in smallest concentration, was made 1000 times the concentration of the growth medium. Ten ml of this solution was pipetted into a liter volumetric flask to which was added the remainder of the mineral salts, except for Na_2EDTA and $FeSO_4 \cdot 7H_2O$, in ten times the concentration of the growth medium. Double distilled water was added to bring to volume. A stock solution of $Na_2FeEDTA$ was prepared by adding 264 mg of $Na_2CO_3 \cdot H_2O$ and 647 mg of EDTA to 100 ml of double distilled water. This was brought to a boil and 557 mg of $FeSO_4 \cdot 7H_2O$ were added slowly, with stirring. A stock solution of 3-indoleacetic acid (IAA) containing 1 mg per ml was obtained with heating on a steam bath. A stock solution of kinetin containing 0.1 mg per ml was made in the same manner. The kinetin and IAA solutions could be stored, with care, up to 3 months in the refrigerator. Kinetin forms a fine precipitate under these conditions and was therefore pipetted as a suspension. Then 20 mg

of thiamine·HCl were dissolved in 100 ml of double distilled water. Five ml aliquots, each containing 1 mg of thiamine·HCL, were stored in the freezer in 25 ml Erlenmeyer flasks.

TABLE 2

COMPOSITION OF TOBACCO TISSUE CULTURE MEDIUM

MINERAL SALTS					
NH_4NO_3	1650	mg/l	H_3BO_3	6.2	mg/l
KNO_3	1900	mg/l	$MnSO_4 \cdot 4H_2O$	22.3	mg/l
$CaCl \cdot 2H_2O$	440	mg/l	$ZnSO_4 \cdot 4H_2O$	8.6	mg/l
$MgSO_4 \cdot 7H_2O$	370	mg/l	KI	0.83	mg/l
KH_2PO_4	170	mg/l	$NaMoO_4 \cdot 2H_2O$	0.25	mg/l
Na_2EDTA	37.3	mg/l	$CuSO_4 \cdot 5H_2O$	0.025	mg/l
$FeSO_4 \cdot 7H_2O$	27.8	mg/l	$CoCl_2 \cdot H_2O$	0.025	mg/l
ORGANIC CONSTITUENTS					
Sucrose	30	g/l	Kinetin	0.2	mg/l
Agar	10	g/l	Thiamine·HCl	1	mg/l
3-Indoleacetic acid	2	mg/l	myo-Inositol	100	mg/l

The following procedure was followed in order to prepare 1 liter of medium:

1. To 886 ml of double distilled water were added 100 ml of the 10x concentration mineral salts, 5 ml of the $Na_2FeEDTA$ stock solution and

30 g of sucrose.

2. The pH of the solution was adjusted to 5.6 with 1 N KOH.

3. Ten g of agar was added and the mixture was autoclaved 5 minutes in order to melt the agar.

4. To the solution were then added 100 mg of myo-inositol, 5 ml of the thiamine·HCl solution, 2 ml of the kinetin solution and 2 ml of the IAA solution.

5. After swirling, the medium was equally dispensed into 20 Erlenmeyer flasks (125 ml) with a 50 ml pipet from which the tip had been removed in order to facilitate rapid pipetting.

6. The flasks were plugged with cotton and autoclaved 12 minutes at 121°C and 15 pounds of pressure.

7. Hardening of the agar requires approximately 30 minutes after which the flasks were inoculated with tissue.

Replicate flasks were planted with discs of firm, white callus from 4-5 week old stock cultures. Each flask was planted with three discs obtained by cutting a cylinder of callus tissue with a number 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 discs were placed with forceps equidistant from each other on the surface of the agar. Any portion of tissue beneath the agar will not grow because of anaerobic conditions. Cultures were prepared under a plexiglass hood which had been swabbed inside with 70 per cent ethanol immediately before use. Cultures were placed on a shelf in diffuse light at room temperature (26°C) until harvested 5 weeks later.

Test materials were introduced into the medium in the following

ways. Rubber dust, equivalent in weight to the selected percentages of the total materials in the flask, was added to each Erlenmeyer flask. After adding medium and autoclaving, each flask was swirled periodically so that the dust did not settle to the bottom before the agar had hardened.

In order to obtain a suspension of the carbon blacks, they had to be blended into the medium minus agar with a Waring blender for 10-15 seconds. After pipetting 50 ml of medium containing the carbon black suspension into each 125 ml Erlenmeyer flask, 0.5 g of agar was added. The cotton-plugged flasks were then autoclaved 12 minutes at 121°C and 15 pounds of pressure. After autoclaving, the flasks were swirled periodically so that the carbon particles did not settle before the medium had hardened.

The cigaret smoke tar was introduced into each empty flask in solution in methanol-benzene (1:9 v/v). After the solvent had been evaporated under an air stream, the medium was added and the flasks were sterilized by autoclaving as before.

After a 5-week growth period, treated flasks and control flasks containing no test materials were harvested. Tissue from replicate flasks were combined, weighed, placed in an Erlenmeyer flask and boiled in a minimal amount of isopropanol-water azeotrope (88:12 w/w) in order to inactivate all enzymes.

Tissues were extracted according to a procedure devised by Winkler (93) to remove quantitatively all flavonoids from tobacco tissue. It was found that all ninhydrin and isatin-positive materials are also quantitatively extracted. The procedure follows:

1. The harvested tissues and the liquid were rinsed into a blender with enough methanol to cover the blades of the blender.

2. This mixture was homogenized for 1 minute.

3. The homogenized mixture was rinsed with methanol into a Soxhlet thimble of suitable size, which was contained in a funnel built in the shape of the thimble. All solvents from the funnel were collected in a 1-liter round bottom flask.

4. The following liquids, brought to a boil on a steam bath, were poured in succession over the solids in the thimble: isopropanol-water (50:50 v/v), isopropanol-benzene-methanol-water (IBMW) (2:1:1:1 v/v/v/v) and isopropanol-water azeotrope. The volume of each solvent was 5 times (in ml) the fresh weight (in g) of the tissue at harvest or enough solvent to fill the thimble, whichever was greater.

5. The solids remaining in the thimble were extracted for 24 hours in a Soxhlet extractor with isopropanol-water azeotrope and for a second 24 hours with isopropanol.

6. All washings and extracts were combined and reduced to a heavy syrup in a rotary evaporator. Reduced pressure was supplied by a water aspirator and the round bottom flask containing the extracted material was heated on a water bath at approximately 50°C or less.

The extract was dissolved in a volumetric flask in isopropanol-benzene-water (IBW) (3:1:1 v/v/v). The final volume was approximately 2.5-3 times (in ml) the fresh weight (in g) of the harvested tissue. To get the extracted material into solution was sometimes difficult but could be readily accomplished in the following manner:

1. One-third of the benzene and one-third of the water that were

to be contained in the final solution was added to the round bottom flask containing the syrupy extract. This emulsion was swirled until all material came loose from the walls of the flask.

2. The emulsion was then poured into a volumetric flask and the first step was repeated twice.

3. The round bottom flask was rinsed several times with small quantities of isopropanol and these rinses were added to the volumetric flask.

4. Sufficient isopropanol was added to the volumetric flask to bring the solution to volume. The extract was stored in a cold room at 0°C. Usually a small amount of solid precipitated. This precipitate could be minimized or eliminated by decreasing the benzene and increasing the water concentration in the IBW. This precipitate was not fluorescent and did not produce color on thin-layer plates when sprayed with 0.2 per cent ninhydrin in acetone or 0.2 per cent isatin in n-butanol-acetic acid water (90:5:5 v/v/v). This precipitate was thought to be glucose.

Plant extracts were quantitatively analyzed for proline content by densitometry of the isatin-reacted proline spot on thin-layer chromatographic plates. Glass plates (20 cm x 20 cm) were spread with a layer of Avicel-SF microcrystalline cellulose (FMC Corporation) of approximately 400 μ in thickness. It was found that the thicker the layer the greater the sensitivity but if the layer was much thicker than 400 μ, cracks will develop. The Avicel-SF layers were prepared by weighing approximately 3.5 g of Avicel-SF per plate, adding 4.5 ml of water per g of Avicel-SF and blending at high speed for 30 seconds. This time interval is critical because less blending yielded a stringy mixture and longer blending

thickened the material so that micro air bubbles were permanently trapped. These bubbles burst as the material dried on the plate, resulting in a large number of minute holes which gave blips on the densitometer. Following blending, the material was stirred for 5-10 minutes to eliminate air bubbles, and then it was spread on the glass plates with a Desaga spreader. Plates were allowed to air dry after spreading, and these were used within several weeks. Plates allowed to stand 6 months before using produced pronounced streaking.

It was found to be important that the thickness of the layer on the plate be as uniform as possible. When thick or thin areas occurred, an uneven recorder base line was obtained. If a thick or a thin streak occurred within the area of a spot which was being recorded densitometrically, an invalid reading resulted. Several mixtures were examined in an effort to achieve more uniformity, to eliminate pin holes and to allow the spreading of layers thicker than 500 μ without cracking. These efforts were unsuccessful. When 20 per cent Woelm polyamide was incorporated into the mixture, no advantage was obtained, but the fragility of the layer was increased. When 20 per cent soluble potato starch was incorporated into the mixture, the tendency of the layer to crack was increased and the resolution of amino acids was decreased. Highly uniform plates were obtained with 20 per cent silica gel in Avicel-SF, but the color yield of proline after its reaction with isatin was diminished and the color faded rapidly.

Each plate was spotted with nine spots which were 0.75 of an inch apart and on a line 0.75 of an inch from the edge of the plate. On each plate an unknown was spotted in triplicate and duplicate spottings were

made of a proline standard solution in three different quantities. The L-proline (Nutritional Biochemicals Company) standard was dissolved in water-isopropanol (4:1 v/v) at a concentration of 0.1 µg per µl. The linear range was found to be from 0.1 µg to 0.8 µg of proline. Spotting was accomplished with a micrometer syringe and buret (Roger Gilmont Instruments, Inc.) with a capacity of 200 µl and calibrated in 0.2 µl divisions. The buret was fitted with a 24 gauge needle which was 0.75 of an inch long. A hair dryer reduced the amount of time required to spot.

Plates were developed in water-formic acid (98 per cent)-n-butanol-methanol-phenol (1:1:3:9:9 v/v/v/v/w) for 3.5 hours. Best results were obtained when the solvent was freshly made immediately prior to use and when the components were added in the order listed above. The plates were developed at a temperature of approximately 70°C in a chamber which had not been equilibrated with the solvent system. An unequilibrated chamber seems to give better resolution of amino acids. Following development, the plates were dried under an air stream at room temperature for 1 hour and at 110°C for 30 minutes. This step removes the phenol which lowered sensitivity.

After the plates had cooled, they were dipped in 2 per cent isatin in acetone-n-butanol-acetic acid-water (9:9:1:1 v/v/v/v). Dissolution of the isatin was facilitated if it was first dissolved in the acetone component by heating and then adding the other components. The color yield could be increased somewhat by increasing the amount of water and acetic acid and by increasing the isatin concentration. Dipped plates were heated for 25 minutes at 110°C and densitometric readings were made

as soon as the plates had cooled. Proline appeared as a bluish-green spot (R_f, 0.50) on a deep yellow background. The density of the color in the spot did not diminish to any measurable extent within the first 2 hours after the plate was dipped and heated, but density readings on the followng day were always lower in value.

Densitometry readings were made on a Model 530 Photovolt TLC densitometer equipped with a Photovolt multiplier photometer model 520-M, Varicord variable response recorder model 42-B and Integraph integrator model 49. The sensing arm was fitted with a Wratten 22 filter above an incident slit of 16 mm length. The plates were placed on the thin-layer stage with the Avicel-SF layer up and were scanned in a direction which was 90 degrees to the direction of solvent development. The photometer decade was set at 10x and the response setting on the recorder was 6. Plates were scanned with the densitometer covered with a wooden frame which was additionally covered with several layers of black cloth. Room lights were also turned off and the shades were closed since the photometer is very sensitive to extraneous light.

It was found necessary to prepare a standard curve each time the proline concentration was determined in a plant extract because of the many variables involved. The most important variable seemed to be the Avicel-SF layer on the plates. It has been found impossible to consistently obtain a plate on which the layer was of uniform thickness throughout. Also, one group of plates spread by one individual were usually of somewhat different thickness than another group of plates spread by a different individual. Differing densitometric values resulted when the same quantity of proline was compared on two such groups

of plates. On each series of analyses, all readings of replicate known concentrations were averaged, plotted and the best straight line was fitted. At no time in the analyses reported was a point obtained which was more than 1.5 integrator counts from a straight line. This is well within the error of precision of the densitometer. A sample standard curve is given in Figure 1. In this figure each point is an average of the density readings of 14 spots, each of which contained the same quantity of proline. The range of readings which was obtained is indicated. Quite frequently, all points fell on a straight line. Most of the standard curves had approximately the same slope but when extended to zero proline concentration they crossed the ordinate between zero and seven integrator counts. The cause for this base line effect is not known but it seemed to be affected by the thickness of the Avicel-SF layer, the shape of the spot that was scanned and the amount of fading of the color in the spot. If plates on which known concentrations of proline had been spotted were scanned immediately and then on the following day, two curves were obtained which were approximately parallel but the values obtained on the later day fell on the lower line.

Tobacco callus extracts were examined in a number of different solvent systems on Avicel-SF thin layers in order to find a system which gave a good separation of proline from the other amino acids which were present. In one system a peculiar phenomenon was noted. In 2-butanol-acetone-formic acid (98 per cent)-water (10:10:2:5 v/v/v/v) (SBAFW), the proline standard had an R_f of 0.40 (all reported R_f's were measured from the center of the spot), but the material in the plant extracts which was thought to be proline separated into two spots with R_f's of 0.37 and 0.46.

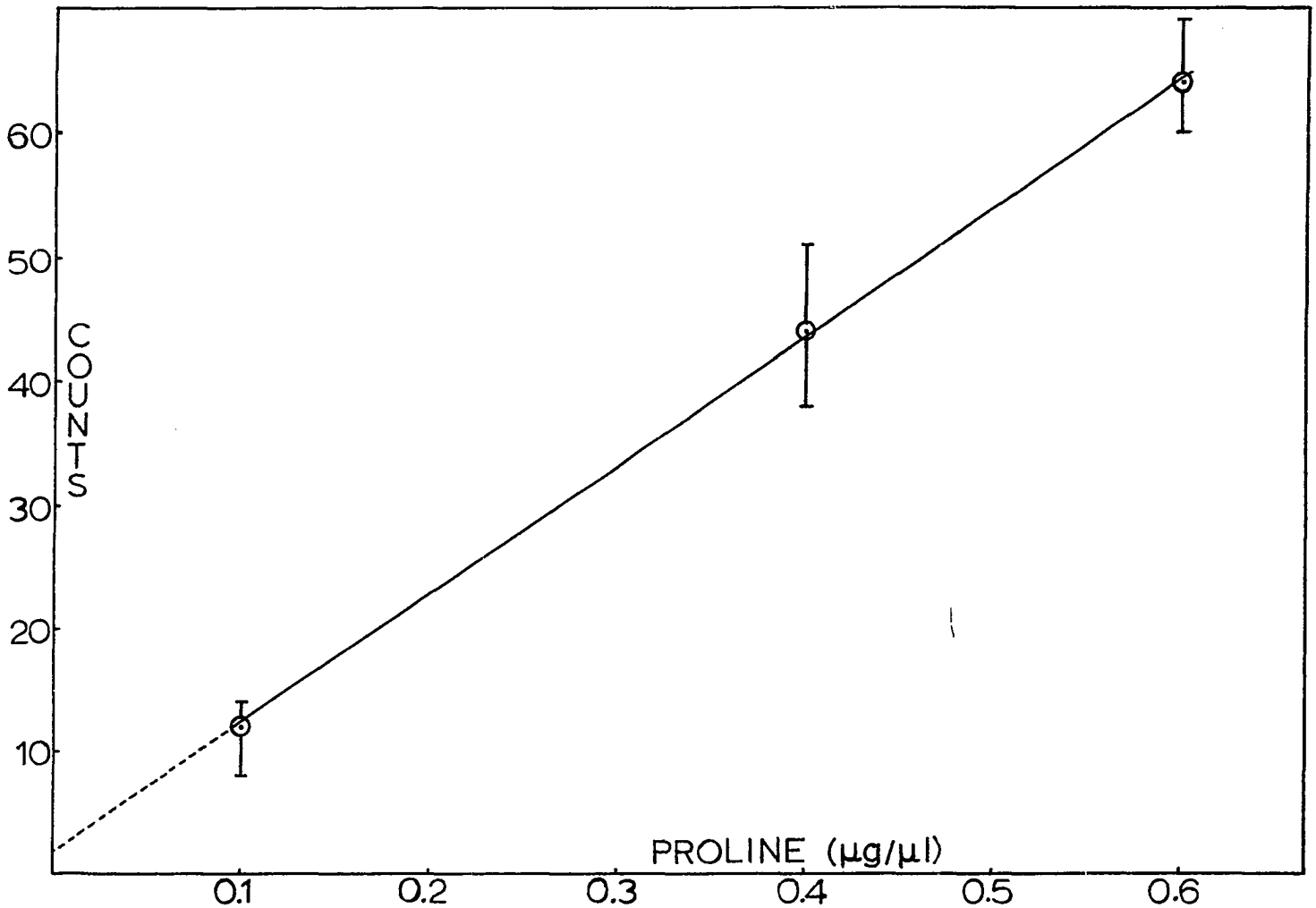

Figure 1. A standard curve for quantitating proline by direct densitometry on thin-layer plates of Avicel-SF.

The plates were sprayed with 0.2 per cent isatin in acetone-water-acetic acid (75:50:2 v/v/v). The color of both these spots could not be distinguished from the color of the proline spot whereas all other amino acids, even hydroxyproline, that have been examined in this laboratory give a tint with isatin which is distinguishable from proline. The material in the extracts which was thought to be proline was homogeneous in all other solvent systems which were investigated and its R_f matched that of proline. When the proline standard was spotted at the origin on top of the tissue extracts and developed in SBAFW, two spots were again obtained with R_f's of 0.37 and 0.46 but no spot appeared between these two with an R_f matching that of the proline standard (0.40). The added proline was divided between the two spots because both spots increased in color density as a result of such over-spotting with proline. The majority of the added standard proline traveled with the spot having the higher R_f.

To investigate this phenomenon further, 1 ml of an extract of tobacco callus tissue grown on medium containing 15 per cent rubber dust was streaked across the bottom of an Avicel-SF thin-layer plate. The plate was developed in SBAFW. The strips of Avicel-SF containing the high R_f and the low R_f material were scraped from the plate and separately eluted with water. When the high R_f material and the low R_f material were separately spotted on Avicel-SF and rechromatographed in SBAFW, both exhibited the splitting phenomenon. Both materials gave two spots with R_f's of 0.37 and 0.46. The high R_f and the low R_f material were then chromatographed in water on a Polyclar column (General Analine and Film Corporation) which had a packed volume of 1.5 x 5 cm.

The splitting phenomenon was still present in both materials so the materials, dissolved in water, were next placed on an ion exchange column (1.5 x 5 cm packed volume) of AG-50-W x 12 (Biorad Laboratories), 100-200 mesh in the hydrogen cycle. After the column was washed with 25 ml of water it was stripped with 1 N ammonium hydroxide. The solvent was removed in a rotary evaporator. The residue was repeatedly taken up in small quantities of water and the water was distilled *in vacuo* to eliminate ammonium hydroxide. The high R_f material was dissolved in 2 ml of water and the low R_f material was dissolved in 1 ml of water. Upon chromatography on Avicel-SF thin-layer plates in SBAFW, neither exhibited the splitting phenomenon. A proline standard had an R_f of 0.40, the high R_f material now gave only one spot with an R_f of 0.40 and the low R_f material gave only one spot with an R_f of 0.39. This indicates that both of these materials were proline and that there was some factor or factors in the extracts which cause the proline to separate into two spots in this solvent system.

To make certain it was proline being quantitated in the tissue extracts, a bioassay was performed on both materials after ion exchange chromatography and on the original tissue extract from which these materials were derived. A proline-requiring *E. coli* mutant that had been isolated by Dr. John H. Lancaster of the University of Oklahoma Department of Microbiology was used. Duplicate cultures were incubated at 37°C for 24 hours with skaking in Vogel's minimum medium (90) plus proline. Growth was measured turbidimetrically in a Spectronic 20 (Bausch and Lomb) spectrophotometer at 420 mμ. The test materials were autoclaved for 15 minutes at 121°C and 15 pounds of pressure. The crude

extract, the low R_f material and the high R_f material all supported growth. Control cultures containing Vogel's minimum medium without proline exhibited no growth. Hydroxy-L-proline, L-glutamic acid, L-ornithine, L-citrulline and L-arginine were not able to support growth at levels of 50 μg per ml of medium. Proline, even at a concentration of 0.5 μg per ml, gave measurable growth (see Figure 2). It appeared certain that both the high R_f material and the low R_f material were proline.

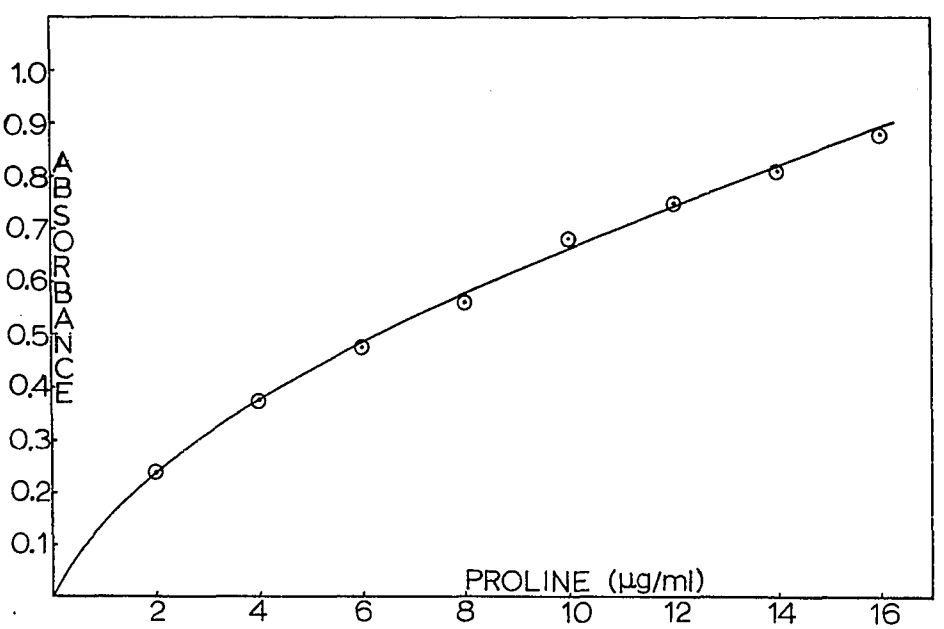

Figure 2. The growth curve for a proline-requiring E. coli mutant grown on Vogel's minimum medium plus proline.

Animal Cell Culture Experiments

The materials, essential to the experiments, together with their source and purity are as follows:

HeLa cells were obtained from Dr. L. V. Scott of the University of Oklahoma Medical School. HeLa is a heteroploid cell line derived from carcinoma of the human cervix.

WI-38 cells (passage 18) were obtained from Baltimore Biological Laboratories, Baltimore, Maryland. This is the Wistar strain of human embryonic female lung cells and is capable of serial propagation *in vitro* while retaining a normal diploid karyotype of 46 chromosomes.

Hank's balanced salt solution, without sodium bicarbonate, was obtained from Flow Laboratories, Rockville, Maryland.

Cigaret smoke particulate materials (tar) was obtained by Philip Schafer in the manner previously described under Plant Tissue Culture Experiments. Two different tar solutions were used; the first, designated tar-1, was obtained from the smoke of 50 filter-tip, 100 mm cigarets of a popular brand. The tar was dissolved in 25 ml of methanol-benzene (1:9 v/v) and was stored in the refrigerator 2.5 months before use. The solution contained 45.3 mg tar per ml. The second tar solution, designated tar-2, was obtained from the smoke of two packs each of regular, non-filter-tip Chesterfields, Lucky Strikes and Camels. Six puffs were taken from each cigaret. The tar was dissolved in 60 ml of benzene-methanol (9:21 v/v) and contained 26.6 mg tar per ml. This material was used immediately.

Scopoletin was synthesized (13) and purified on a silicic acid column in this laboratory.

Eagle's minimum essential medium (20) containing Earle's balanced salt solution (24) and 10 per cent calf serum (CulturSTAT) was obtained from Baltimore Biological Laboratories. This medium was used to cultivate HeLa cells and its composition is given in Table 3. For brevity, this medium is referred to as growth medium-1 (GM-1).

TABLE 3

EAGLE MINIMUM ESSENTIAL MEDIUM WITH EARLE BASE (GM-1) FOR CULTURING HELA CELLS

INGREDIENTS	CONCENTRATION (Per Liter)	INGREDIENTS	CONCENTRATION (Per Liter)
L-Arginine·HCl	126.98 mg	Ca-Pantothenate	1 mg
L-Cystine	24 mg	Pyridoxal·HCl	1 mg
L-Glutamine	292 mg	Thiamine·HCl	1 mg
L-Histidine·HCl·H_2O	41.88 mg	Nicotinamide	1 mg
L-Leucine	52 mg	Riboflavin	0.1 mg
L-Isoleucine	52 mg	Sodium chloride	6.8 g
L-Lysine·HCl	58 mg	Potassium chloride	0.4 g
L-Methionine	15 mg	Calcium chloride	0.2 g
L-Phenylalanine	32 mg	Magnesium sulfate	97.7 mg
L-Threonine	48 mg	Monosodium phosphate·H_2O	0.14 g
L-Tryptophan	10 mg	Glucose	1 g
L-Tyrosine	36 mg	Sodium bicarbonate	2.2 g
L-Valine	46 mg	Phenol red	0.02 g
Choline chloride	1 mg	Pooled calf serum	100 ml
Biotin	1 mg	Penicillin	100,000 units
Folic acid	1 mg	Streptomycin	100 mg
myo-Inositol	2 mg	Water	to volume

Eagle's basal medium (21) containing Earle's balanced salt solution (24) and 10 per cent calf serum (CulturSTAT) was obtained from Baltimore Biological Laboratories. WI-38 cells were propagated in this medium and its composition is given in Table 4. For brevity this medium is referred to as GM-2.

TABLE 4

EAGLE BASAL MEDIUM WITH EARLE BASE (GM-2)
FOR CULTURING WI-38 CELLS

INGREDIENTS	CONCENTRATION (Per Liter)	INGREDIENTS	CONCENTRATION (Per Liter)
L-Arginine·HCl	21.04 mg	Ca-Pantothenate	1 mg
L-Cystine	11.6 mg	Pyridoxal·HCl	1 mg
L-Glutamine	292 mg	Thiamine·HCl	1 mg
L-Histidine·HCl·H_2O	10.81 mg	Nicotinamide	1 mg
L-Isoleucine	25.8 mg	Riboflavin	0.1 mg
L-Leucine	25.8 mg	Sodium chloride	6.8 g
L-Lysine·HCl	29.1 mg	Potassium chloride	0.4 g
L-Methionine	7.5 mg	Calcium chloride	0.2 g
L-Phenylalanine	15.9 mg	Magnesium sulfate	97.7 mg
L-Threonine	25.8 mg	Monosodium phosphate·H_2O	0.14 g
L-Tryptophan	4 mg	Glucose	1 g
L-Tyrosine	18.3 mg	Sodium bicarbonate	2.2 g
L-Valine	25.8 mg	Phenol red	0.02 g
Choline chloride	1 mg	Pooled calf serum	100 ml
Biotin	1 mg	Penicillin	100,000 units
Folic acid	1 mg	Streptomycin	100 mg
myo-Inositol	2 mg	Water	to volume

The carbon blacks that were used have been described previously on page 15.

Rubber dust was obtained from the buffing wheel of a local tire recapping shop and screened through a number 40 sieve (0.417 mm). This dust represented both passenger and truck tire rubber.

Five per cent carbon dioxide in air, biological atmosphere, was obtained from the Matheson Company.

All chemicals and solvents were analyzed reagent grade.

Sterile transfers were accomplished in a large enclosed hood sterilized with ultraviolet radiation and provided with rubber gloves.

HeLa stock cultures were carried in milk dilution bottles. Cultures were subcultivated in the following manner when the cell sheet became confluent. The medium was decanted and 5 ml of 0.05 per cent pronase (California Biochemical Company) in Hank's balanced salt solution was added with the cell sheet up. The pronase solution had been brought to pH 7.4 with 7.2 per cent sodium bicarbonate and filter sterilized before using. After allowing the pronase solution to rinse over the cells, the flask was inverted and the pronase solution was decanted. The cells were observed until they had sloughed from the glass surface. Ten ml of GM-1 were added. The cells were aspirated vigorously at least ten times by means of a cotton-plugged 10 ml pipet and a rubber bulb. The resultant cell suspension was evenly distributed between two milk dilution bottles. This is called a 1:2 split. Sufficient fresh medium was added to bring the total volume per bottle to 20 ml. Cultures were sealed with silicone stoppers (The West Company, Phoenixville, Pennsylvania) and incubated at 37°C. All operations were carried out aseptically.

In experiments involving test materials, subcultivations involved a 1:3 or 1:4 split so that cells could be exposed to the test materials for a longer period of time. Large Leighton tubes (28 x 110 mm) were used in some experiments and milk dilution bottles were used in the others. In each experiment, control cultures were inoculated and treated in a manner identical to those containing test materials. All cultures were inoculated from a common pool of cells so that the cell number per flask would be identical. The cultures were gassed for approximately 10 seconds with 5 per cent carbon dioxide in air before they were incubated. This brought the pH to approximately 7.4 and reduced the lag

period in the growth cycle. In order to obtain enough cells to analyze, replicate cultures were utilized for each test material. The cultures were established for 24 hours at 37°C, at which time the spent medium was replaced with fresh medium and 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.

WI-38 cultures were treated in basically the same manner except for minor differences. They were subcultivated alternately every third and fourth day on a strict schedule. Although HeLa cells will not proliferate as rapidly in plastic as in glass, WI-38 cells were more conveniently cultured in plastic. WI-38 cells will not tolerate a pH above 7.4 for any extended period so cultures were always gassed with 5 per cent carbon dioxide in air at the time of inoculation and whenever medium changes were made. Since WI-38 cells replicate more slowly than HeLa cells, subcultivation for an experiment involving test materials was always a 1:2 split. All flasks were inoculated from a common pool of cells.

Human diploid cell strains have a finite limit of approximately 50 subcultivations (30), therefore, all excess cultures were frozen in the following manner:

1. After the confluent cell sheet had sloughed from the surface of the flask and had been aspirated in 10 ml of medium as described above, the cell suspension was centrifuged for 2 minutes at 195 x g. The sterile centrifuge tube was plugged with cotton to prevent contamination. The supernatant was decanted and 2 ml of GM-2 containing 10 per cent

glycerine (sterilized by autoclaving) was added.

3. The cells were aspirated a few times with a sterile 5 ml syringe fitted with a 20 gauge needle 2 inches long in order to obtain a good suspension. The pH of the medium was initially 7.0 so that it would not rise above 7.4 during manipulations.

4. Using the syringe, 1 ml of the cell suspension was dispensed into each of two ampules (2 ml). Care was taken not to touch the necks of the ampules because heat sealing later will cause the medium to char.

5. The ampules were sealed rapidly in a Fisher burner by use of a glass rod. The seal is assured if the tip of the ampule collapses upon heating. All ampules were tagged to indicate the date, cell strain, passage number, medium, etc.

6. The ampules were placed in the refrigerator overnight at 4°C.

7. In the morning the cells were resuspended by agitation of the ampules and the temperature was lowered from 4°C to -25°C at the rate of 1.5° per minute. This was accomplished by placing the ampules in a 125 ml beaker which in turn was inside a 250 ml beaker. The top of the inner beaker was sealed with styrofoam and sufficient insulation was placed around the inner beaker to give the proper rate of cooling when placed in a freezer at -65°C.

8. Ampules were stored in a freezer (Revco, Inc. ultralow temperature model SZB-659) at -65°C.

WI-38 cells were frozen according to the above procedure, stored 4 months at -65°C, and successfully retrieved in the following manner:

1. An ampule to be reconstituted was removed from the -65°C freezer and immediately plunged into a large volume of water at 37°C.

2. The ampule was constantly moved about in the water for 5 minutes to bring the contents of the ampule to 37°C rapidly.

3. After the neck of the ampule was swabbed with 70 per cent ethanol and briefly flamed, it was snapped.

4. The contents were transferred to a 250 ml plastic tissue culture flask by means of a sterile syringe and 20 ml of GM-2 were added.

5. The flask was gassed with 5 per cent carbon dioxide in air, stoppered, and incubated at 37°C.

6. After 24 hours, the medium was changed.

7. When the culture was confluent, it was subcultivated as above. HeLa cells in GM-1 containing 10 per cent glycerine have been successfully frozen, retrieved, and were used in some of the experiments. Mouse L cells were frozen in GM-1 containing 10 per cent glycerine for Dr. Donald C. Cox of the University of Oklahoma Department of Microbiology and have also been successfully recovered by following the above procedure.

To introduce the rubber dust into the test cultures, a quantity of dust sufficient to obtain the required treatment level was weighed into a 250-ml Erlenmeyer flask containing a teflon stirring bar and 5 ml of double distilled water. After autoclaving for 15 minutes at 121°C and 15 pounds of pressure, much of the water is lost but the rubber dust is less hydrophobic than previously. Following autoclaving, the required amount of growth media was added to the Erlenmeyer flask. An even suspension of dust particles in the media was maintained by magnetic stirring while dispensing to test cultures. Upon incubation, the dust particles settled to the cell sheet. If the dust particles

were not uniformly distributed over the bottom of the flask, the medium was swished in the flask until a fairly uniform density of particles was obtained.

The carbon blacks were introduced into the test cultures in the same manner as the rubber dust. Arbitrarily, a concentration of 1 per cent carbon black in the media was utilized. Visually, it could not be determined whether there was uniform dispersion of carbon particles in a flask because 20 ml of media containing a 1 per cent concentration results in an almost complete coverage of the cell sheet with carbon particles.

The cells were exposed to the cigaret smoke tar by pipetting the extract into a 250-ml round bottom flask, removing the solvent in a rotary evaportor and placing the media in contact with the tar for 10-15 minutes before its introduction into treatment flasks. Tar-2 was sterilized by autoclaving in the round bottom flask for 15 minutes at 121°C and 15 pounds of pressure before exposure to the media. Tar-1 was exposed to the media for 15 minutes and the media was then sterilized by filtration. The major portion of the tar was found to be insoluble in the media although at higher levels of exposure, a brownish color was imparted to the media.

Scopoletin was introduced into cultures after sterilization by either autoclaving (no water added) or filtration. The concentration, tested in culture, was 1 mM which is close to the limits of its solubility in growth media. At this concentration, 2-4 hours at approximately 60°C were necessary to effect solution.

At the end of the treatment period, cells were harvested in the

following manner:

1. The spent medium was decanted and the cell sheets were rinsed with 2 ml of 0.85 per cent sodium chloride in double distilled water (1-ml volumes were used in Leighton tube cultures).

2. The saline solution was decanted and 2 ml of saline solution were again added.

3. Using a rubber policeman, the cells were scraped from the surface of the glass and poured into a 40-ml, conical, graduated centrifuge tube.

4. Each flask was rinsed twice with 2 ml of saline solution per rinse. All cells and rinses from replicate treatment flasks were combined.

5. The cells were centrifuged at 195 x g for 2 minutes.

6. The supernatant was carefully removed from the cell "button" with a 10-ml pipet so that 0.5 ml of cells and liquid remained.

7. To the cells were added 10 ml of distilled water. This caused lysis of cells but it was determined experimentally that there was no resulting decrease in the number of cell nuclei present.

8. The cells were resuspended by vortex mixing at high speed for 2 minutes.

9. One-half ml was pipetted and saved for nuclei counting.

10. The remainder of the suspension was transferred to a 100-ml beaker with two rinses (5 ml each) of the centrifuge tube with distilled water.

11. The mixture was placed in boiling water for 2 minutes to stop enzymatic activity and was then sonicated by a Brownwill ultrasonic generator for 2 minutes. The sonicator was set to produce maximum cavitation.

12. The sample was stored in a freezer until it could be deproteinized and analyzed.

Samples were deproteinized with picric acid according to the procedure of Stein and Moore (79) as follows:

1. Each sample was removed from the freezer and allowed to melt. To each sample was then added 5 ml of 1 per cent picric acid in water. In the first deproteinizations, 2 ml of picric acid solution were used per sample but this amount was sometimes insufficient to remove all protein. Some samples contained as many as 29 x 10^6 cells.

2. The mixture was placed on an ion exchange column (1.5 x 3 cm packed volume) of Dowex 2 x 8 (200-400 mesh) in the chloride form. Both the precipitate and the excess picric acid are removed in this step. The beaker which contained the sample was rinsed twice with small quantities of distilled water and the rinsings were added to the column. The flow rate was increased by using 1-5 pounds of pressure on the column.

3. The column was washed with 5 ml of 0.02 N hydrochloric acid.

4. The total effluent from the column was taken to dryness in a 50 ml round bottom flask in a rotary evaporator. Reduced pressure was produced by a water aspirator and distillation was facilitated by a water bath at 50°C.

5. The residue was dissolved in 0.5 ml of distilled water-isopropanol (9:1 v/v) and transferred by means of an eye dropper to a 1-dram vial. The round bottom was rinsed twice with 0.5 ml of the above solvent. This material was also added to the vial.

6. The vials were taken to dryness overnight under an air stream and 0.5 ml of water-isopropanol (9:1 v/v) was added per vial. (Some

samples were 1 ml in volume.)

7. The vials were stoppered and stored in the refrigerator until analyzed.

The deproteinized samples were quantitatively analyzed for proline content by densitometry on Avicel-SF plates according to the procedure previously outlined for the plant tissue extracts.

In order to determine the number of cells being analyzed, nuclei numbers were determined at the termination of each experiment. The nuclei were also counted in a representative flask when the test materials were introduced and a nuclei count was made on the inoculum when the cultures were started. It was assumed that each cell contained only one nucleus. Nuclei were counted according to a modification of the procedure of Sanford et al. (72) as follows:

1. To cells suspended in 5 ml or less of media, 0.85 per cent sodium chloride or distilled water in a 40-ml centrifuge tube, was added sufficient 0.1 M citric acid to bring the volume to 40 ml. A few crystals of thymol were placed in the citric acid stock solution to inhibit growth of molds.

2. The centrifuge tube was stoppered and shaken as vigorously as possible for at least 20 times.

3. The mixture was incubated in air at 37°C for 1 hour.

4. After removing from the incubator, the mixture was again shaken as vigorously as possible for at least 20 times. If shaking was not done, clumps of nuclei occurred which were difficult to count accurately. This problem was greatly diminished if there were few clumps of cells in the original suspension. This was usually the case when the suspension

was obtained by the action of pronase but clumping was more of a problem when the cells were obtained with a rubber policeman. There was also more clumping with WI-38 cells than with HeLa cells.

5. The nuclei were collected by centrifugation at 292 x g for 20 minutes.

6. The supernatant was carefully withdrawn (saved for step 7) with a pipet to the 1-ml level and the nuclei in the sediment were stained by adding 4 ml of 0.1 per cent crystal violet in 0.1 M citric acid. After mixing well on a vortex mixer for several seconds, the mixture was allowed to stand at least 5 minutes at room temperature.

7. To the stained nuclei were added 20 ml of 0.5 per cent methyl cellulose (in 0.1 M citric acid) and enough of the citric acid supernatant (saved in step 6) to bring the mixture to 40 ml in volume. If relatively small numbers of cells were present as in the 0.5 ml sample taken from the harvested cells, all quantities were halved or quartered so that final volumes were 20 or 10 ml.

8. In a hemocytometer, 8-10 counts were made on each nuclei suspension except those cells harvested after treatment with carbon black. In these samples, the many carbon particles present complicated and lengthened the counting procedure; consequently, only four counts were made on each sample containing carbon particles. It is almost impossible to count WI-38 cells directly because upon harvest with either a rubber policeman or a proteolytic enzyme it is extremely difficult to distinguish, under a microscope, cells from debris. This is not such a problem with HeLa cultures but for uniformity and greater accuracy, all cell numbers reported, except where specified, are based on nuclei counts

rather than cell counts.

The number of total nuclei in the sample being counted can be calculated from the average hemocytometer count. There are nine large squares in a hemocytometer field. Each of these large squares is 1 mm on a side and the nuclei suspension is 0.1 mm thick. Therefore the number of nuclei in 1 mm^3 would be 10 times the average number counted in one large square. Usually all nine large squares were counted so that the count must be divided by nine to obtain the average count per large square and multiplied by 10 in order to obtain the number of nuclei in 1 mm^3. In essence, one is multiplying by 10/9 or 1.11. If large numbers of nuclei were present, only four large squares were counted and the factor was 10/4. Since 1 cm^3 is equivalent to 10^3 mm^3, one must multiply the average count per mm^3 by 10^3 to obtain the number of nuclei per ml. If the total nuclei suspension is 10 ml, it is then necessary to multiply by 10 in order to obtain the total number of stained nuclei in the sample. If this in turn is only a sample from a harvested cell suspension to be analyzed, the total nuclei number must be multiplied by another factor to obtain the number of cells that were subjected to analysis. For example, harvested cells were usually suspended in 10.5 ml of distilled water and 0.5 ml was withdrawn for nuclei counting. The 10 ml cell suspension remaining was analyzed for proline content, so that the total number of stained nuclei (contained in 10 ml) was multiplied by 20 to obtain the number of cells that were analyzed. The above information can be condensed to the following formula:

Total cells analyzed $\sim$ average hemocytometer count$(1.11)(10^3)(10)(20)$

when: 1. All 9 large squares are counted in the hemocytometer,

2. the total volume of stained nuclei is 10 ml and

3. the cell suspension aliquot was 0.5 ml of 10.5 ml total.

All glassware used in animal cell cultures was cleaned according to the following procedure:

1. A 25 per cent solution of amphyl was introduced into all flasks which had contained cells.

2. After pouring out the amphyl or other contents, the glassware was brushed in a 1 per cent solution of 7X detergent.

3. Glassware was brought to a boil in a 1 per cent solution of 7X in a large stainless steel pan over an open Fisher burner.

4. After the detergent was rinsed off, the glassware was brought to a boil in a very dilute solution (approximately 0.1 per cent) of acetic acid.

5. Following a thorough rinse with distilled water, glassware was brought to a boil in distilled water.

6. Glassware was then rinsed in distilled water and allowed to air dry.

CHAPTER IV

EXPERIMENTAL RESULTS

Plant Tissue Culture Experiments

Tobacco callus cultures were treated with rubber dust at six different levels. The concentrations are reported as a percentage of rubber in the medium. At each treatment level there were five replicate flasks. The tissues were harvested after a 5-week growth period, weighed, extracted and quantitatively analyzed for free proline content. The results, reported in ng (nanograms) per mg tissue, are presented in Table 5.

It can be seen that concentrations of rubber dust in the medium greater than 0.5 per cent are quite toxic and cause marked inhibition of growth. At the same time, there is a striking increase in free proline in the tissue exposed to increasing amounts of rubber dust.

In the control tissue and in the tissue exposed to 0.5 per cent rubber dust, the free proline content is so low that relatively large quantities of the extracts must be spotted on the thin-layer plates to obtain a quantity which will fall within the range of the standard curve. In such quantities, other amino acids begin to appear and may interfere with the analyses if they are not well separated from proline. There was complete separation in the analyses reported in Table 5 but in a

previous set of analyses of the same extracts there was poor separation in the extracts of untreated tissue and in the extracts obtained from the 0.5 per cent rubber dust cultures. A comparison of the values obtained in these two sets of analyses of the same tissue (Figure 3 and Table 6) gives some idea concerning the reproducibility of direct densitometry for determining proline.

TABLE 5

THE FREE PROLINE CONTENT AND TISSUE YIELD OF TOBACCO CALLUS CULTURES GROWN ON MEDIUM CONTAINING RUBBER DUST[a]

PERCENTAGE RUBBER IN MEDIUM	AVERAGE FRESH WEIGHT[b] (g Tissue/Flask)	PERCENTAGE OF CONTROL TISSUE[c]	FREE PROLINE CONTENT (ng/mg Tissue)	RATIO[d]
0	13.05	(100)	19	(1.0)
0.5	13.37	102	28	1.5
1	6.74	51	111	5.8
5	0.92	7	370	19.4
10	0.98	7	565	29.7
15	0.72	5	575	30.2
20	0.74	5	543	28.6

[a]Data obtained after 5 weeks of growth.

[b]Weight of tissue only and does not include weight of medium.

[c]Weight in g of treated tissue x 100 divided by weight in g of control tissue.

[d]Proline per mg treated tissue:proline per mg control tissue.

These two sets of data were obtained 2 months apart on a different set of plates using different standard curves. Otherwise conditions were as similar as it was possible to make them. At higher levels of proline concentration the reproducibility was very good except with 20

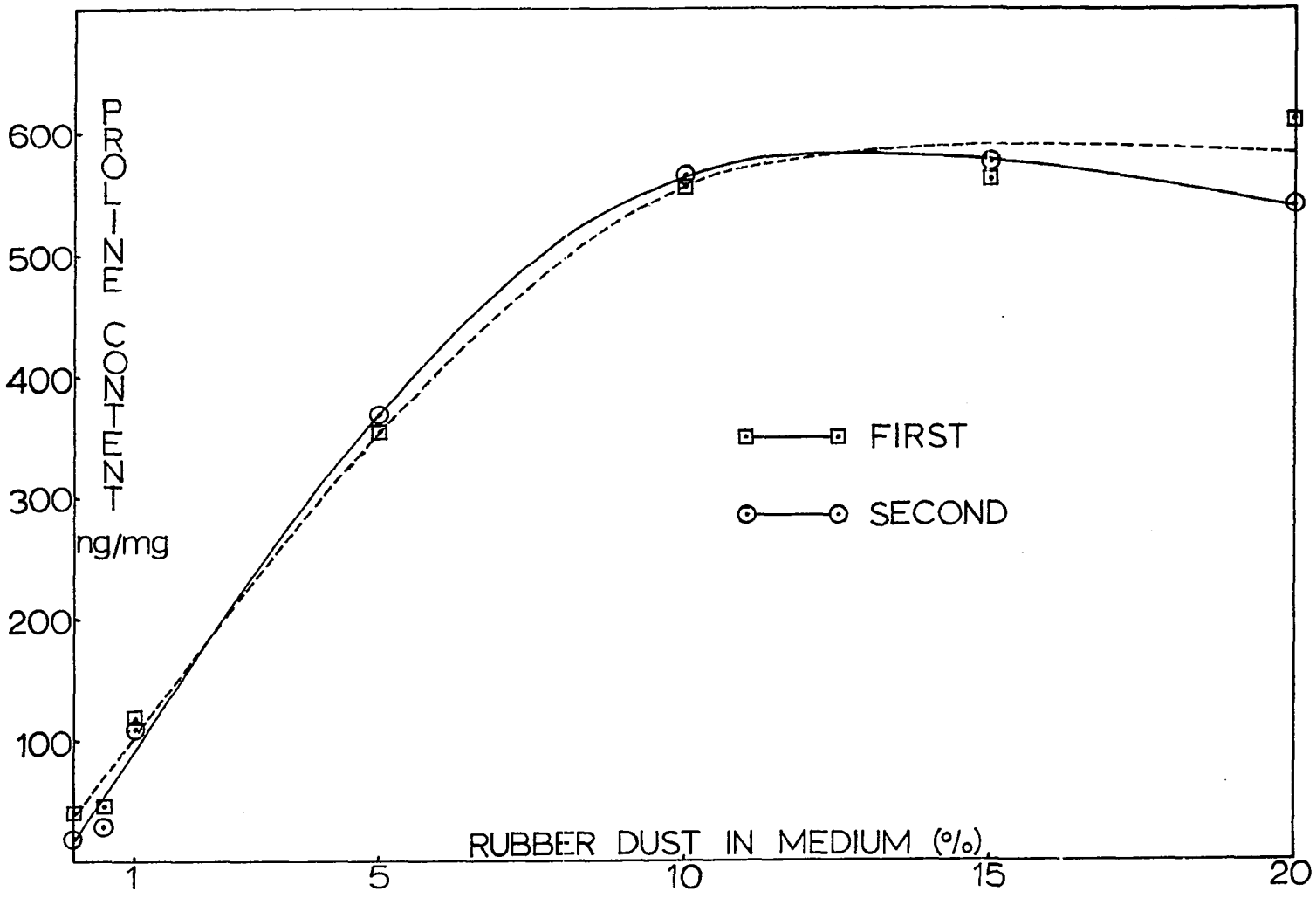

Figure 3. Duplicate direct densitometric determinations of proline in tobacco callus tissue grown on medium containing rubber dust.

TABLE 6

DUPLICATE DETERMINATION OF THE FREE PROLINE CONTENT[a] OF TOBACCO CALLUS TISSUE GROWN ON MEDIUM CONTAINING RUBBER DUST[b]

PERCENTAGE RUBBER IN MEDIUM	FREE PROLINE		DIFFERENCE
	FIRST DETERMINATION	SECOND DETERMINATION	
0	40	19	21
0.5	47	28	19
1	120	111	9
5	357	370	13
10	558	565	7
15	561	575	14
20	613	543	70

[a] Reported in ng of proline per mg fresh weight of tissue.

[b] Data obtained after 5 weeks of growth.

per cent rubber. At the level of proline in the tissue treated with 10 per cent rubber, a difference in one integrator count was found to be equivalent to 10 ng of proline. The large difference in the two determinations on tissue treated with 20 per cent rubber dust is unaccounted for.

From visual observation on thin-layer plates it was determined that the free amino acid in greatest quantity in tobacco callus grown in vitro is glutamine (R_f, 0.09). This amino acid was identified by Innerarity (33). Glutamine gave a purple spot with isatin of the same tint as the unknown spot and the R_f's matched in three solvent systems. When the unknown was isolated in quantity from thin layer plates, hydrolyzed with hydrochloric acid and chromatographed, the R_f of the resulting

compound matched that of glutamic acid and its color produced with isatin was the same as that of glutamic acid plus isatin. Although free glutamine in the tissue was not quantitated, from visual observation of plates free glutamine appeared to increase substantially when the tissue was treated with increasing levels of rubber dust.

TABLE 7

THE TISSUE YIELD AND FREE PROLINE CONTENT OF TOBACCO CALLUS CULTURES GROWN ON MEDIUM CONTAINING WHOLE CIGARET SMOKE TAR[a]

TREATMENT[b] (Tar equivalent to:)	AVERAGE FRESH WEIGHT[c] (g Tissue/Flask)	PERCENTAGE OF CONTROL TISSUE[d]	FREE PROLINE CONTENT (ng/mg Tissue)	RATIO[e]
0 cigaret	5.43	(100)	32	(1.0)
0.25 cigaret	8.30	152	10	0.3
0.5 cigaret	10.90	200	17	0.5
1 cigaret	7.88	145	16	0.5
2 cigarets	3.32	61	29	0.9
3 cigarets	0.57	10	27	0.8

[a]Data obtained after 5 weeks of growth.

[b]Tar was incorporated into the medium.

[c]Weight of tissue only and does not include weight of medium.

[d]Weight in g of treated tissue x 100 divided by weight in g of control tissue.

[e]Proline per mg treated tissue:proline per mg control tissue.

Extracts of tobacco callus tissue treated with whole cigaret smoke tar gave, on thin-layer plates, a poor separation of proline from a material which produced a gray color with isatin. Therefore, all values which are reported in Table 7 under the heading, free proline

content, are probably high. Table 7 indicates that growth was stimulated at lower treatment levels of tar but was severely inhibited at the highest level.

TABLE 8

THE TISSUE YIELD AND FREE PROLINE CONTENT OF TOBACCO CALLUS CULTURES GROWN ON MEDIUM CONTAINING CARBON BLACKS[a]

PERCENTAGE CARBON IN MEDIUM	AVERAGE FRESH WEIGHT[b] (g Tissue/Flask)	PERCENTAGE OF CONTROL TISSUE	FREE PROLINE CONTENT (ng/mg Tissue)	RATIO[d]
Thermal				
0	4.83	(100)	13	(1.0)
1	4.62	95	18	1.4
5	3.39	70	25	1.9
10	4.47	92	17	1.3
15	3.18	65	20	1.5
20	2.65	54	30	2.3
Furnace				
0	4.83	(100)	13	(1.0)
1	8.17	169	23	1.8
5	5.75	119	40	3.1
10	0.96	19	60	4.6
15	0.58	12	128	9.8
20	0.49	10	152	11.7

[a]Data obtained after 5 weeks of growth.

[b]Weight of tissue only and does not include weight of medium.

[c]Weight in g of treated tissue x 100 divided by weight in g of control tissue.

[d]Proline per mg treated tissue:proline per mg control tissue.

Tobacco cultures were treated with two different carbon blacks (Table 8). Thermal black produced some inhibition of growth but no

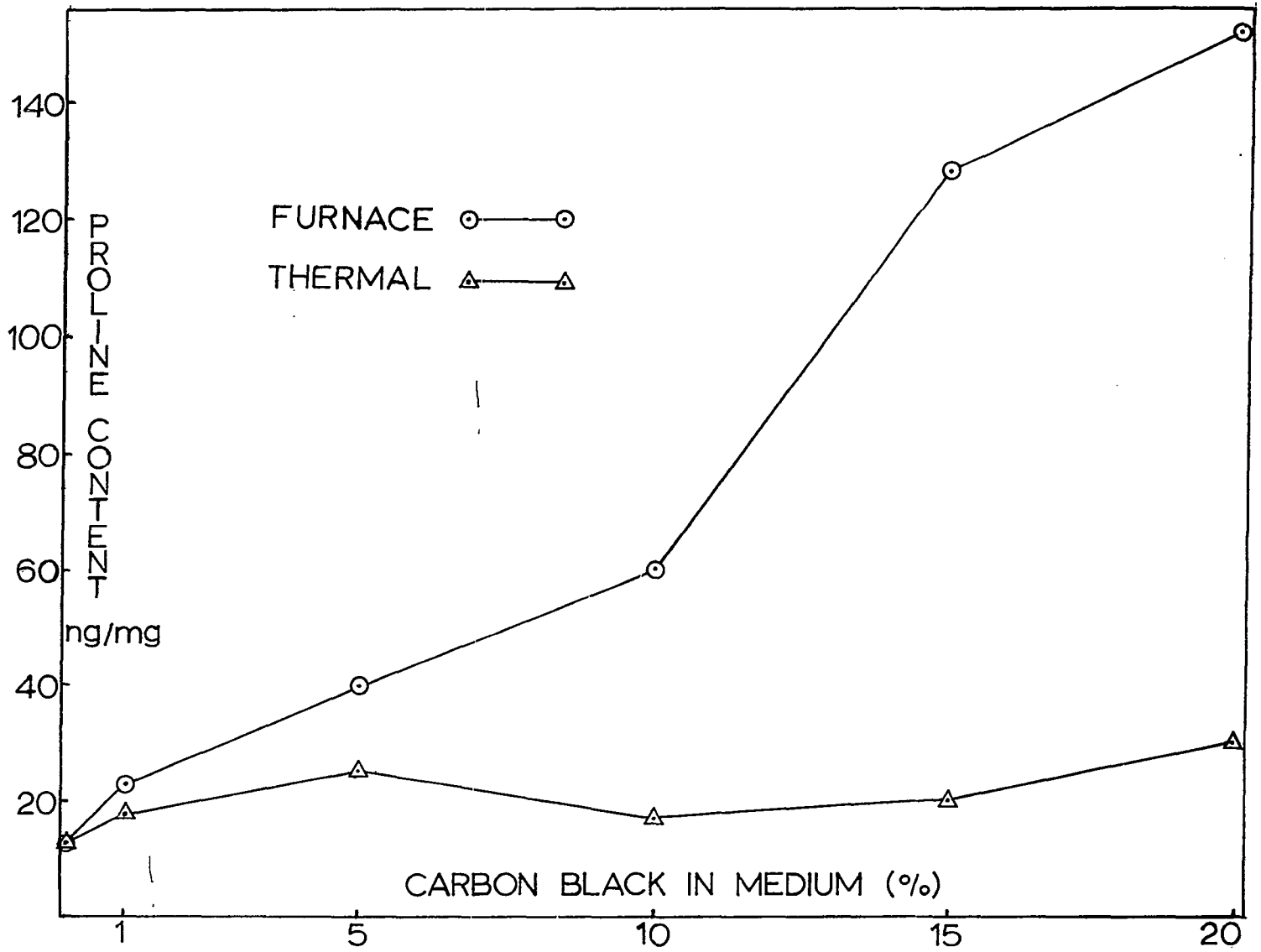

Figure 4. The free proline content of tobacco callus tissue grown separately on media containing two different carbon blacks.

marked change in the free proline content of the tissues. Furnace black produced a much greater inhibition of growth at high treatment levels but enhanced growth at relatively low concentrations. There was a dramatic increase in free proline in the tissues exposed to increasing levels of the furnace black. The increase in free proline, presented in Table 8, is graphically shown in Figure 4.

Animal Cell Culture Experiments

HeLa Cells

HeLa cells in culture were exposed separately for varying periods of time to each of the following materials: rubber dust, carbon blacks, the particulate phase (tar) of cigaret smoke, and scopoletin, which is a coumarin occurring in cigaret smoke (94). The following tables (9-12) summarize the information obtained concerning cell proliferation and the change in the free proline concentration in the cells when exposed to the test materials. The total number of sticking cells that were present when the treatment started is given in millions under the heading: initial cells. It was assumed that only the sticking cells were viable, therefore, no floating cells were included in cell numbers. All cell numbers are based on nuclei counts in a hemocytometer except where otherwise noted. In every case, treatment began after cells had been established for 24 hours in culture. The total number of cells at the termination of the experiments which had been exposed to the test materials is given and the number of hours exposure is designated at the top of the column. An indication of the inhibition of proliferation produced by the test materials is given by dividing the number of treated cells at harvest by the number of control cells at harvest and

multiplying by 100. All harvested cells were quantitatively analyzed for free proline content and this is reported in ng of proline per 10^6 cells.

TABLE 9

THE PROLIFERATION AND FREE PROLINE CONTENT OF HELA CELLS GROWN IN MEDIUM CONTAINING CARBON BLACKS

PERCENTAGE CARBON IN MEDIUM	INITIAL CELLS[a] (x 10^6)	TERMINAL CELLS[b] (x 10^6)	PERCENTAGE OF CONTROL CELLS[c]	FREE PROLINE CONTENT[d]	RATIO[e]
		Experiment 1			
	(0 hours)	(48 hours)			
0	11.4	20.7	(100)	278	(1.00)
1 Thermal	11.4	13.6	66	367	1.32
1 Furnace	11.4	14.1	68	402	1.45
		Experiment 2			
	(0 hours)	(72 hours)			
0	0.5	21.6	(100)	131	(1.00)
1 Thermal	0.5	17.6	82	173	1.35
1 Furnace	0.5	15.8	73	490	3.82

[a]Total cells present at the beginning of the experiment.

[b]Total cells present at the termination of the experiment.

[c]Total treated cells at the termination of the experiment x 100 divided by the total control cells at the termination of the experiment.

[d]In ng of proline per 10^6 cells.

[e]Proline per 10^6 treated cells:proline per 10^6 control cells.

In experiments 1 and 2 in which HeLa cells were treated with scopoletin (Table 12), the cultures were wrapped in aluminum foil to protect the scopoletin against degradation by light. In experiment 3,

cultures were not protected from light. In no case were control cultures protected from light. Cultures were grown in a walk-in incubator which contained a light but the time this light was on was extremely variable. No explanation is known for the decrease in free cellular proline in the cells treated with scopoletin in experiment 1.

TABLE 10

THE PROLIFERATION AND FREE PROLINE CONTENT OF HELA CELLS GROWN IN MEDIUM CONTAINING RUBBER DUST

TREATMENT (mg/100 ml medium)	INITIAL CELLS[a] (x 10^6)	TERMINAL CELLS[b] (x 10^6)	PERCENTAGE OF CONTROL CELLS[c]	FREE PROLINE CONTENT[d]	RATIO[e]
		Experiment 1			
	(0 hours)	(48 hours)			
0	11.4	20.7	(100)	278	(1.00)
200	11.4	9.4	45	520	1.87
500	11.4	10.4	50	441	1.57
800	11.4	0.8	3	1430	5.16
		Experiment 2			
	(0 hours)	(72 hours)			
0	0.5	21.6	(100)	131	(1.00)
400	0.5	12.1	56	320	2.50
600	0.5	9.5	43	374	2.92
800	0.5	8.1	37	240	1.87

[a]Total cells present at the beginning of the experiment.

[b]Total cells present at the termination of the experiment.

[c]Total treated cells at the termination of the experiment x 100 divided by the total control cells at the termination of the experiment.

[d]In ng of proline per 10^6 cells.

[e]Proline per 10^6 treated cells:proline per 10^6 control cells.

TABLE 11

THE PROLIFERATION AND FREE PROLINE CONTENT OF HELA CELLS GROWN IN MEDIUM CONTAINING THE SOLUBLE PORTION OF CIGARET SMOKE TAR

TREATMENT (mg tar/ml medium)	INITIAL CELLS[a] (x 10^6)	TERMINAL CELLS[b] (x 10^6)	PERCENTAGE OF CONTROL CELLS[c]	FREE PROLINE CONTENT[d]	RATIO[e]
		Experiment 1			
(Tar-2 used)	(0 hours)	(48 hours)			
0	11.4	20.7	(100)	278	(1.00)
0.1	11.4	25.1	121	504	1.81
0.5	11.4	6.6	32	402	1.44
1.0	11.4	4.5	22	510	1.84
		Experiment 2			
(Tar-2 used)	(0 hours)	(72 hours)			
0	0.5	21.6	(100)	131	(1.00)
0.2	0.5	5.0	23	541	4.22
0.6	0.5	0.8	4	insufficient cells	
		Experiment 3[f]			
(Tar-1 used)	(0 hours)	(68 hours)			
0	7.8	29.0	(100)	169	(1.00)
0.5	7.8	21.4	74	289	1.71
1.0	7.8	14.4	50	389	2.30

[a]Total cells present at the beginning of the experiment.

[b]Total cells present at the termination of the experiment.

[c]Total treated cells at the termination of the experiment x 100 divided by the total control cells at the termination of the experiment.

[d]In ng of proline per 10^6 cells.

[e]Proline per 10^6 treated cells:proline per 10^6 control cells.

[f]Cell numbers in this experiment were based on cell counts, not on nuclei counts.

TABLE 12

THE PROLIFERATION AND FREE PROLINE CONTENT OF HELA CELLS GROWN IN MEDIUM CONTAINING SCOPOLETIN

CONCENTRATION IN MEDIUM (mM)	INITIAL CELLS[a] (x 10^6)	TERMINAL CELLS[b] (x 10^6)	PERCENTAGE OF CONTROL CELLS[c]	FREE PROLINE CONTENT[d]	RATIO[e]
		Experiment 1			
	(0 hours)	(48 hours)			
0	11.4	20.7	(100)	278	(1.00)
1	11.4	8.8	42	175	0.63
		Experiment 2			
	(0 hours)	(72 hours)			
0	0.5	21.6	(100)	131	1.00
1	0.5	20.2	94	200	1.56
		Experiment 3[f]			
	(0 hours)	(68 hours)			
0	7.8	29.0	(100)	169	(1.00)
1	7.8	18.6	64	289	1.71

[a]Total cells present at the beginning of the experiment.

[b]Total cells present at the termination of the experiment.

[c]Total treated cells at the termination of the experiment x 100 divided by the total control cells at the termination of the experiment.

[d]In ng of proline per 10^6 cells.

[e]Proline per 10^6 treated cells:proline per 10^6 control cells.

[f]Cell numbers in this experiment were based on cell counts, not on nuclei counts.

WI-38 Cells

The exposure of WI-38 cells to the test materials and the analyses for proline were performed in a manner identical to that used with HeLa cells. The data also are reported in tables 13-16 in a similar manner. Because WI-38 cells multiply more slowly than HeLa cells, all cultures were established for 48 hours before the introduction of test materials, unless specified otherwise. When WI-38 cells, treated with rubber dust (Table 13), were harvested, the cell population in the control flasks after a 48 hour growth period was essentially the same numerically to what it had been when the dust was introduced. WI-38 cells also seem to be more sensitive to rubber dust than are HeLa cells. All treatments of WI-38 cells caused a reduction in cell population when compared to the untreated cells except for furnace carbon black (Table 15). Furnace black was also the only test material which caused an increase in free proline in WI-38 cells. All other materials which were tested, contrary to the results obtained with HeLa cells, caused a reduction in free proline in WI-38 cells. The following tables (13-16) present this information in concise form.

TABLE 13

THE PROLIFERATION AND FREE PROLINE CONTENT OF WI-38 CELLS GROWN IN MEDIUM CONTAINING RUBBER DUST

TREATMENT (mg/100 ml medium)	INITIAL CELLS[a] (x 10^6)	TERMINAL CELLS[b] (x 10^6)	PERCENTAGE OF CONTROL CELLS[c]	FREE PROLINE CONTENT[d]	RATIO[e]
	(0 hours)	(48 hours)			
0	8.7	8.1	(100)	208	(1.00)
200	8.7	4.0	41	95	0.37
600	8.7	1.0	12	insufficient	cells
800	8.7	no viable cells		-	-

[a]Total cells present at the beginning of the experiment.

[b]Total cells present at the termination of the experiment.

[c]Total treated cells at the termination of the experiment x 100 divided by the total control cells at the termination of the experiment.

[d]In ng of proline per 10^6 cells.

[e]Proline per 10^6 treated cells:proline per 10^6 control cells.

TABLE 14

THE PROLIFERATION AND FREE PROLINE CONTENT OF WI-38 CELLS GROWN IN MEDIUM CONTAINING THE SOLUBLE PORTION OF CIGARET SMOKE TAR

TREATMENT (mg tar/ml medium)	INITIAL CELLS[a] (x 10^6)	TERMINAL CELLS[b] (x 10^6)	PERCENTAGE OF CONTROL CELLS[c]	FREE PROLINE CONTENT[d]	RATIO[e]
		Experiment 1			
(Tar-2 used)	(0 hours)	(48 hours)			
0	8.7	8.1	(100)	208	(1.00)
0.2	8.7	3.2	40	120	0.58
0.6	8.7	3.6	44	178	0.86
1.0	8.7	2.5	31	181	0.87
		Experiment 2[f]			
(Tar-2 used)	(0 hours)	(120 hours)			
0	2.4	3.6	(100)	375	(1.00)
0.2	2.4	1.8	50	207	0.65
0.4	2.4	1.6	44	insufficient cells	

[a]Total cells present at the beginning of the experiment.

[b]Total cells present at the termination of the experiment.

[c]Total treated cells at the termination of the experiment x 100 divided by the total control cells at the termination of the experiment.

[d]In ng of proline per 10^6 cells.

[e]Proline per 10^6 treated cells:proline per 10^6 control cells.

[f]Established in culture 24 hours before introduction of test materials.

TABLE 15

THE PROLIFERATION AND FREE PROLINE CONTENT OF WI-38 CELLS GROWN IN MEDIUM CONTAINING CARBON BLACKS

PERCENTAGE CARBON IN MEDIUM	INITIAL CELLS[a] (x 10^6)	TERMINAL CELLS[b] (x 10^6)	PERCENTAGE OF CONTROL CELLS[c]	FREE PROLINE CONTENT[d]	RATIO[e]
		Experiment 1			
	(0 hours)	(48 hours)			
0	8.7	8.1	(100)	208	(1.00)
1 Thermal	8.7	6.9	85	116	0.51
1 Furnace	8.7	7.6	94	277	1.32
		Experiment 2			
	(0 hours)	(120 hours)			
0	2.0	6.4	(100)	394	(1.00)
1 Thermal	2.0	5.6	88	345	0.88
1 Furnace	2.0	6.4	100	939	2.38

[a]Total cells present at the beginning of the experiment.

[b]Total cells present at the termination of the experiment.

[c]Total treated cells at the termination of the experiment x 100 divided by the total control cells at the termination of the experiment.

[d]In ng of proline per 10^6 cells.

[e]Proline per 10^6 treated cells:proline per 10^6 control cells.

TABLE 16

THE PROLIFERATION AND FREE PROLINE CONTENT OF WI-38 CELLS GROWN IN MEDIUM CONTAINING SCOPOLETIN

CONCENTRATION IN MEDIUM (mM)	INITIAL CELLS[a] (x 10^6)	TERMINAL CELLS[b] (x 10^6)	PERCENTAGE OF CONTROL CELLS[c]	FREE PROLINE CONTENT[d]	RATIO[e]
		Experiment 1			
	(0 hours)	(48 hours)			
0	8.7	8.1	(100)	208	(1.00)
1	8.7	3.0	37	246	1.18
		Experiment 2[f]			
	(0 hours)	(120 hours)			
0	2.4	3.6	(100)	375	(1.00)
1	2.4	2.8	75	246	0.66

[a]Total cells present at the beginning of the experiment.

[b]Total cells present at the termination of the experiment.

[c]Total treated cells at the termination of the experiment x 100 divided by the total control cells at the termination of the experiment.

[d]In ng of proline per 10^6 cells.

[e]Proline per 10^6 treated cells:proline per 10^6 control cells.

[f]Established 24 hours in culture before introduction of test materials.

CHAPTER V

DISCUSSION

All test materials (rubber dust, carbon blacks and cigaret smoke tar) exhibited toxicity in both plant and animal tissue cultures. At higher treatment levels, toxicity was demonstrated by inhibition of cell proliferation. With the possible exception of cigaret smoke tar, the levels of exposure reported in this work are higher than would usually be encountered physiologically. In certain industries, however, for example in tire recapping and carbon black plants, it is possible that the exposure levels may at times approach those levels which were tested in this investigation. Admittedly, tissue culture itself is an artificial system yet this technique can be useful in obtaining hints as to what molecular events may be taking place in vivo. In the same way, increased levels of environmental pollutants in cultures may give some indication of the direction of physiological changes which take place in an organism exposed to lesser levels. Also it should be noted that although in vivo exposure to these materials may be at a lower concentration level, the length of the exposure in vivo is usually for a much longer period of time than is practical in culture. Hopefully, the increased concentrations in culture are balanced by the decreased time period.

At lower levels of exposure, growth was found to be stimulated at times. When callus was treated with cigaret smoke tar equivalent to that obtained from half a cigaret there was almost a two-fold increase in fresh tissue weight over the control tissue. The same phenomenon was observed in the tobacco tissue cultures exposed to furnace carbon black. The only case of stimulation of growth observed in animal cells was in HeLa cultures which were grown in medium which had been exposed to 0.1 mg tar per ml of medium. This proliferative effect in animal cell cultures has been reported previously for cigaret smoke tar (27, 51) and for carcinogens (3, 50, 57, 59) when exposures were at low concentrations. Stimulation of growth with the other test materials might have been observed if a wider range of concentrations had been examined. Hyperplasia may also be a toxic symptom. Well known examples are the treatment of plants with certain herbicides and the exposure of epithelium to carcinogens.

The macroscopic effects of toxicity can be readily observed but determining the sequence of events at the molecular level which produces these effects is much more difficult. It was at first thought that an increase in the cellular proline pool might be an indication of toxicity at the molecular level.

The increase in free proline in the callus treated with rubber dust roughly paralleled the degree of inhibition of growth but no correlation between free proline content and inhibition of growth was observed when callus was treated with cigaret smoke tar. At the highest levels of tar that were tested growth was virtually nil, but the free proline content of the treated tissue was approximately that of the untreated.

At low treatment levels of tar, growth is greatly stimulated and there appears to be a drop in the concentration of free proline in the treated tissue as compared to the untreated tissue. On the other hand, there was a steady increase in free proline in callus tissue exposed to increasing concentrations of furnace carbon black in spite of the fact that at low concentrations of furnace black there was a great stimulation of growth and at high concentrations there was severe inhibition of growth. In HeLa cells treated with cigaret smoke tar there was an increase of free proline in the cells at all treatment levels even though there was stimulation of growth at the lowest concentration tested and a reduction in the number of cells at higher levels.

It is interesting that whereas the neoplastic cells (HeLa) were found to contain increased quantities of free proline after exposure to the test materials, the normal cell strain (WI-38) showed a drop in free proline content after treatment. The only exception to this is in furnace carbon black cultures where an increase in the proline pool resulted in both HeLa and WI-38 cultures. It seems unusual that furnace carbon black should show no effect on cell numbers in WI-38 cultures, a reduction in cell numbers in HeLa cultures, and either a great stimulation or a severe inhibition of growth in tobacco callus cultures, depending on the concentration of carbon black. However, only one concentration level was tested in animal cells. If a test material enhanced cell growth at low levels and inhibited cell growth at high levels, it might be possible to select an intermediate level of treatment in which there would be no apparent effect on the number of cells in the population.

Thermal carbon demonstrated effects quite different from those of furnace carbon, especially in the tobacco cultures. No stimulation of growth was observed at any treatment level and there was not the severity of growth inhibition in callus cultures encountered with furnace carbon even when the tissue was exposed to 20 per cent thermal carbon in the medium. Carbon blacks are known to vary widely in their chemical and physical properties (60, 77). The amount of benzene-extractable material which is adsorbed to the particles also varies (60, 77). Such differences in carbon blacks could explain the different results obtained with these two in culture.

The variability demonstrated by scopoletin in culture remains unexplained. The only conclusion which can be drawn is that scopoletin inhibits proliferation of human cells in vitro at a concentration level of 1 mM. Scopoletin is of interest because it is a component of tobacco smoke and its presence can be demonstrated in the tar fraction (94). Furthermore, many phenols exhibit co-carcinogenic activity but scopoletin, a phenolic compound, has never been adequately tested in this respect.

In the animal cell cultures, a question might arise concerning the proline concentration of the liquid medium which is bathing the cells. Neither GM-1 nor GM-2 contain proline although these media necessarily contain certain amino acids which the cells can readily synthesize. This apparent anomaly was resolved by Eagle and Piez (23) who found that there is a rapid (15-30 minutes) equilibrium attained between the concentrations of certain amino acids in the cells and their concentrations in the medium. The requirement for these amino acids which

could be synthesized by the cells was found to be dependent upon the cell density in the medium. In dense cell populations the amino acid was not required but in sparse populations the cells would die if the amino acid was not provided in the medium. This is not the case with proline. The free proline concentration in human cells in vitro was found to be essentially independent of the proline concentration in the media. Eagle and Piez (23) reported that the free proline concentration in HeLa cell water was 0.80 mM whereas the proline concentration in GM-1 in which these cells were proliferating was less than 0.005 mM. The ratio of the proline concentration in the cell water to the proline concentration in the medium was therefore greater than 160. In the same publication, Eagle and Piez reported that they found no end-product (negative feedback) inhibition of proline synthesis in human cells.

The significance of increased levels of free proline in living cells exposed to the tested environmental pollutants is not known but some interesting possibilities can be suggested. It is possible that increased levels of free proline in cells might indicate a modification of collagen metabolism. Various collagens contain 10-13 per cent proline and 5-10 per cent hydroxyproline which is derived from proline. The epidemic proportions that emphysema has assumed and the increasing importance of so-called collagen diseases in recent years makes this an important consideration. It has been shown that animal cells in vitro can produce collagen (22), therefore this problem could be investigated in cell cultures.

The significance of a large proline pool may be its ready con-

vertibility into glutamic acid and glutamine. The latter two compounds play central roles in the nitrogen metabolism of the cell and a large proline pool might be of great value to the cell as an augment to its supply of glutamic acid and glutamine. In tobacco callus cultures exposed to rubber dust there was an increase in glutamine as well as proline. Glutamine was visually observed on thin-layer plates to be the amino acid occurring in largest concentration in the free amino acid pool of tobacco callus, although at higher levels of rubber dust treatment, proline may assume this distinction. Saunier et al. (73) obtained similar results when creosote bush was subjected to drought conditions. They found 3.5 times as much proline and twice as much glutamic acid (analysis did not distinguish between the acid and the amide) in the water stressed plants as compared to non-stressed plants. It is of interest that they also found that arginine, which is convertible to glutamic acid, had more than doubled in quantity in the stressed plants. Some workers have found that there is a net increase in proteolysis of protein when plants are subjected to various stress conditions with a resultant increase in release of proline from protein and also an increase in proline synthesis. However, proteolysis of protein does not explain the experiments reported herein in which an increase in free proline was accompanied by a striking stimulation of growth.

Glutamic acid and glutamine are interconvertible in most biological systems. Not only do they play a central role in nitrogen metabolism (by deamination, transamination and decarboxylation), but they serve in other ways. Both occur in proteins. Glutamic acid is utilized in the biosynthesis of arginine. It alsc serves as an important link in the

intermediate metabolism of proteins and carbohydrates by virtue of its conversion to α-ketoglutaric acid. Glutamine plays a crucial role in the biosynthesis of purines. It is possible that one of the most important functions of the proline pool is to act as a bank for glutamic acid and glutamine which can be readily drawn upon when conditions become such that rapid growth is possible.

CHAPTER VI

SUMMARY AND CONCLUSIONS

The growth and free proline content of tobacco callus and human cells exposed in culture to rubber dust, furnace carbon black, thermal carbon black and cigaret smoke tar, was investigated.

In order to facilitate this study, a new and rapid procedure for the quantitation of proline by direct densitometry on thin-layer plates was developed. Plant extracts or deproteinized mammalian cell sonicates were spotted on thin layers of Avicel-SF microcrystalline cellulose (400 μ thick) with the aid of a micrometric syringe. The materials to be quantitated were spotted in triplicate together with standard solutions of proline. The plates were developed in water-formic acid-n-butanol-methanol-phenol (1:1:3:9:9 v/v/v/v/w) for 3.5 hours. After thorough drying, the plates were dipped in 2 per cent isatin in acetone-n-butanol-acetic acid-water (9:9:1:1 v/v/v/v) and heated at 110°C for 25 minutes. The absorbance of the bluish-green proline spots was determined on a densitometer fitted with a Wratten 22 secondary filter. The average percentage absorbance of the material being analyzed was compared to that of the standard solutions spotted on the same plates. A linear response was obtained between 0.1-0.8 μg proline per spot. At the lower limits of detection (0.1 μg), the reproducibility is

±0.03 µg of proline per spot or about ± 30 per cent. At mid-range on the standard curve the reproducibility is approximately ± 15 per cent while at the upper limits of the linear response it is ± 7 per cent. The precision can be greatly improved if a standard curve is drawn for each plate. The lower limit of detection is 0.1 µg of proline per sample which is a sensitivity ten-fold greater than the methods commonly employed for quantitating proline.

Using this method of analysis, proline was quantitated in tobacco callus and human cells treated in culture with rubber dust, carbon blacks and cigaret smoke tar. Human cells were also treated with scopoletin, a coumarin which occurs in cigaret smoke.

Tobacco callus treated with increasing levels of rubber dust in the medium exhibited an inhibition of growth and dramatic increases in free proline content. The free proline content of treated tissue increased as much as thirty times that of the control cultures. When callus tissue was treated with whole cigaret smoke tar there was a stimulation of growth at low concentrations of tar in the medium and an inhibition of growth at high concentrations. There was a decrease in proline content of the tissue at all treatment levels of tar. Increasing levels of thermal carbon black in the medium produced some inhibition of growth and moderate increases of proline in tobacco tissue. Furnace carbon black produced a stimulation of callus growth at low concentrations and an inhibition of growth at high levels. All treatment levels of furnace carbon black resulted in increased quantities of free proline in callus, the highest concentration being eleven times that found in untreated tissue.

When HeLa cells (human carcinoma of the cervix) were exposed in turn to rubber dust, thermal carbon black, furnace carbon black and the portion of cigaret smoke tar soluble in media, an inhibition of cell proliferation was always obtained except in one instance. When cells were grown in medium exposed to 0.1 mg of tar per ml medium, there was a stimulation of proliferation. A rise in free proline content of the treated cells compared to the untreated cells was found in every case. The proliferation of HeLa cells exposed to scopoletin was inhibited but the free proline response was variable. In two experiments, the free proline content of the treated cells was increased while in one experiment it was decreased.

WI-38 (human fetal diploid cells derived from lung) cultures treated in turn with rubber dust, thermal carbon black and the portion of cigaret smoke tar which was soluble in media, exhibited inhibition of proliferation accompanied by a decrease in free proline content. WI-38 cells exposed to furnace carbon exhibited no inhibition of proliferation but an increase in free proline was found in the treated cells. Treatment with scopoletin also produced a reduction in cell population but the free proline response, as in HeLa cultures, was again variable. In one experiment there was a slight increase in free proline but in another experiment there was a decrease in the proline content of the cells.

The possible significance of changes in the proline pool of cells exposed to these environmental pollutants may be summarized as a cellular response to stress, an upset in collagen or collagen-like protein metabolism, a bank for supplementing the supply of free glutamic

acid and glutamine or a combination of these.

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