EFFECT OF DIETARY VITAMIN A AND CYCLIC NO_2 EXPOSURE ON THE HAMSTER LUNG

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ELOISE R. SCOTT CARLISLE

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ABSTRACT

EFFECT OF DIETARY VITAMIN A AND CYCLIC NO_2 EXPOSURE ON THE HAMSTER LUNG

By

Eloise R. Scott Carlisle

The effect of dietary vitamin A and cyclic NO_2 exposure on the hamster lung was evaluated by histopathology, electron microscopy and liquid scintillation techniques.

Hamsters were maintained on a deficient (0 µg), adequate (100 µg) and high (200 µg) dose level of vitamin A while being exposed cyclically to 10 ppm NO_2 for 5 hours once a week, over an 8-week period.

Hamsters of the deficient group exhibited clinical and morphologic changes characteristic of vitamin A deficiency. Animals maintained on adequate and high dose levels of vitamin A were not similarly affected.

Hypertrophy and hyperplasia of the epithelial cells of the terminal bronchiole-alveolar region of adequate and high dose animals as a result of NO_2 exposure were greater than that observed in the deficient animals. Ultrastructural changes observed were hypertrophy and hyperplasia of bronchiolar epithelial cells, diffuse loss of cilia, membrane damage, and mitochondrial damage manifested by calcium deposition.

Eloise R. Scott Carlisle

Limited tritiated thymidine uptake studies of lungs of animals in the 3 groups revealed a variation in cell kinetics following NO_2 exposure.

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INTRODUCTION

Recent public awareness has directed considerable attention toward the importance of environmental contaminants in relation to the health and continued survival of man and animals. The scope of the adverse effects that these man-made contaminants may have on a host has not been fully established. However, there is evidence to suggest that environmental contaminants originating from such varied sources as automobile combustion and exhaust fumes, industrial waste and cigarette smoke may play an important role in inhalation carcinogenesis. The importance of irritating air pollutant gases, such as ozone, nitrogen and sulfur dioxide, cannot be over-emphasized because of their known irritating effects on mucous membranes of the respiratory tract.

It is essential to understand a single factor and its biological effects before we are able to investigate the association of all other possible factors. Experimental studies done to date unequivocally demonstrate the injurious effects of nitrogen dioxide; however, very few studies have been directed toward host factors which modify its injurious effect.

Vitamin A has been shown to play an important role in host response following exposure to 10 parts per million nitrogen dioxide gas (Kim et al., 1976).

The objective of this study was to determine pathologic changes that occurred in hamsters fed a deficient (0 µg), adequate (100 µg)

and high dose (200 μg) level of vitamin A while being cyclically exposed to 10 parts per million (ppm) nitrogen dioxide gas. This mode of exposure not only reflects industrial pollution found in an urban-suburban environment but also the exposure of the respiratory tract of a habitual smoker. Experimental data simulating such a situation are not available. The biologic effects of nitrogen dioxide and the modifying role of vitamin A were evaluated by histopathology, electron microscopy, and liquid scintillation techniques.

REVIEW OF LITERATURE

Vitamin A

Vitamin A and Its Biological Role

Vitamin A is one of the fat soluble vitamins required for several highly specialized functions. In 1913 two groups of investigators reported that certain fats contained an essential nutrient for rats (Follis, 1969). Since that time, numerous investigators have advanced our knowledge of this vitamin. A brief review of pertinent information relevant to this work is presented.

There are three active forms of vitamin A. They are vitamin A alcohol (retinol), aldehyde (retinal) and acetate (retinoic ester). One International Unit (IU) of vitamin A is equivalent to the activity of 0.344 µg vitamin A acetate, 0.3 µg vitamin A alcohol or 0.6 µg carotene. Vitamin A is found in fish, fish oils and dairy products. Substances that act as provitamins are found in plants and are referred to as carotenoids. The carotenoids are converted to vitamin A as they are absorbed through the intestinal wall. The absorption of vitamin A is a function of the digestion and absorption of fat (Ullrey, 1972). Vitamin A is stored primarily in the liver and released as needed. It is transported via the blood plasma for biological utilization (Follis, 1959; Sebrell and Harris, 1967; Ames, 1969).

The vitamin A requirements for man and animals vary based on the quantity stored in the liver. Vitamin A is necessary for vision, reproduction (spermatogenesis, development of the fetus) and growth (maintenance and development of epithelial tissue, bone growth and development) (Wolbach and Howe, 1925, 1928; Tilden, 1930; Salley and Bryson, 1957; Smith et al., 1972; DeLuca, 1975).

Vitamin A acetate is the form believed to be most active in biological utilization, particularly growth and differentiation of epithelial tissue (Ames, 1969; DeLuca, 1975). Wald and Hubbard (1950) showed vitamin A aldehyde to be the active form necessary for the formation of opsin which is utilized in the visual process. Deprivation of vitamin A results in a degeneration of the rods and cones and subsequent nyctalopia (Follis, 1959; Smith et al., 1972).

Hypovitaminosis A

All mammals including man are subject to vitamin A deficiency with little variation in symptoms (Lehninger, 1970). A significant decline in growth is observed in animals increasingly deficient in vitamin A. There is loss of weight, a rough, dull hair coat, and generalized unthriftiness (Wolbach and Howe, 1925, 1928; Tilden, 1930; Salley and Bryson, 1957).

Vitamin A deficiency affects reproduction by causing testicular atrophy and aspermatogenesis in the male. The mechanism by which vitamin A plays a role in spermatogenesis is not known. Keratinization of the mucosa of the uterus and oviducts occurs in the female; however, ovulation does not appear to be affected (Wolbach and Howe, 1925, 1928; Salley and Bryson, 1957).

The most profound pathologic effect of vitamin A deficiency is seen in the mucus secreting epithelial cells. Observation with the light microscope reveals keratinization and squamous metaplasia of the mucous cells of the trachea and bronchi. The submaxillary and paraocular glands atrophy. There is cellular disintegration, transient edema and infection manifested by abscess formation (Wolbach and Howe, 1925, 1928; Salley and Bryson, 1957).

Vitamin A and Infection

During vitamin A deficiency, animals are more susceptible to infection. Green et al. (1928) observed an increase in infections of a pyogenic nature especially in the salivary glands, gastrointestinal tract, prostate and seminal vesicles of rats. Bang and Foard (1971) found an increased susceptibility to Newcastle and influenza virus in chicks. They attributed this to impaired immune mechanism due to vitamin A deficiency. Tvedten et al. (1973) noted vitamin A deficient rats showed an increased susceptibility to *Mycoplasma pulmonis* under germfree and conventional conditions. They stated that this may be due to a decrease in the functional protective capacity of epithelial tissues.

Hypervitaminosis A

In man, hypervitaminosis A is most often seen with oversupplementation. Accidental toxicity in man and animals can occur following the ingestion of polar bear liver and the liver and flesh of other marine animals possessing a high vitamin A content (Follis, 1959; Sebrell and Harris, 1967). Toxicity can be induced experimentally in cattle and other animals (Jones, 1965).

Symptoms of vitamin A toxicity include loss of weight, inappetence, hyperesthesia, rapid bone growth and increased bone fragility. Significant changes are seen at the epiphyseal plate manifested by an increased rate of consumption of the epiphyseal cartilage and replacement by osseous tissue.

A review of vitamin A by DeLuca (1975) states that several physiologic functions can be shown by the fact that vitamin A acid is not critical for visual or reproductive functions, whereas it is for growth and differentiation of epithelial tissue. It has been found that vitamin A acid is an abundant and normal metabolite of vitamin A alcohol. This suggests that the acid form or a further metabolite may be the active form of vitamin A.

The mode of action of vitamin A in cell differentiation is not known. However, DeLuca et al. (1972) have shown vitamin A essential in forming intermediates which function as carriers in the biosynthesis of epithelial mucins and of surface glycoproteins.

In vitro Studies

Using tracheal organ cultures, Marchok et al. (1975) demonstrated the change of normal tracheal epithelium to keratinizing squamous epithelium using culture nutrients deficient in vitamin A. A reversal was observed when vitamin A was added. Changes in cell populations of the trachea of hamsters were observed by Boren et al. (1974) using two groups of experimental hamsters. The hamsters maintained on a high level of vitamin A showed an increase in ciliated cells and a slight decrease in mucous cells. The deficient hamsters showed an increase in basal cells and a decrease in ciliated cells.

Ultrastructural Studies

An electron microscopic study by Wong and Buck (1971) elucidated the ultrastructural changes that take place in rats maintained on a vitamin A deficient diet. The first evidence of metaplasia was the appearance of clusters of hyperplastic basal cells. As the deficiency progressed, the most superficial cells were desquamated and the hyperplastic cells differentiated into flatter cells. Cornifying features such as keratohyaline granules and keratin fibrils were observed. Intercellular spaces began to shrink and there was further development of desmosomes. It was concluded that metaplastic transformation rather than a result of de-differentiation was a result of the differentiation of generative cells in a new direction.

Vitamin A and Carcinogenesis

Investigators have postulated that squamous metaplasia may be a preneoplastic stage in the histogenesis of squamous cell carcinoma of the lung in man (Auerbach et al., 1961) and hamsters (Harris et al., 1972). Harris and associates (1972) described the similarities of lesions produced in vitamin A deficient hamsters and hamsters treated by the tracheal instillation of a carcinogenic agent, benzo (a) pyrene ferric oxide. Squamous metaplasia seen was the same under the light microscope. However, examination by electron microscopy showed the morphology of the cells to be different in hamsters given the carcinogenic agent. There were pleomorphic nucleoli, enlarged nuclei and focal defects in the basement membrane. They cautioned that the ultrastructural differences cited cannot as yet be regarded as specific for respiratory carcinogenesis. Low levels of vitamin A may increase the susceptibility of rats to lung cancer both prior to

and after tracheal instillation of methyl cholanthrene (Nettesheim et al., 1975).

Recently emphasis has been placed on the role of vitamin A in cancer chemotherapy. Saffioti et al. (1967) observed a reduction of squamous cell tumors developed in hamsters after ten intratracheal instillations of benzo (a) pyrene and hematite, and subsequently given twice weekly feedings of vitamin A palmitate (5 mg) for life. Vitamin A has a systemic inhibitory effect on the induction of squamous changes in the columnar mucous epithelium of the respiratory tract.

Studies have been done (Bollog, 1970; Sporn et al., 1976) using natural as well as synthetic retinoids as therapeutic agents for epithelial tumors in experimental animals. The potential use of vitamin A as a cancer prophylaxis appears to lie with the use of the synthetic retinoids because of their high potency and low toxicity (Sporn et al., 1976).

Nitrogen Dioxide (NO_2)

Source

Air pollution is one of the major concerns of large urban communities. The important irritant air pollutants are ozone, oxides of nitrogen and sulfur dioxide. Solar photochemical energy induces reactions in smoggy atmospheres between oxides of nitrogen and oxygen to give nitrogen dioxide and ozone. Nitrogen dioxide is a product of industrial waste and motor vehicle combustion and exhaust fumes, and it is a serious health hazard since it is found not only in air pollution but also in ciagarette smoke (Hagen-Smith et al., 1949; Freeman et al., 1968a; Environmental Protection Agency, 1971; U.S.

Department of Health, Education and Welfare, 1972; Johnson et al., 1973; Goldstein, 1975). Incidents of NO_2 toxicity have been reported in silo fillers and cattle exposed to high concentrations from fermenting silage (Lowery, 1956). A recent concern has been the role air pollutants might play in chronic respiratory disease and lung cancer, particularly at low dose levels (National Cancer Institute, 1970a).

Effects of NO_2 in Laboratory Animals

Freeman and Haydon (1964) exposed rats to 100 parts per million (ppm) of NO_2 and observed that these animals died within 24 hours. Acute pulmonary edema, marked vascular congestion and focal areas of hemorrhage were seen in the lungs at necropsy. Subsequent studies with 50 ppm showed similar changes with death occurring in 48 to 68 days. At 25 ppm the lungs were described as air containing and voluminous, resembling emphysema (Freeman and Haydon, 1964). Microscopically, moderate hypertrophy and hyperplasia of the bronchial and bronchiolar epithelium and increased activity of the goblet cells was noted. Proliferation of connective tissue stroma, free macrophages and desquamated cells were observed in the alveolar spaces. Similar changes were seen in rats exposed to 12.5 ppm.

Using an exposure level of 0.8 ppm of NO_2, Freeman et al. (1966) observed no lesions that could be unequivocally related to NO_2, although the animals exhibited tachypnea. Experiments with rats at 2 ppm of NO_2 resulted in cells of the bronchiolar epithelium being shortened and widened in addition to reduced or absent cilia (Freeman et al., 1968a,b). Experimental evidence indicates lesions induced by NO_2 may be a function of concentration and time within the subacute range (Freeman et al., 1969).

Goldstein (1975) reviewed the effect of NO_2 on a wide variety of animal models. It was concluded from these experiments that brief exposures to high concentrations of NO_2 tend to be more toxic than equivalent exposures to low concentrations of pollutants for prolonged time periods. Acute exposure at high levels causes airway irritation, vascular congestion, edema formation, bronchiolitis, tissue destruction, and enhanced susceptibility to respiratory infection in several species. For rodents (mice, rats and guinea pigs) a one-hour exposure at 40 to 50 ppm resulted in acute death. Monkeys were found to be affected the same as rats in an eight-hour experiment at 65 ppm. Rabbits and dogs were found to be more resistant.

Various animal models (rats, guinea pigs, primates, rabbits and dogs) have been shown to survive continuous exposure of one year or more of NO_2 above ambient levels, generally considered to be less than 1 ppm (Goldstein, 1975). Physiologic changes of severe airway obstruction, hyperinflation and arterial oxygen desaturation were seen. However, these abnormalities would revert to normal after exposure ceased.

Since distal airways of primates have been found to be morphologically similar to those of man in contrast to those of rodents, Mellick et al. (1977) exposed rhesus monkeys to ambient levels of ozone. They observed hyperplasia and hypertrophy of nonciliated bronchial epithelial cells and intraluminal accumulations of macrophages after exposure. Large conducting airways showed damage to ciliated cells while mucous producing cells were unaltered. An intermediate cell type (between type 1 and 2 cells) was observed.

The hamster is a suitable animal model for inhalation studies because it is relatively resistant to pulmonary infection and free from spontaneous tumors of the respiratory tract. Additionally, the hamster respiratory tract has been shown to more closely resemble that of man than other murine species (Nettesheim, 1972; Kleinerman, 1972), although few studies have been made with hamsters.

Hamsters intermittently exposed to 10 ppm of NO_2 for a period of 10 weeks have shown changes consistent with those of other murine species. Animals observed to be tachypneic during exposure returned to normal breathing at the end of the exposure period. Microscopic study revealed marked hypertrophy and hyperplasia of the cells of the terminal bronchial area and thickening of the alveolar walls (Creasia et al., 1972; Kim, in press 1977a).

Similar studies have been done using low levels (0.5 to 3 ppm) of ozone (O_3) in dogs (Freeman et al., 1973) and rats (Stephens, 1974a,b; Schwartz et al., 1976). Lesions produced by O_3, a deep irritating agent, were observed distal to the terminal bronchiole but comparable to the NO_2 lesion in morphology.

Investigators have observed an increased susceptibility to infection following exposure to NO_2 with ensuing challenge using pathogenic bacteria (Purvis and Ehrlich, 1963; Ehrlich, 1966). This susceptibility is related to the inhibition of the function of the alveolar macrophage (Goldstein et al., 1973, 1974). The mucus ciliary transport system does not appear to be affected by NO_2 exposure (Goldstein et al., 1974).

Ultrastructural Studies

Breeze et al. (1976) reviewed the characteristic structure and function of cells lining the trachea, bronchi and bronchioles. Ultrastructural morphology in the hamster varies little from descriptions of other mammals (dog, rat, mouse and man) (Kleinerman, 1972; Breeze et al., 1976). Ciliated and nonciliated cells make up the bulk of the tracheobronchial epithelium. The nonciliated (Clara) cell is most abundant in the bronchioles. It contains large amounts of agranular endoplasmic reticulum, long filamentous mitochondria and occasionally dense staining spherical inclusions. Microvilli are present on the luminal surface. Respiratory bronchioles closely resemble terminal bronchioles. The bronchiolar epithelial cells at the alveolar duct junction are attached to membranous pneumocytes by tight junctions and a continuous basement membrane underlying both cell types. The type 1 pneumocyte has an extended (flattened) cytoplasm and a small number of organelles. Granular (type 2) pneumocytes occupy a large portion of the alveolar epithelial lining. These cells possess microvilli, a dense cytoplasm, numerous laminated osmiophilic inclusions and many organelles. They are believed to be responsible for the production of surfactant (Askin and Kuhn, 1971). The alveolar macrophages lie on the surface active film on the epithelial cells lining the pulmonary alveoli (Evans et al., 1973).

The vascular surface is thicker and more membranous than the alveolar surface, which is lined by squamous endothelial cells. Epithelial and endothelial cells are closely associated with the basement membranes which join to form a single membrane in areas of close proximity between the two cell types. The septal interstitium contains collagen fibers.

Studies by electron microscopy have shown that exposure to NO_2 and O_3 affects the ciliated epithelium of the terminal bronchiolar area and the type 1 cells of the alveoli (Evans et al., 1971; Stephens et al., 1972, 1974b). Hypertrophy and hyperplasia of ciliated epithelial cells was seen. The type 1 pneumocyte showed swelling of the cell as well as mitochondrial swelling, rupture of the plasma membrane and cell disintegration. Death and subsequent desquamation of the type 1 cells was followed by proliferation of type 2 cells. The type 2 daughter cells then migrated across the basement membrane thus replacing the damaged epithelium (Evans et al., 1974). Using tritiated thymidine (^{3}H-TdR) and autoradiography, the cycle of the cells was determined following exposure to low levels of NO_2 (Evans et al., 1972, 1974). Respiratory cells were affected within the first 24 hours. There was an increase in the number of dividing cells during the first 48 hours following exposure. Mitosis then declined until dividing cells approached control levels by 4 days after NO_2 exposure.

Nutritional Modifying Factors for Air Pollutant Gases

Combined Effects of Air Pollutants and Vitamin E as an Antioxidant

Many factors must be considered in order to determine the effect air pollutants may have on the respiratory system. These factors include nutritional status (dietary protein and fat, trace minerals and vitamins), age, sex, smoking habits and individual genetic variance (Anderson and Ferris, 1965; Shakman, 1974).

Stephens (1971) has suggested that in the respiratory tract NO_2 may react to form nitric acid, or act as an agent causing oxidation

of unsaturated lipids with the formation of free radicals. Antioxidants such as vitamin E (alpha tocopherol) may protect against peroxidation by disrupting this sequence (Tappel, 1973; Thomas et al., 1967). In contrast, Ramazzotto and Engstrom (1975) observed rats supplemented, adequate and deficient with vitamin E and exposed to NO_2 show a diminished percent of lipids for all groups. This indicated that alpha tocopherol may not protect lipids from breakdown when exposed to NO_2 or similar pollutants.

Sato et al. (1976) observed ultrastructural changes in rats deficient in vitamin E and exposed to 0.3 ppm of ozone. The surfaces of alveolar ducts and walls showed scattered areas of cytoplasmic swelling, cilia damage and round electron dense bodies.

Combined Effects of NO_2 and Vitamin A

Since the modifying role of vitamin A on injurious effects of NO_2 had not been done, studies were begun to compare the response of the lung tissues obtained from non-gas exposed, vitamin A deficient hamsters and gas exposed hamsters fed a regular commercially prepared diet (Kim et al., 1976). Electron microscopic studies of the hamsters on the regular commercially prepared diet exposed to NO_2 showed hypertrophy and focal hyperplasia in the epithelium of the terminal bronchioles and loss of cilia. Lung tissues obtained from hamsters on a vitamin A deficient diet for 4 weeks showed a critical morphologic difference. Thickening of the basement membrane, collagen proliferation and edema were characteristic. Alveolar necrosis was observed. Variable-sized lipid droplets were observed within the alveolar walls; in addition, electron dense bodies and budding

virus-like particles were observed along the inner and outer aspects of the basement membrane (Kim et al., in press 1977a).

MATERIALS AND METHODS

Animals

Pregnant, Golden Syrian hamsters (*Cricetus mesocricetus*) were procured from a commercial source.[a] Immediately upon arrival, the animals were placed one to a cage with paper nesting material and wood chip bedding and given vitamin A free pellet diet[b] (Appendix A) and water *ad libitum*. The hamsters were received at approximately 10 days of gestation and were undisturbed for the remainder of gestation and parturition. The pups were weaned at 21 days of age, separated according to sex and randomly placed 5 per cage. All cages were covered with filter tops. The weanling hamsters were given vitamin A free food and water *ad libitum*. A total of 20 animals was used for group 1 (Figure 1).

The subsequent groups of hamsters (groups 2 and 3) were received from the same source[a] at 21 days of age. They were sexed and randomly placed 5 per cage and given vitamin A deficient food and water *ad libitum*. A total of 21 and 19 animals for groups 2 and 3, respectively, were used (Figure 1). The hamsters were maintained on a vitamin A free diet throughout the experiment.

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[a]Courtesy of the National Institutes of Health, Sprague-Dawley, Madison, WI.

[b]Tekland Mills, Division of Mogul Inc., Madison, WI.

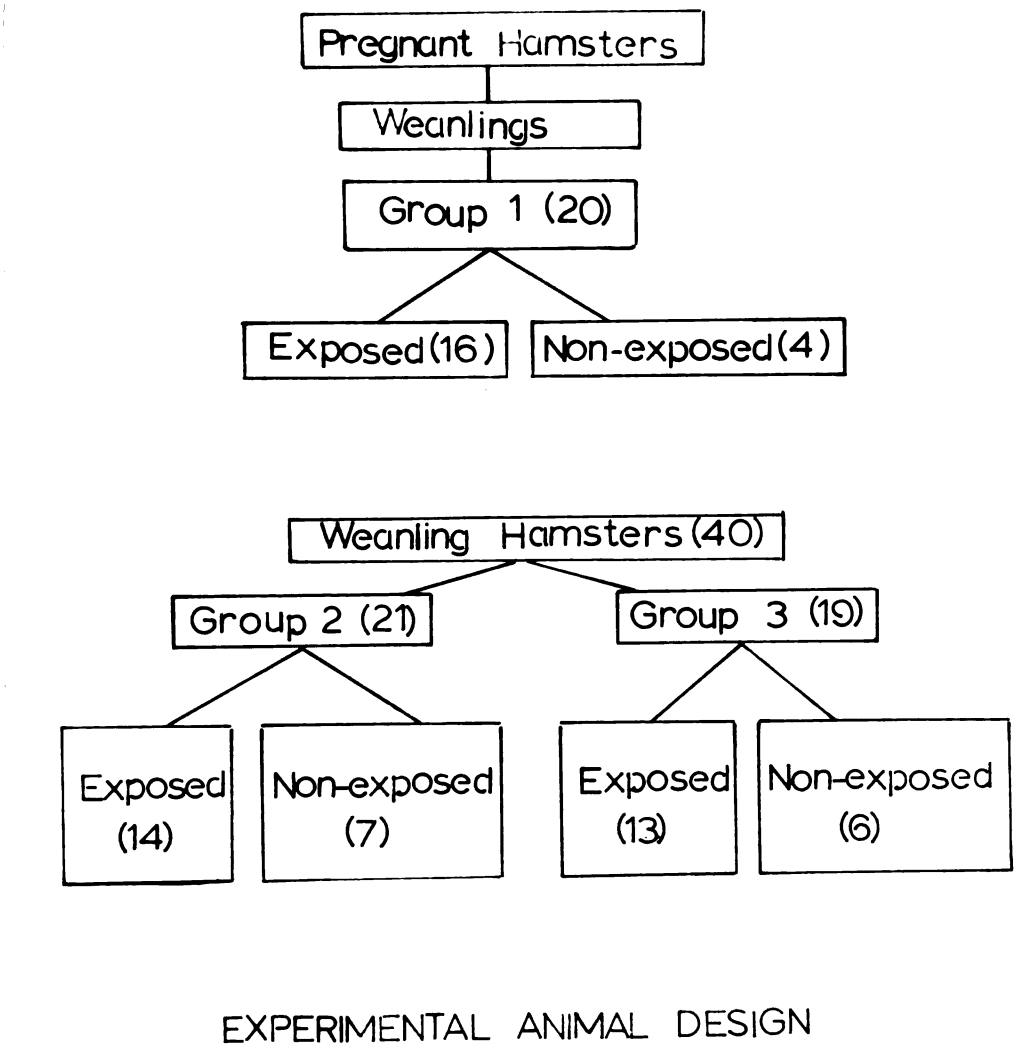

Figure 1. Experimental animal design.

Weighing

Bedding material and cages were changed once a week. The hamsters were weighed to the tenth gram prior to the weekly NO_2 gas exposure. The weekly weight of the exposed and control animals was taken and recorded.

NO_2 Exposure

A plastic exposure chamber measuring 52 x 33 x 18 cm equipped with a sampling outlet was used (Figure 2). The hamsters were exposed to 10 ppm NO_2 for a period of 5 hours, once a week, for a total of 8 weeks. Due to space limitation, a maximum of 10 animals were exposed each time.

Nitrogen dioxide gas at a concentration of 2,040 ppm was purchased from a commercial source.[c] This was further diluted to 10 ppm with an air and flow meter prior to entering the exposure chamber. The relative humidity of the chamber was approximately 50%; the temperature range was 70-72 F. Approximately 14 air changes were achieved per hour. Concentration of the final mixture was colorimetrically tested at 90-minute intervals during exposure according to the Griess-Saltzman method (Appendix B) and a commercial NO_2 detection kit.[d]

Administration of Vitamin A

Animals in group 1 were not given vitamin A during the experiment. They were observed during the 8-week period and allowed to reach a

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[c]Matheson Gas Products, Division of Will Ross, Inc., Chicago, IL.

[d]Kitagawa[R], Matheson Gas Products, Division of Will Ross, Inc., Chicago, IL.

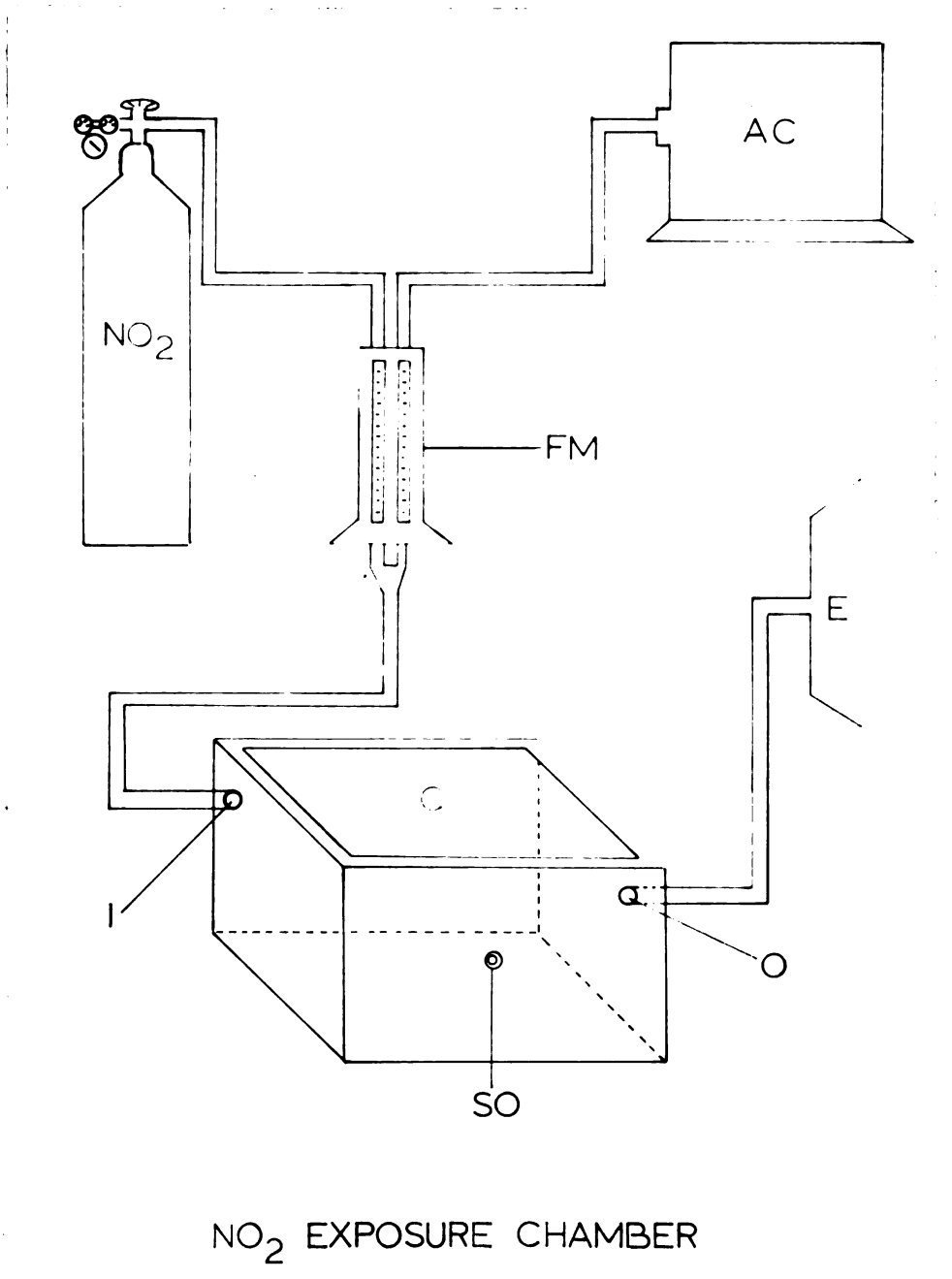

Figure 2. Nitrogen dioxide exposure chamber. Air compressor (AC), flow meter (FM), chamber (C), inlet (I), sampling outlet (SO), outlet (O) and exhaust (E).

deficient state. For groups 2 and 3, retinyl acetate[e] in crystal form was obtained and used in making an oral preparation to maintain the desired vitamin A level (Appendix C). This preparation was given 24 hours before each weekly exposure. Each hamster received 0.1 ml using a 1-ml tuberculin syringe and dosing needle via the cheek pouch.

Group 2 was designated vitamin A adequate. Each hamster was given 50 µg retinyl acetate once a week for 5 weeks.

Group 3 was designated vitamin A high. Each hamster was given 100 µg retinyl acetate once a week for 5 weeks. The dose levels were later altered to 100 and 200 µg twice weekly for groups 2 and 3, respectively, due to loss of body weight.

Necropsy Procedure

The experiments were terminated after the eighth week of exposure. The hamsters were chosen at random and killed 24, 48, 72, 96 and 120 hours after the final gas exposure. Two hamsters in each time period in groups 2 and 3 were injected intraperitoneally with tritiated thymidine[f] at 0.2 µC per gram of body weight, 55 minutes prior to an overdose of sodium pentobarbital.[g] They were then placed on a necropsy board. The abdomen was opened and the aorta and vena cava were cut. The pooled blood was collected with a 5-ml syringe and a blood smear was made for a differential leukocyte count.

The larynx and upper trachea were exposed before opening the thorax and 2 ml of universal fixative for both light and electron

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[e]Eastman Kodak, Rochester, NY.

[f]Schwartzmann, Division of Becton, Dickinson and Company, Orangeburg, NY.

[g]Haver-Lockhart Laboratories, Shawnee, KS.

microscopy were slowly infused into the lungs (McDowell and Trump, 1976). The thorax was then opened and the respiratory system was removed *en bloc*.

The trachea was tied with suture material to prevent drainage of the fixative. Other tissues collected and placed in universal fixative were stomach, liver, kidney, urinary bladder, spleen, ileum, heart, eyes, and upper respiratory tract. Pancreas and salivary gland were infrequently taken. Tissues for light microscopy were routinely processed, cut at 6 μ and stained with hematoxylin and eosin.

The liver of each animal was taken *en bloc* except for a small piece taken for light microscopic examination. The liver was assayed for vitamin A content according to the Neeld method (Appendix D).

Electron Microscopy

Using the method described by Stephens and Evans (1973), leaf sections of the right lung were cut under a dissecting microscope so that small airway passages could be selectively taken for embedding. The sections of lung which were originally fixed in universal fixative were placed in 4% formaldehyde-1% glutaraldehyde and phosphate buffer, pH 7.2 (McDowell and Trump, 1976). The specimens were then washed in cacodylate buffer with 4.5% sucrose and osmicated in osmium tetroxide. Tissues were dehydrated and embedded in Epon. Sections were cut with an LKB ultramicrotome, stained with uranyl acetate and lead citrate, and examined with a Phillips 300 electron microscope operating at 60 kv. The negatives were processed according to standard photographic procedure.

Liquid Scintillation

Lung tissue from animals in each group injected with ^{3}H-TdR at 24, 48, 72, 96 and 120 hours post-NO_2 exposure were used for the liquid scintillation procedure. The detailed procedure followed is outlined in Appendix E.

RESULTS

Clinical Observations

Antemortem and Postmortem Findings

Nitrogen dioxide exposure for group 1 began at 28 days of age. All hamsters were clinically normal. Exposure was carried out during daylight hours (8 a.m. to 6 p.m.). The animals were usually quite active for the first 20 to 30 minutes of the exposure; thereafter they huddled together and slept. At this point respiration was often rapid and shallow. This type of breathing was maintained throughout the exposure period; however, the animals did not appear to be in distress. At the termination of the exposure period, normal respiration resumed within 5 minutes. The first 4 exposures proceeded as described above. At the fifth exposure the rapid breathing appeared early and recovery time at the termination of exposure lengthened to 5 to 10 minutes. By the sixth exposure period, rapid and often labored breathing appeared immediately and continued throughout the 5 hours. Recovery was slow (10 to 15 minutes). For the remaining 2 exposures over half of the group experienced dyspnea and distress during exposure. At the end of the exposure period respiration improved but continued to be rapid and shallow with some degree of discomfort.

All animals were closely observed each week at the time of weighing, feeding and cage changing. Appetite and general appearance

were good for the first 4 weeks, reflected by an average weight gain of 5 gm per week (Figure 3). The hamsters were alert and active. At the fifth week weight gains began to diminish. Appetite remained normal; however, food consumption appeared to decrease. A drying of the skin around the eyes was noticed. A comparison with non-exposed hamsters on regular commercially prepared food showed a weight difference of 25 gm. During the succeeding 3 weeks, weight gain in the experimental animals declined until there was a net loss of 15 gm. There was generalized unthriftiness evidenced by lethargy, anorexia, rough, dull hair coats and focal alopecia. The eyes were dry and often sealed shut by an accumulation of dried purulent exudate. The nose was also crusty. The testicles of the males were much smaller in size. The nonexposed animals exhibited the same signs of vitamin A deficiency; however, labored breathing was not evident.

Supplementation of group 2 with retinyl acetate began at 28 days of age. Each hamster, including nonexposed animals, was given via the cheek pouch, 50 μg of retinyl acetate once a week for 4 weeks. Nitrogen dioxide exposure for group 2 began at 42 days of age. Each hamster at this time had received a total of 150 μg of retinyl acetate. All the animals were normal. At the onset of exposure the hamsters were active and curious of their new surroundings. However, they were usually settled and sleeping within 30 minutes. Respiration was increased and shallow during exposure; however, distress was not observed. At the termination of the exposure period, normal respiration resumed quickly. Observations during and after exposure proceeded as above throughout the 8-week experiment.

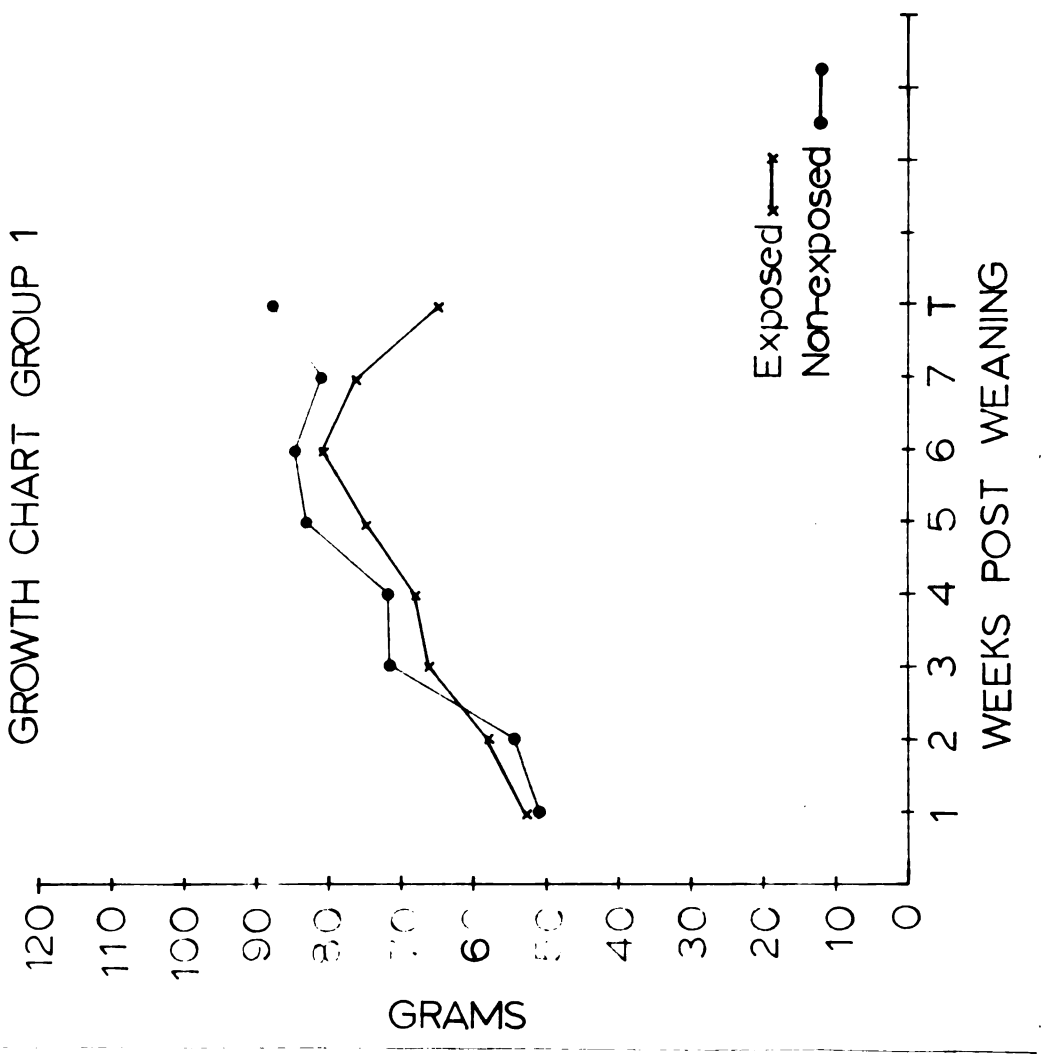

Figure 3. Growth chart - group 1 vitamin A deficient.

All animals were in good condition with normal behavior and appetite for the first 2 exposures. The weights for the exposed and nonexposed animals had increased. After the second NO_2 exposure the weight gain of the exposed animals lessened and continued to do so for 2 weeks. The retinyl acetate supplement dosage for exposed and nonexposed animals was increased the fifth week of exposure to 100 µg twice a week for the remainder of the experiment. Weight gain began to rise. A total of 950 µg of retinyl acetate was given during the 8-week period. At the termination of the experiment the exposed animals were healthy and alert. However, controls were larger in size, as can be noted in Figure 4.

Beginning at 28 days of age, hamsters in group 3 were each given 100 µg of retinyl acetate by way of the cheek pouch. Nitrogen dioxide exposure for group 3 was begun at 42 days of age. Each hamster had received a total of 300 µg retinyl acetate at the time of the first exposure. Exposure proceeded the same as described for group 2.

All animals appeared to be in good condition at the time of weighing. Weight gain continued normally until after the second exposure, at which time weight gain of the exposed animals began to decrease. At the fifth week the vitamin A dosage for exposed and nonexposed animals was increased to 200 µg twice a week because of weight loss. Weight gain began to rise as seen in Figure 5. A total of 2,100 µg of retinyl acetate was given during the 8-week period. At the termination of the experiment data obtained were similar to that seen with group 2. The nonexposed and exposed animals were healthy and active; however, the nonexposed animals were larger in size (Figure 5).

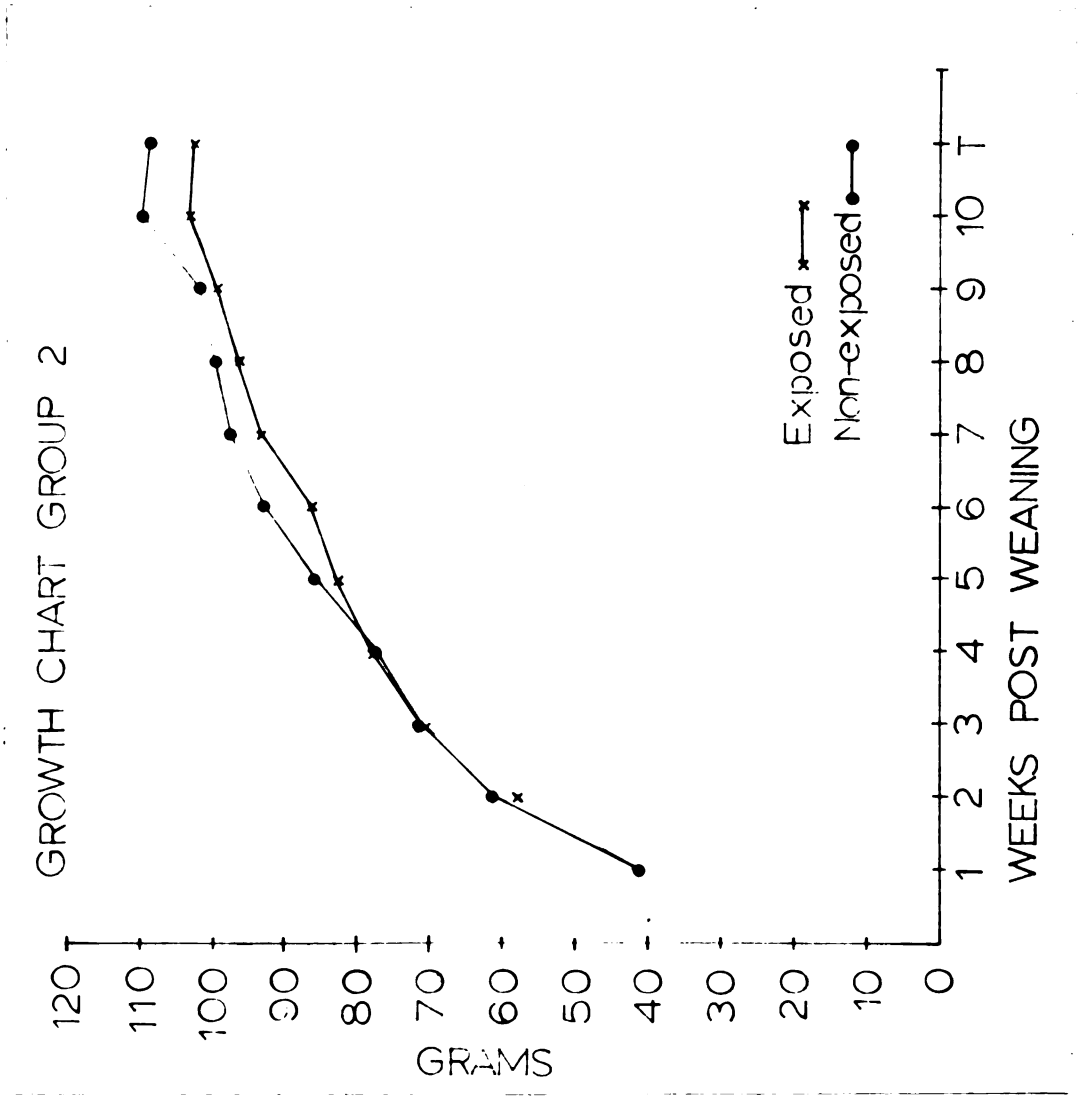

Figure 4. Growth chart - group 2 vitamin A adequate.

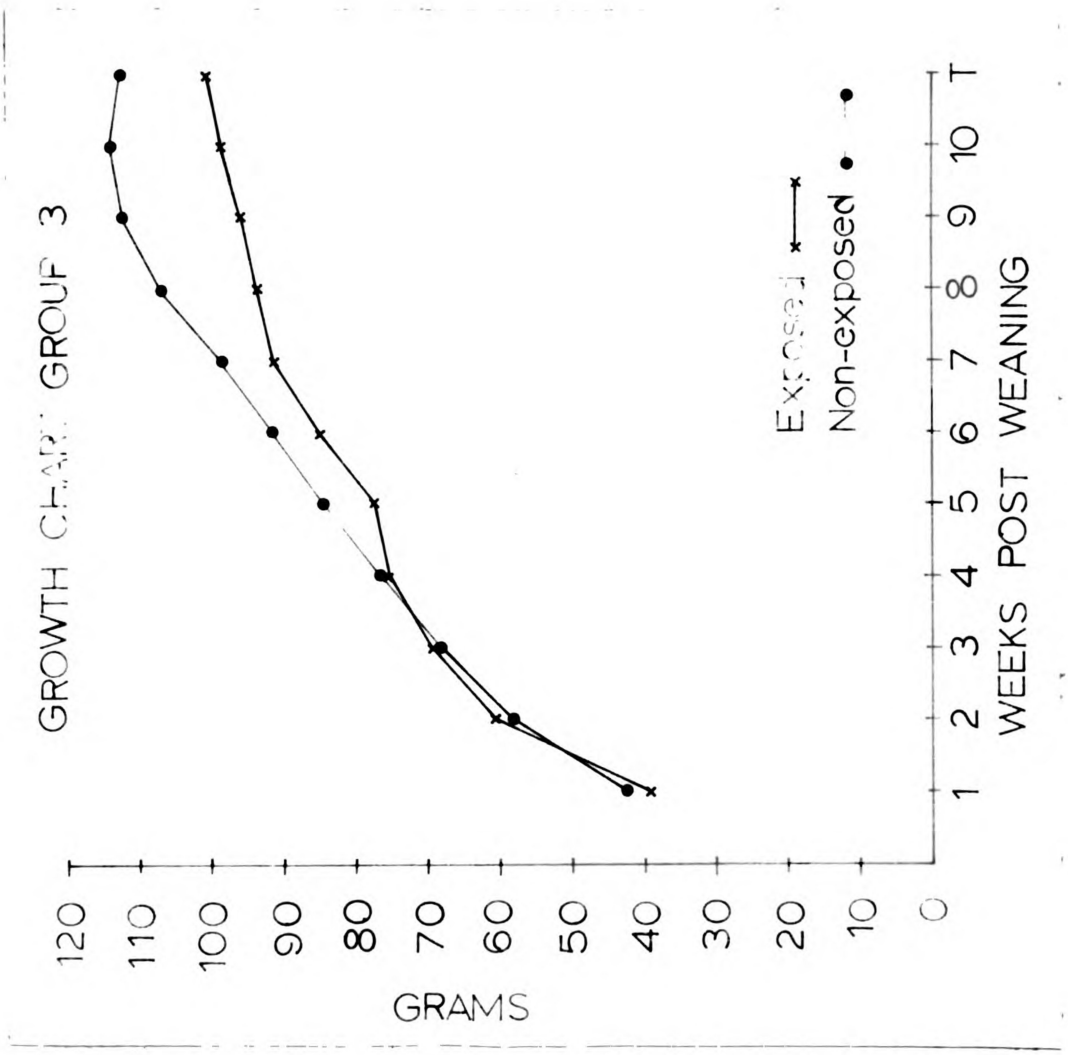

Figure 5. Growth chart - group 3 vitamin A high.

Group 1. The hamsters in group 1 were killed at the eighth week. All exposed animals without exception were in poor condition. Emaciation, dehydration, poor hair coat, and purulent ocular and nasal discharges were evident. Two animals had rectal prolapse. The testicles of the males were small and shrunken. The nonexposed hamsters showed identical signs of vitamin A deficiency; however, respiratory difficulty was not noted. At necropsy, lesions consistent with the gross antemortem observations were seen. The mucous membranes were pale. The nasal and ocular openings were clogged with purulent exudate. The tissues of the orbit were atrophic, abscessed or both in all animals. The cheek pouches were empty and frequently dry. The gastrointestinal tract was empty of ingesta and the large intestine was ballooned. In the males the abdominal viscera was frequently adhered. The livers were grossly shrunken and fibrotic. The lungs in several animals exhibited small focal pneumonic lesions. The right lung of 1 hamster was consolidated. This hamster also had a head tilt and was circling to the left. The kidneys were pale without other obvious gross lesions. Involvement of the urinary bladder was associated with adhesions of abdominal viscera in the males. This was not seen in the females. Seminal vesicles in the males were frequently abscessed with extension of infection to the testicles.

Group 2. The hamsters in group 2 were killed according to the method described for group 1. All animals were in good condition. One hamster had its right rear leg amputated at the knee; otherwise it was normal. No gross lesions were observed.

Group 3. Euthanasia was performed on group 3 hamsters following the same procedure as for groups 1 and 2. All animals were in good condition. No gross lesions were observed.

Liver Vitamin A Assay

Liver vitamin A assay was performed on all animals in the 3 experimental groups (Appendix D). Chemical analysis cannot establish with complete accuracy the bio-potency of vitamin A (Ullrey, 1972); however, in this experiment it was used as a broad indicator of liver vitamin A levels. Tables 1, 2 and 3 present vitamin A values for exposed and nonexposed hamsters in each group. The mean for exposed animals in group 1 was 0.138 µg per gram of wet liver, indicating a deficient state. Mean vitamin A level for exposed and nonexposed animals in groups 1, 2 and 3 are shown in Table 4. Group 3 shows slightly higher values, associated with the higher dose level of vitamin A fed, although individual values in both groups overlap. However, both groups are within adequate range for normal body function. Comparison of exposed and nonexposed vitamin A values using the 24-hour termination intervals was similar. The marked differences in values in groups 2 and 3 can be attributed to individual variation. In addition, interactions between vitamin A and other important nutrients such as protein, vitamin E and fat have been shown to have a marked effect on vitamin utilization (Ames, 1969; Ullrey, 1972).

Hematology

Differential leukocyte counts for groups 1, 2 and 3 are shown in Tables 5, 6 and 7. The normal differential leukocyte count of the hamster shows a high lymphocyte-neutrophil ratio in comparison to

Table 1. Liver vitamin A assay, group 1 (vitamin A deficient)

Animal No.	µg vitamin A/gm wet liver
Exposed	
149110	.015
148111	.248
148112	.552
148113	.093
148114	.058
148115	.019
148116	.085
148117	.085
148119	.067
148120	.159
Nonexposed	
148109	.017
148118	.194

Table 2. Liver vitamin A assay, group 2 (vitamin A adequate)

Animal No.	µg vitamin A/gm wet liver
Exposed	
149302	34.047
149303	48.909
149304	41.002
149305	41.678
149307	44.194
149308	38.028
149310	25.373
149311	31.900
149313	28.311
149314	70.191
149317	97.676
149318	35.191
149319	21.619
149339	10.554
Nonexposed	
149300	56.933
149301	47.217
149306	32.534
149309	36.241
149312	27.469
149315	22.537
149316	37.700

Table 3. Liver vitamin A assay, group 3 (vitamin A high)

Animal No.	µg vitamin A/gm wet liver
Exposed	
149322	52.913
149323	120.135
149324	61.463
149325	30.470
149327	112.928
149328	49.824
149330	102.639
149331	47.925
149334	76.098
149335	35.063
149336	53.267
149337	75.747
149338	37.475
Nonexposed	
149320	81.871
149321	64.929
149326	23.955
149329	50.064
149332	40.500
149333	53.976

Table 4. Mean liver vitamin A levels - groups 1, 2 and 3

	Deficient	Adequate	High
Exposed	0.138 µg	41.380 µg	68.725 µg
Nonexposed	0.106 µg	37.233 µg	52.549 µg

Table 5. Differential leukocyte count, group 1 (vitamin A deficient)

Animal No.	Neutrophils		Lympho-cytes	Eosino-phils	Mono-cytes	Baso-phils
	Seg.	Nonseg.				
148110	58	11	31	-	-	-
148111						
148112	71	1	28	-	-	-
148113	61	2	35	-	2	-
148114	72	6	22	-	-	-
148115	56	3	41	-	-	-
148116	61	3	36	-	-	-
148117	53	6	42	-	-	-
148119	79	0	21	-	-	-
148120	72	1	26	-	1	-
148109*	38	0	62	-	-	-
148118*	55	2	42	1	-	-

*Nonexposed.

Table 6. Differential leukocyte count, group 2 (vitamin A adequate)

Animal No.	Neutrophils		Lympho-cytes	Eosino-phils	Mono-cytes	Baso-phils
	Seg.	Nonseg.				
149302	42	1	30	1	1	-
149303	26	-	73	1	1	-
149304	49	-	51	-	-	-
149305	39	-	58	3	-	-
149307	25	-	53	3	-	-
149308	56	-	43	1	-	-
149310	62	-	37	1	-	-
149311	32	-	68	-	-	-
149313	35	-	62	3	-	-
149314	20	-	76	3	1	-
149317	30	-	69	-	1	-
149318	32	-	65	2	-	1
149319	37	1	60	2	-	-
149339	44	-	52	2	2	-
149300*	26	-	70	3	1	-
149301*	22	-	76	2	-	-
149306*	19	-	77	3	1	-
149309*	33	-	63	2	2	-
149312*	25	-	73	1	1	-
149315*	45	1	50	4	-	-
149316*	45	-	52	2	1	-

*Nonexposed.

Table 7. Differential leukocyte count, group 3 (vitamin A high)

Animal No.	Neutrophils		Lympho-cytes	Eosino-phils	Mono-cytes	Baso-phils
	Seg.	Nonseg.				
149322	38	1	53	2	6	-
149323	51	-	47	-	2	-
149324	24	-	74	1	1	-
149325	37	-	58	2	4	-
149327	37	-	62	-	1	-
149328	17	-	81	-	2	-
149330	26	1	73	-	-	-
149331	43	2	51	1	3	-
149334	20	1	77	2	-	-
149335	34	2	61	1	1	-
149336	24	-	76	-	-	-
149337	25	1	71	2	-	1
149338	23	-	70	-	7	-
149320*	36	6	54	2	2	-
149321*	46	1	50	2	1	-
149326*	49	2	48	1	-	-
149329*	44	-	54	-	2	-
149332*	39	-	58	1	2	-
149333*	29	1	67	2	1	-

*Nonexposed.

other mammals expressing a high neutrophil-lymphocyte ratio (Schermer, 1967). In the rodent, the peripheral blood picture reverses due to the increased numbers of neutrophils needed to combat bacterial infection and inflammation. The counts in groups 2 and 3 are within normal range. However, group 1 shows a reversal in lymphocytes to neutrophils. This was expected in view of the gross and histopathologic findings.

Morphologic Observations

Light and Electron Microscopy

Group 1. Upon microscopic examination of group 1 the respiratory tracts of the exposed animals were severely affected. A large accumulation of neutrophils, other inflammatory cells and debris was seen in the upper respiratory tract. Inflammatory cells were observed in the submucosa and lamina propria of the nasal mucosa. There was goblet and epithelial cell hyperplasia. The mucus secreting cells of the epithelium were degenerating and squamous metaplasia was often seen. In the middle of the nasal scrolls cilia were often shortened and patchy. The olfactory epithelium was disrupted and degenerating. One hamster exhibited hemorrhage in the orbit with chronic inflammatory changes. The layers of the retina appeared normal.

Several tracheal sections examined exhibited varying degrees of squamous metaplasia and absence of cilia (Figure 6).

The lungs with few exceptions exhibited varying degrees of focal pneumonia (Figure 7). The cellular infiltrate was predominantly neutrophilic. In 2 animals this inflammation was seen around bronchi.

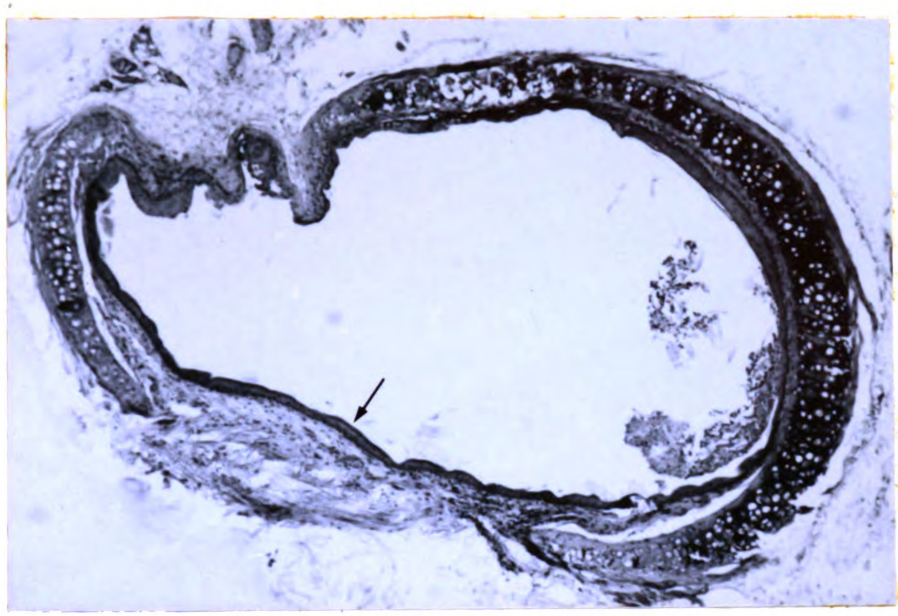

Figure 6. Squamous metaplasia of the tracheal epithelium (arrow) of a vitamin A deficient hamster. Inflammatory cells and debris are present in the lumen. H&E; X 60.

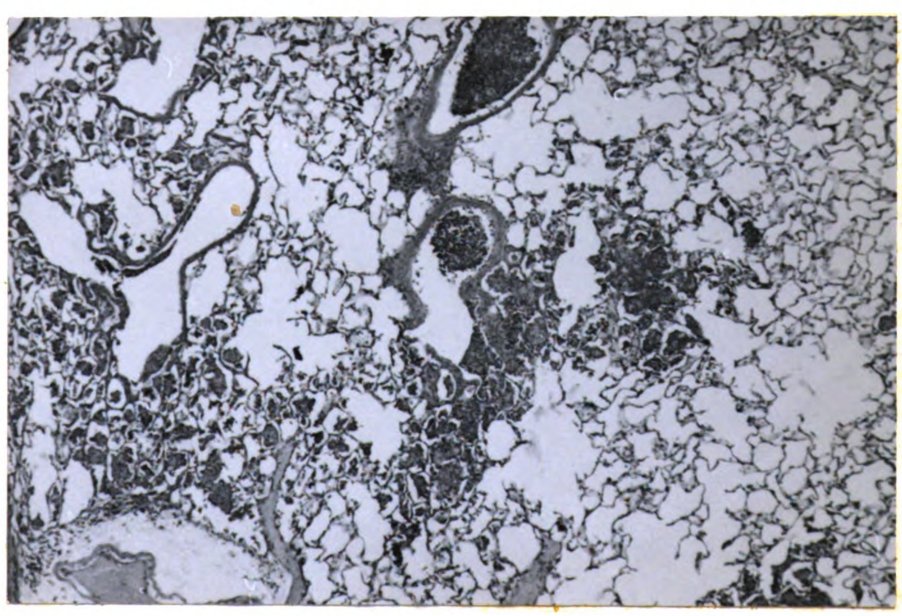

Figure 7. Focal pneumonia in the lung of a vitamin A deficient, NO_2 exposed hamster. H&E; X60.

Alveolar cells appeared to be cornified. The epithelial cells were flattened and had undergone squamous metaplasia. Although the cilia were many times difficult to see, they were occasionally patchy and shortened. Epithelial hypertrophy and hyperplasia were minimal as compared with the lungs of hamsters given a single 5-hour NO_2 exposure (Figure 8) (Creasia et al., 1972). A section of normal hamster lung is shown for comparative purposes (Figure 9).

Ultrastructural changes in the lung of the exposed hamsters in group 1 were significant. Swelling and necrosis of epithelial cells with infiltration of inflammatory cells was observed. Separation of the basement membrane and loss of cilia were characteristic alterations (Figure 10). Occasionally fine calcium deposits within swollen mitochondria were found. Hypertrophy and hyperplasia was less than that observed in the other groups.

The stomach was difficult to assess. The cells of the glandular portion stained more eosinophilic on the surface. However, this could have been a normal regenerative process. Sections of ileum showed changes similar to those of the stomach.

Various stages of hepatitis with fibrosis and fatty change were observed in the liver. In several livers a chronic inflammatory reaction was observed in the parenchyma and in the capsule.

No lesions were observed in the kidney of the majority of animals. In 3, neutrophils and eosinophilis could be seen in the pelvis. Neutrophils and small numbers of other inflammatory cells could be seen in the submucosa of the urinary bladder of 2 hamsters.

In the males aspermatogenesis was evidenced by vacuolation and a reduced number of spermatogonia, abscessation, spermatic giant cells, and lack of mature sperm. The female reproductive tract was

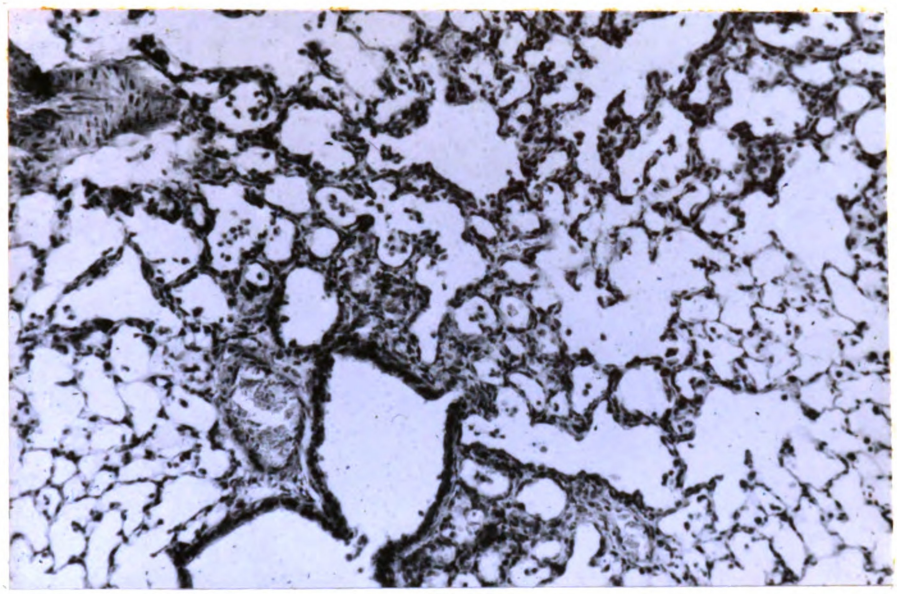

Figure 8. Marked hypertrophy and hyperplasia of the epithelium of the terminal bronchiole seen in hamsters fed a regular commercial diet and exposed to 10 ppm NO_2 for 5 hours. H&E; X 140.

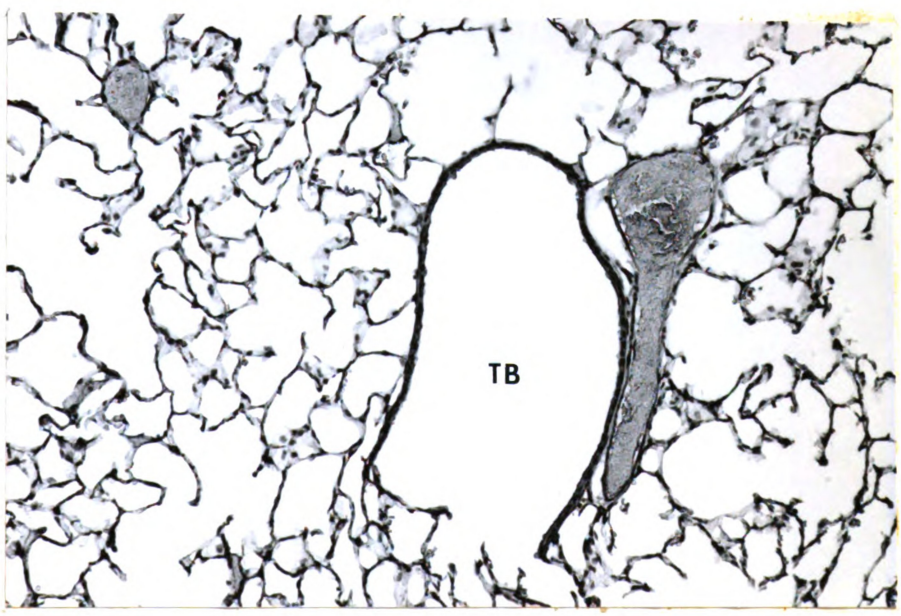

Figure 9. Terminal bronchiolar area in normal, nonexposed hamster. Terminal bronchiole (TB). H&E; X 140.

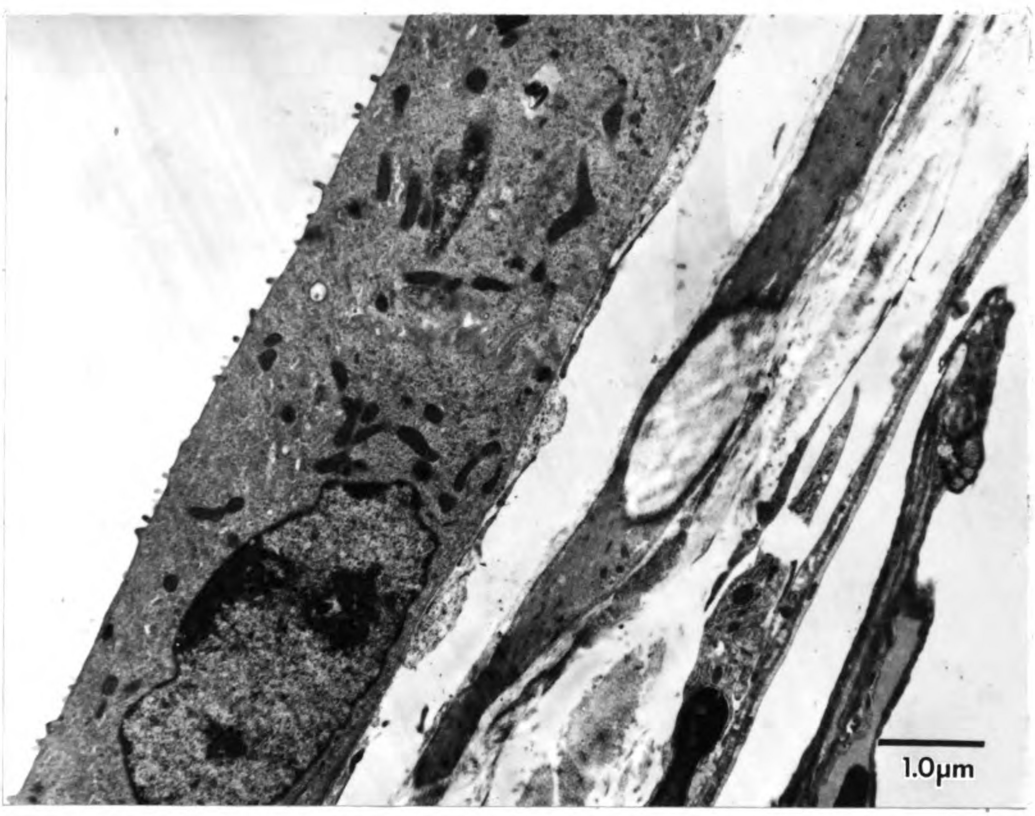

Figure 10. Separation of the basement membrane and diffuse loss of cilia were characteristic alterations observed in the exposed vitamin A deficient hamsters. Uranyl acetate and lead citrate; X 13,000.

not routinely taken. Sections of salivary and Harderian gland showed atrophy and abscessation. No lesions were observed in the heart.

The nonexposed animals of group 1 showed similar lesions of vitamin A deficiency; however, pneumonia was not seen.

Group 2. On microscopic examination of group 2 the upper respiratory tract showed patchy and/or shortened cilia. A few sections had small focal areas of inflammatory cells in the submucosa. The tracheal epithelial cells were more cuboidal and occasionally patchy cilia were observed. Hypertrophy and hyperplasia of the epithelial cells of the terminal bronchiole of the lung was seen (Figures 11 and 12).

Electron microscopic examination of the lung revealed the primary site of pulmonary damage to be the terminal bronchiole and the adjacent bronchiolar alveolar region. Hypertrophy and hyperplasia of epithelial cells was seen (Figure 13). Additionally, there was cytoplasmic swelling and desquamation of epithelial cells. Interstitial edema was present in the form of low density spaces around clumps of collagen fibers in perivascular and peribronchiolar connective tissue as well as in the interalveolar interstitium (Figure 14). The ciliated and nonciliated cells appeared to be equally affected.

Nonexposed animals showed focal interstitial edema in the subepithelial lining cells of the alveoli.

Fatty change was the most significant lesion found in the liver, although one section had a focal granulomatous reaction.

The urinary bladder in several cases exhibited focal calcification of the epithelium and, in one, calcification extended into the smooth muscle fibers (Figure 15). Some surface cells were sloughed.

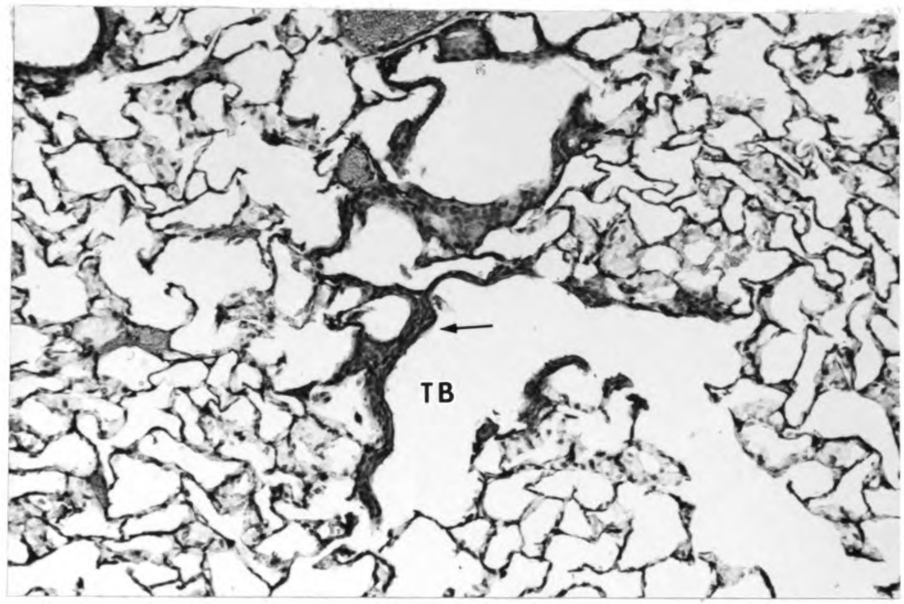

Figure 11. Terminal bronchiole lesion following NO_2 exposure in a vitamin A adequate hamster. Hypertrophy and hyperplasia of the epithelial cells are seen (arrow). Terminal bronchiole (TB). H&E; X 140.

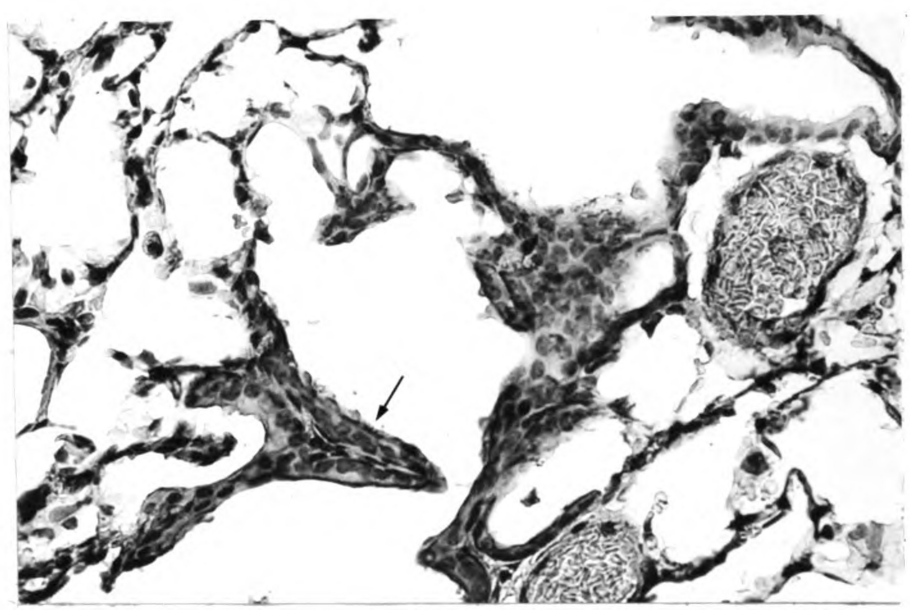

Figure 12. Higher magnification of a similar lesion as shown in Figure 11 depicting hypertrophy and hyperplasia of the terminal bronchiolar epithelium (arrow). H&E; X 350.

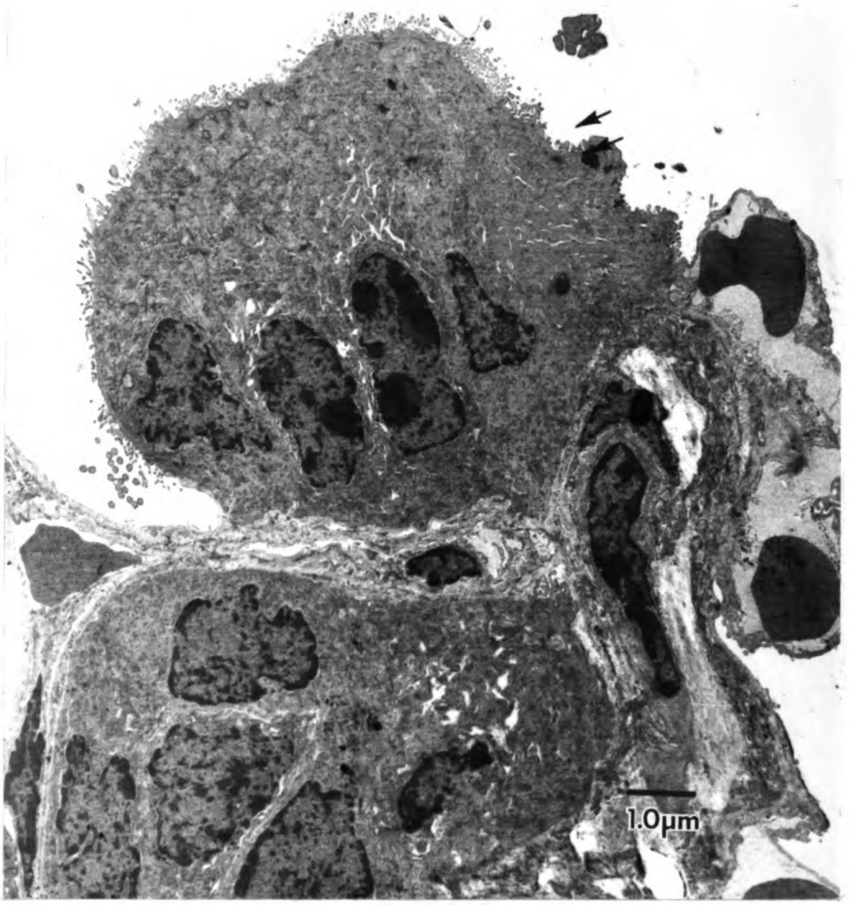

Figure 13. Ciliated bronchiolar epithelial cell hypertrophy and hyperplasia observed in the bronchiolar alveolar region of exposed, vitamin A adequate and high dose hamsters. Uranyl acetate lead citrate; X 9,000.

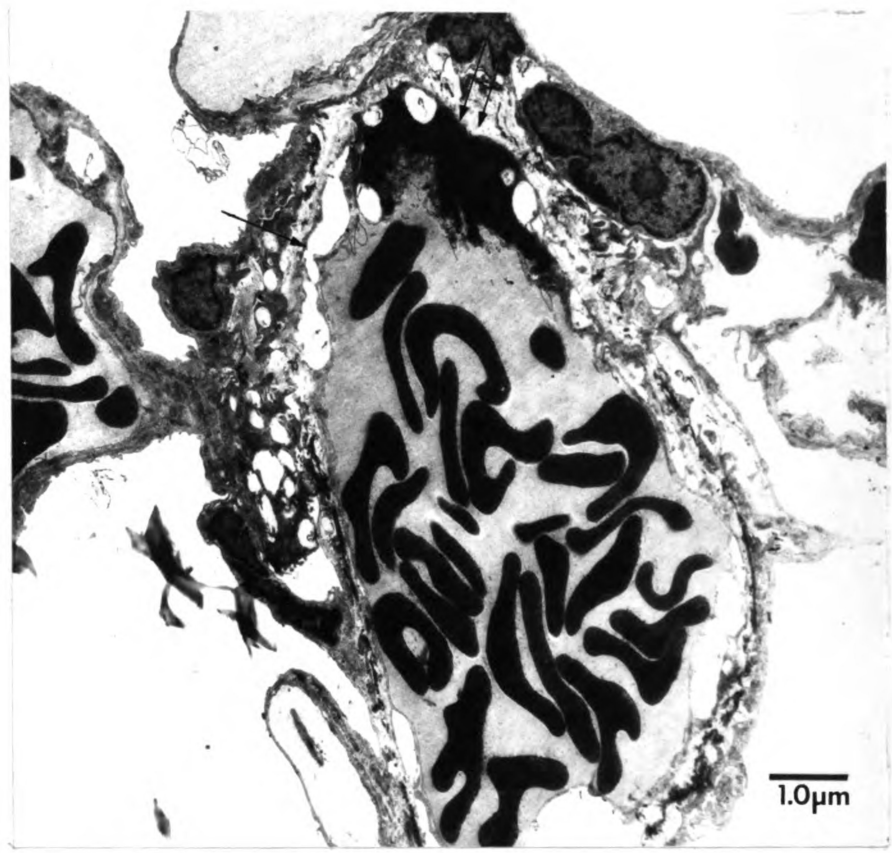

Figure 14. Interstitial edema around clumps of collagen fibers can be seen in the perivascular connective tissue (arrow) of vitamin A adequate hamster. Electron dense material was observed within the blood vessel (double arrows). Uranyl acetate lead citrate; X 11,000.

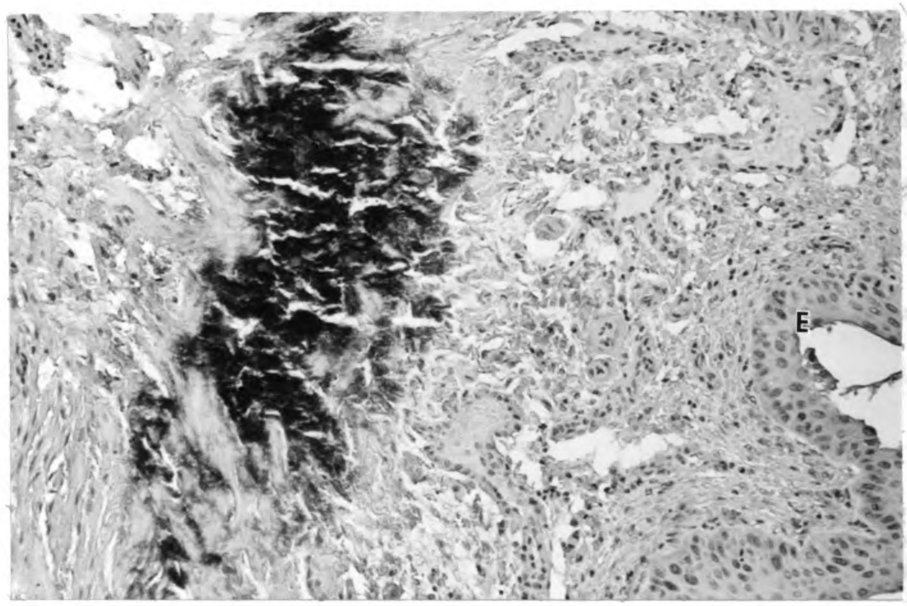

Figure 15. Light micrograph of the urinary bladder wall of a vitamin A adequate hamster showing focal calcification. Epithelium (E). H&E; X 140.

No lesions were observed in the gastrointestinal tract, spleen, heart and testicles.

Group 3. The most significant microscopic lesion found in group 3 was in the respiratory system. In the middle nasal scrolls, cilia were shortened. Focal inflammation and mucus cell hyperplasia occurred in 1 animal, respectively. Cilia were not prominent and epithelial cells appeared flattened in the trachea.

In the lungs the lesions were the same as those seen in group 2. There was hyperplasia and hypertrophy of the epithelial cells at the terminal bronchiole.

Ultrastructural observations of the terminal bronchiolar area in group 3 also revealed hypertrophy and hyperplasia of the epithelial cells. Loss of cilia and fragmentation of ciliary bodies was the same as seen in the previous groups (Figure 16). Degenerative changes of type 2 cells were observed (Figure 17). Desquamation and cytoplasmic swelling occurred with much less intensity. Perivascular edema and thickening of the interalveolar septa occurred more frequently than seen in previous groups of hamsters. Calcium deposits were found in the lining of the epithelial cells of exposed animals. Mitochondrial degeneration characterized by swelling and loss of cristae were also seen.

The liver showed fatty change.

Focal calcification of the epithelium of the urinary bladder was seen in 2 animals.

Lesions were not observed in the gastrointestinal tract, kidney, spleen, testicles and heart.

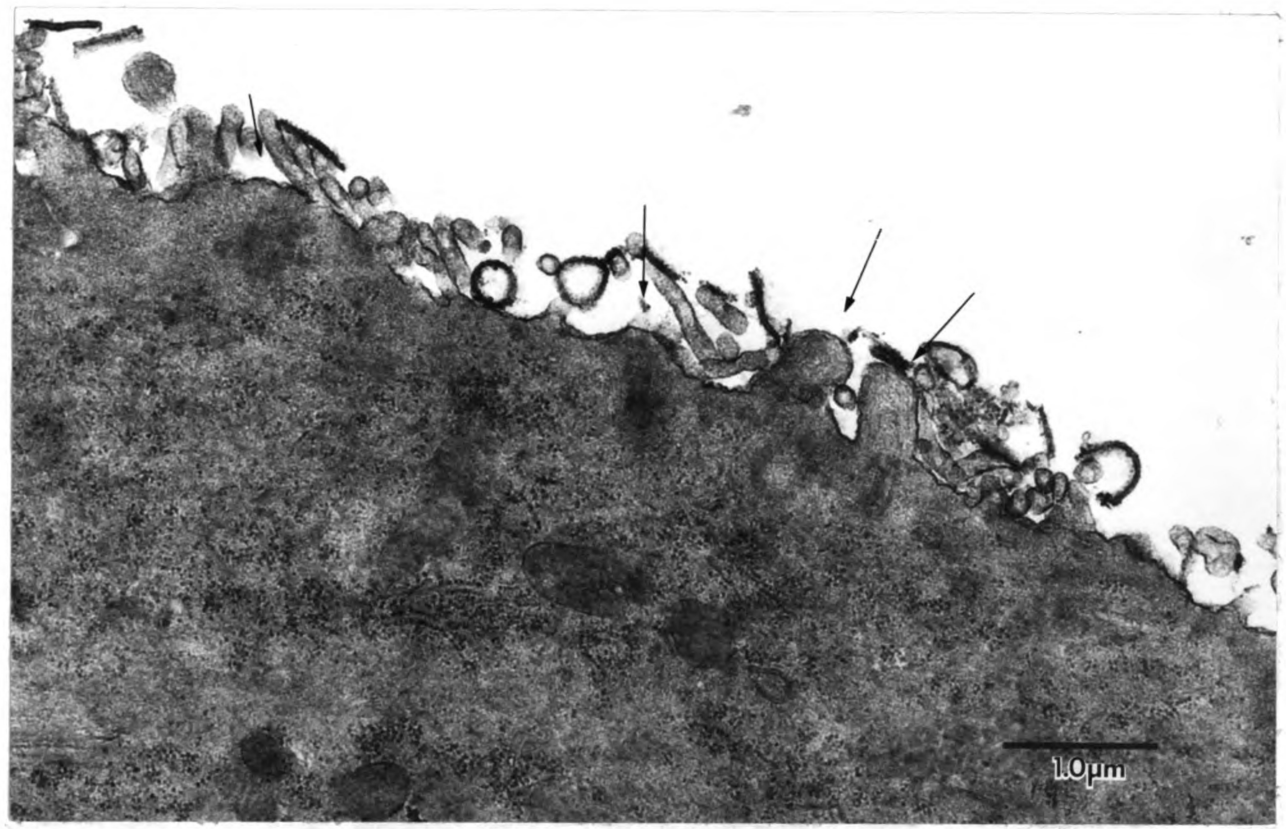

Figure 16. Thickening, fragmentation and loss of cilia in the terminal bronchiolar epithelium following NO_2 exposure. The cytoplasm appears to have a reduced number of organelles and increased electron density. Uranyl acetate lead citrate; X 20,000.

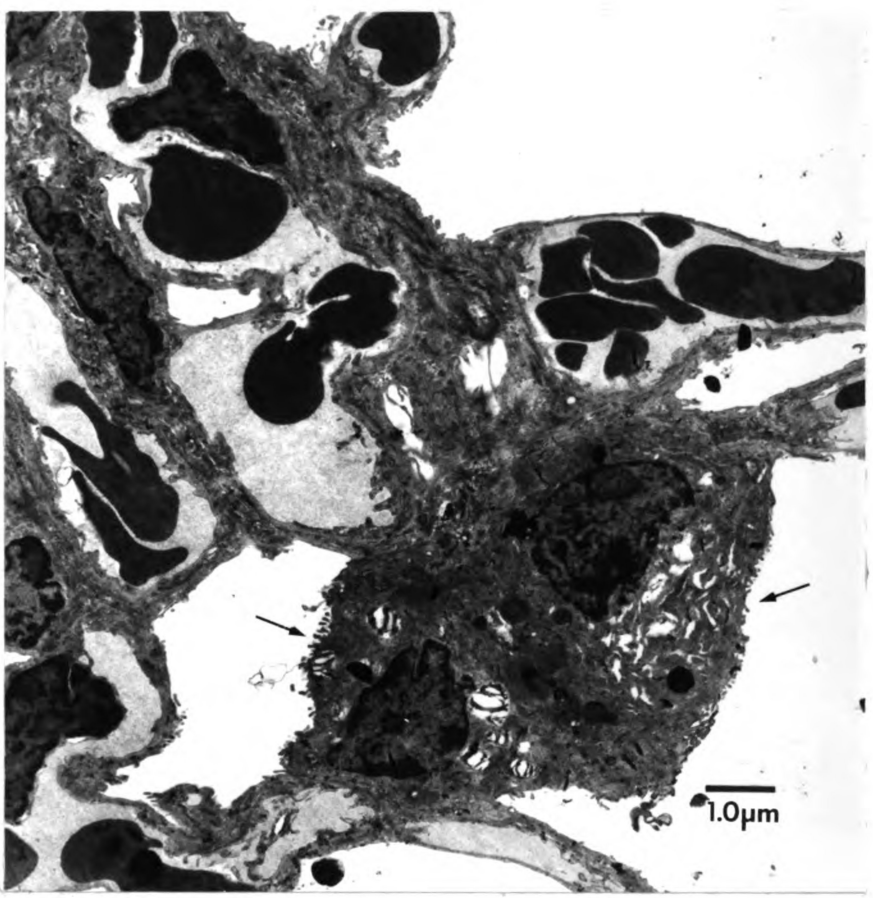

Figure 17. Type 2 cells showing degenerative changes following NO_2 exposure of vitamin A high dose hamster (arrows). Uranyl acetate lead citrate; X 9,000.

Liquid Scintillation

Cellular regeneration in the lung tissue was measured by the uptake of ^{3}H-TdR during DNA synthesis. The radioactivity observed in the lung of exposed and nonexposed animals of groups 2 and 3 is shown in Figure 18. Although animals in group 1 were not thymidine injected, data from hamsters vitamin A deficient, NO_2 exposed and ^{3}H-TdR injected in the same manner from a separate study (unpublished data) were included for comparative purposes. The mean of all non-exposed animals is represented by the horizontal dotted line.

By linear regression the mean peak labeling occurred in vitamin A deficient animals at 24 hours and thereafter declined as time increased ($P<0.01$). The mean peak labeling for vitamin A adequate animals peaked at 24 hours, appeared to decline at 48 hours and subsequently increased. However, by polynomial regression, the apparent decline was not statistically significant ($P>0.10$). The mean labeling for animals on high dose vitamin A levels did not change significantly over the 120-hour period ($P>0.10$).

Figure 19 depicts the mean and standard deviation disregarding time intervals. Despite the overlap of standard deviations, a notable difference in means can be seen between group 1 and groups 2 and 3. Analysis of variance between group 1 and groups 2 and 3 was significant ($P<0.01$). A comparison of groups 2 and 3 showed no significant change ($P<0.01$).

There appears to be an increase in cell regeneration in animals supplemented with vitamin A in contrast to those not supplemented. Even though a significant change was not statistically observed between groups 2 and 3, a variation in the cell kinetics is suspected.

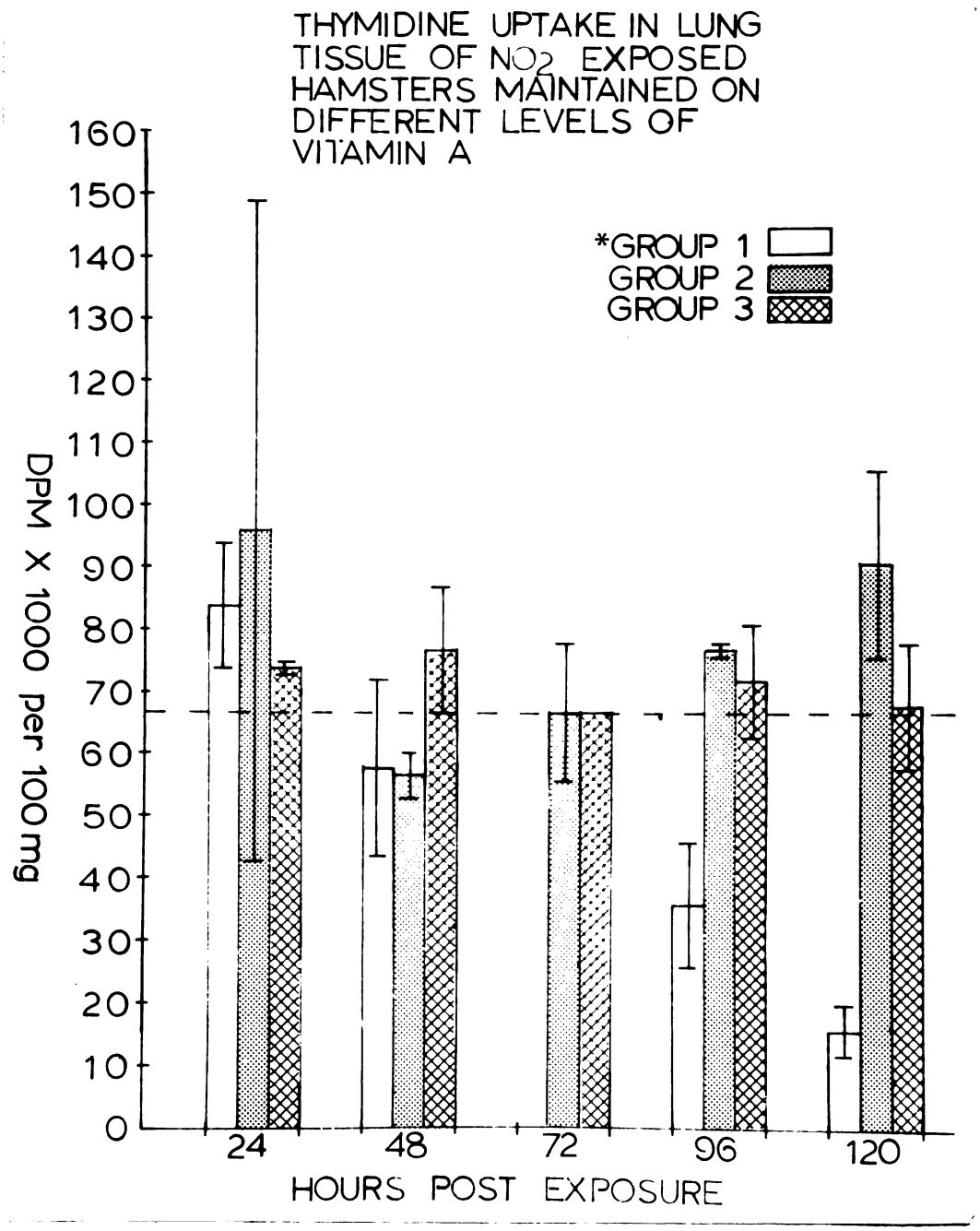

Figure 18. Thymidine uptake mean ± standard deviation in lung tissue of NO_2 exposed hamsters maintained on different levels of vitamin A. *Vitamin A deficient hamsters treated similarly from a separate study (unpublished data).

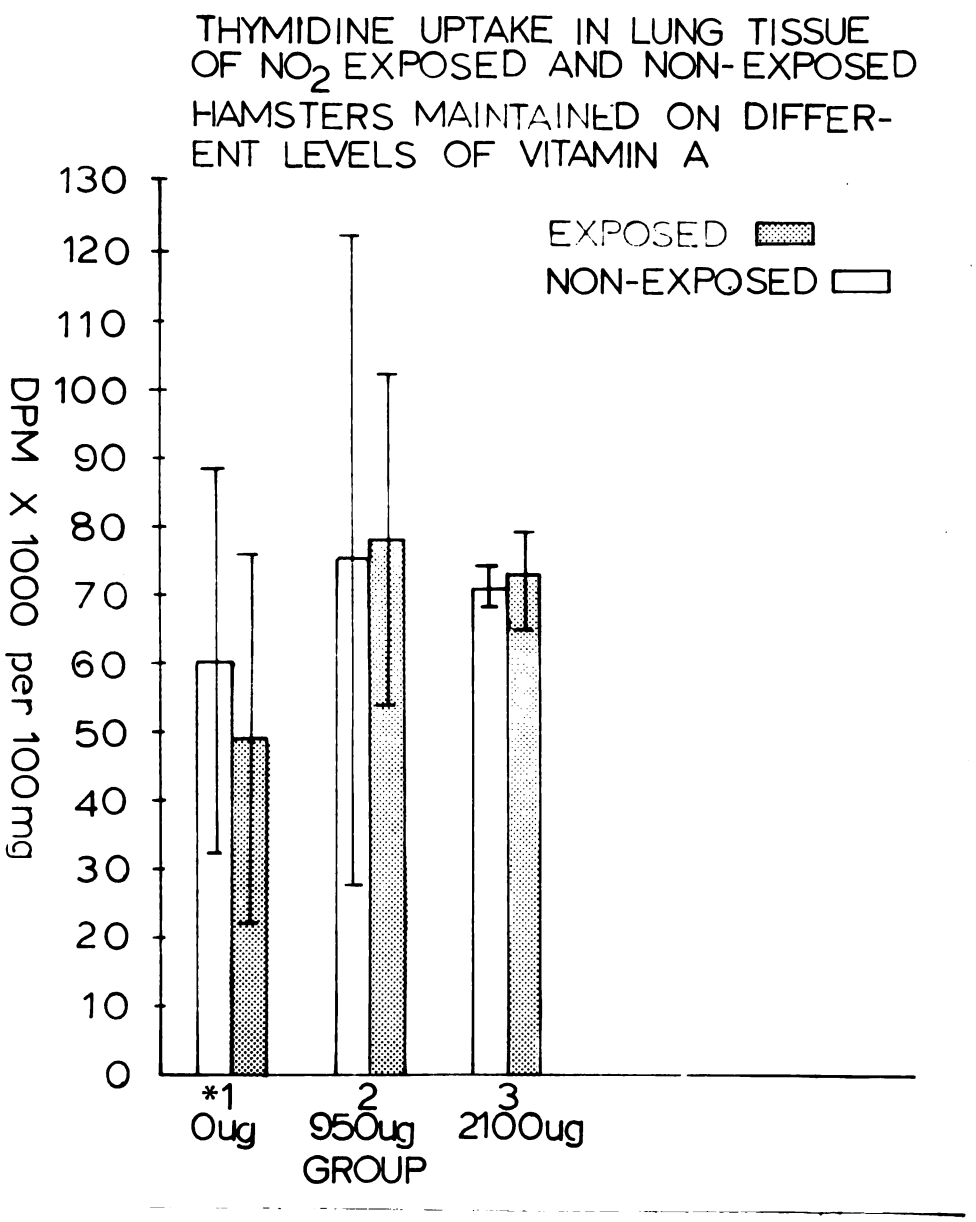

Figure 19. Thymidine uptake mean ± standard deviation in lung tissue of NO_2 exposed and nonexposed hamsters maintained on different levels of vitamin A. *Vitamin A deficient hamsters treated similarly from a separate study (unpublished data).

The size of the sample may be a major consideration in increasing the significance of variation observed for groups 2 and 3.

DISCUSSION

Signs of vitamin A deficiency in group 1 were consistent with those observed by others (Wolbach and Howe, 1925; Salley and Bryson, 1957). However, the additional stress of NO_2 exposure appeared to have a profound clinical effect.

In this experiment weight gain in animals of group 1 continued throughout the first 6 weeks of exposure. During the last 2 weeks a rapid decline was noted resulting in death of several hamsters. Nonexposed animals showed a weight difference of 4 grams. However, they too showed a decline. The increase in weight shown for the nonexposed at the termination of the experiment represents a significant difference in weight of the 2 remaining nonexposed animals at necropsy.

Vitamin A levels and NO_2 exposure also affected groups 2 and 3. Animals in both groups progressed at the same rate for 3 weeks prior to the first exposure. At that time retinyl acetate had been given on 3 occasions. After the first exposure, animals of both exposed groups began to show a decline in the amount of weight gained over the next 2 weeks. Based on this weight loss, the vitamin A dosage level was doubled. It was obvious that the initial retinyl acetate dose level of 50 and 100 μg for groups 2 and 3, respectively, was not adequate for normal growth. Frequency of NO_2 exposure as well as utilization and storage in this instance appeared to influence the

metabolism of vitamin A. Dosage level adjustment therefore was difficult.

With the increase in retinyl acetate levels the adequate animals receiving 100 µg twice a week improved markedly and the high dose animals readily evidenced a large difference over their nonexposed counterparts, as well as the adequate group. It is apparent that a level of at least 200 µg retinyl acetate per week was necessary for growth.

A comparison of groups 1, 2 and 3 reveals a difference in weight gain throughout the experiment. The higher the vitamin A level the greater the growth. Each nonexposed group showed an increase over its exposed group, indicating the important role vitamin A might play in body protein synthesis (DeLuca, 1975). Nitrogen dioxide exposure serves as a stress factor; an important demand is made on the vitamin A levels. Growth was clearly stunted within and among groups.

Liver assay confirmed the vitamin A deficiency state of hamsters in group 1. Average levels at the beginning of the experiment were approximately 20 µg per gram of wet liver. Values for groups 2 and 3 show the tremendous variation in storage and utilization of vitamin A in this population of hamsters, both exposed and nonexposed. This also indicates a variation in the effect of NO_2 exposure on each animal.

As mentioned previously, the reversal of the lymphocyte-neutrophil ratio was expected in the deficient animals because of their increased susceptibility to infection. Hematologic findings in groups 2 and 3 were within normal range for hamsters.

Changes seen on histopathologic examination of group 1 showed most lesions attributable to vitamin A deficiency and secondary

bacterial infection (i.e., upper respiratory infection, abscessation of the seminal vesicles and salivary and Harderian glands, hepatitis and squamous metaplasia) as observed by others (Wolbach and Howe, 1925; Salley and Bryson, 1957; Kim et al., 1976).

Respiratory injury and repair observed by light microscopy in groups 1, 2 and 3 was essentially the same although there was some variation in the extent of the lesions of the terminal bronchiole area between groups. Group 1 showed minimal hypertrophy and hyperplasia, in comparison to groups 2 and 3. Terminal bronchiole changes were minimal in cyclic exposure as compared with those observed in a single exposure (Creasia et al., 1972), suggesting tolerance and adaptation phenomena during repeated exposures. This attests that the lung is capable of adjusting to injury by producing cells that are less differentiated and thereby able to provide protection (Stephens et al., 1972).

Limited electron microscopic examination of the lung of an animal in all 3 groups indicated that this technique provides a more sensitive indication of the changes observed than light microscopy. Epithelial cell damage as well as thickening of the interalveolar septa was seen. Collagen fibers and edema contributed to the thickening of the septa. In man, lipid deposits in the pulmonary connective tissue occur more often among heavy smokers (Bonfiglio et al., 1974). Cyclic NO_2 exposure in vitamin A deficient animals produces similar effects (Kim et al., 1976).

By ^{3}H-TdR uptake by respiratory cells, there appears to be an increased capability for epithelial cell regeneration in animals supplemented with vitamin A than those not supplemented. Because vitamin A is necessary for epithelial cell differentiation (DeLuca

et al., 1972), it is reasonable to assume that adequate and high levels of vitamin A would enhance the ability of an individual to replace cells at a more rapid rate, thereby repairing damage and maintaining function to some degree. However, the adequacy of other nutritional essentials is also paramount in vitamin A utilization (Ullrey, 1972). Whether the administration of vitamin A is responsible for the irregular cell regeneration remains to be elucidated. Changes such as these may protect the respiratory tract of chronic smokers and persons inhaling other irritant gases. However, this may present a serious problem since rapidly dividing cells are more susceptible to chemical and other environmental carcinogens (Saffioti et al., 1967). Because the individual levels of vitamin A required for epithelial cell differentiation are variable, the irregular pattern of epithelial regeneration may be attributed to dietary vitamin A, cyclic exposure, or both.

SUMMARY

The effect of nitrogen dioxide (NO_2) on the lung is well documented; however, little is known of the host factors which may modify its injurious effect. This experimental study was undertaken to determine pathologic changes occurring in hamsters fed deficient, adequate and high dose levels of vitamin A while being exposed cyclically to 10 ppm NO_2 for 5 hours over a period of 8 weeks.

The experimental observations were as follows:

1. A vitamin A deficiency state was produced by feeding a vitamin A deficient diet over a period of 6 weeks.

2. The feeding of synthetic retinyl acetate prevented vitamin A deficiency. However, adjustment was necessary because of individual storage and utilization variation.

3. Differential leukocyte counts showed no significant change in the lymphocyte-neutrophil ratio in adequate and high dose animals. However, a lymphocyte-neutrophil reversal was seen in the deficient animals, most likely due to bacterial infection.

4. Nitrogen dioxide exposure was an apparent stress as reflected in animal growth and body weight.

5. The extent of the lesion in the terminal bronchiole area in cyclically exposed animals was less than that observed in normal animals following a single exposure, suggesting an adaptive phenomenon.

6. Membrane damage was evident in exposed animals with electron microscopy. These morphologic changes were not evident with the light microscope.

7. Limited study of tritiated thymidine uptake by cells of the lung following NO_2 injury indicated the regeneration pattern of epithelial cells was variable in contrast to that seen in normal animals. However, further investigation is needed.

From these observations, it appears that dietary vitamin A is an important factor in the effect of NO_2 on the respiratory tract.

APPENDICES

APPENDIX A

VITAMIN A DEFICIENT HAMSTER DIET*

	g/kg
Casein, Vitamin Free Test, heat-treated	240.0
Sucrose	519.8268
Corn starch	100.0
Cottonseed oil	50.0
Non-nutritive fiber (cellulose)	50.0
Mineral mix, Williams-Briggs Modified (Cat. #170911)	35.0
Ascorbic acid, coated (97.5%)	1.0166
Inositol	0.1101
Choline dihydrogen citrate	3.4969
P-Aminobenzoic acid	0.1101
Niacin	0.0991
Riboflavin	0.022
Pyridoxine HCl	0.022
Thiamine HCl	0.022
Calcium pantothenate	0.0661
Biotin	0.0004
Folic acid	0.002
Vitamin B_{12} (0.1% trituration in mannitol)	0.0297
Vitamin D_2 in corn oil (400,000 U/g)	0.0055
DL alpha tocopheryl acetate (1000 U/g)	0.1211
Menadione	0.0496

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*Tekland Mills, Division of Mogul Corp., Madison, WI. Ref.: Adapted from the National Academy of Sciences, Nutrient Requirements of Laboratory Animals, No. 10, Second Revised Edition, 26 (1972).

APPENDIX B

NITROGEN DIOXIDE MEASUREMENT - GRIESS-SALTZMAN METHOD*

This method is based on the reaction of NO_2 with sulfanilic acid to form a diazonium salt, which couples with N-(1-naphthyl)-ethylene-diamine dihydrochloride to form a deeply colored azo dye. The color produced, which is proportional to the amount of NO_2 sampled, is measured at 550 nanometers.

Reagents used and preparation of the standard are the same as listed in the reference.

1. Ten milliliters of absorbing reagent is pipetted into a 160 x 32 mm gas collection tube.

2. The tube is then connected to the vacuum pump[a] at 26 psi and the stopcock to the exposure chamber is opened.

3. The vacuum is allowed to pull the air sample into the tube for a period of 2 minutes.

4. At the end of 2 minutes the stopcock is closed and the color is allowed to develop for 15 minutes.

5. Zero the spectrophotometer using a 12 x 75 mm cuvette containing fresh absorbing reagent at 550 nm.

6. Place the sample in a clean cuvette, read value and record.

7. Calculate the amount of NO_2 utilizing the dilution factor, percent efficiency and standard curve.

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*Intersociety Committee: Tentative Method of Analysis for Nitrogen Dioxide Content of the Atmosphere (Griess-Saltzman Reaction) 42602-01-68T; adapted from *Selected Methods for the Measurement of Air Pollutants*, PHS Publication No. 99-AP-11, May, 1965.

[a]Research Appliance Company, Gibsonia, PA.

APPENDIX C

RETINYL ACETATE PREPARATION FOR ORAL FEEDING

The following equations may be used to calculate the amount of vitamin A and cottonseed oil needed to reach a predetermined concentration:

retinyl acetate (μg)/0.1 ml cottonseed oil

retinyl acetate (μg) = (# of animals) x (amount of retinyl acetate needed per animal)

cottonseed oil = (# of animals) x (0.1 ml cottonseed oil) - (0.1 ml chloroform)

1. Weigh and tare a 50-ml Erlenmeyer flask to 4 decimal places.
2. Weigh the calculated amount of retinyl acetate to 4 decimal places (e.g., 1.5 mg = .0015 g) in the flask, being careful not to leave any crystals on the side of the flask.
3. Dissolve the retinyl acetate crystals in 0.1 ml chloroform.
4. Add the cottonseed oil as determined above and mix.
5. Feed each animal 0.1 ml of this mixture using a 1.0 ml tuberculin syringe.

NOTE:

1. Because of the viscosity of the mixture, calculate for a few more animals (3 to 5) than you expect to feed.
2. Retinyl acetate must be stored at -20 C (or -80 C) under vacuum in a nitrogen atmosphere. Before doing any calculations, the retinyl acetate should be removed from the freezer and allowed to thaw. Once used it should be promptly returned to a vacuum and nitrogen atmosphere and placed in the freezer.
3. The retinyl acetate preparation should be protected from light and air and fed immediately.

APPENDIX D

LIVER VITAMIN A ASSAY

1. Record weight of wet liver (usually 3 to 6 grams).

2. Homogenate in 10 ml of distilled water for approximately 1 minute using a Sorvall omni mixer at full speed.

3. Transfer the homogenate to a 25 ml graduated cylinder and record the volume.

4. Pipette into a 50-ml ground glass tube the aliquot of homogenate to be used for extraction.

   a. For vitamin A deficient animals use as much of the homogenate as possible.
   b. For adequate animals use 2 ml of homogenate.
   c. For high dose animals use 0.5 ml of homogenate.

5. Add an equal volume of a 1:10 (v/v) mixture of 0.1N KOH:absolute alcohol. Cap the tube and anchor closed with a rubber band.

6. Incubate the tubes for 5 minutes in a 40 C water bath. (Tap water in a dewar flask may be used.)

7. Remove the tubes and cool at room temperature.

8. Under the hood, add 5 ml of petroleum ether to each tube, stopper and replace the rubber band. Shake vigorously by hand for 2 minutes.

9. Balance the tubes and centrifuge for approximately 1 minute at 1500 rpm.

10. If the sample is not from a vitamin A deficient animal, transfer the ether layer to another ground glass tube using a Pasteur pipette, and extract again as specified in steps 8 and 9 above. Using a Beckman double beam spectrophotometer, read values of the ether phase at 450 nm for carotene. For these readings, transfer enough ether to fill approximately 2/3 of the cuvette. This may be accomplished by using a Pasteur pipette. Zero the machine using a petroleum ether blank. After reading at 450 nm, place this amount back in the original tube for further analysis.

11. To obtain absorbancy readings for vitamin A at 620 nm:

    a. Transfer ether to screw-cap tubes. Amounts to be used are discussed in step 12.
    b. Evaporate the ether under the hood using a stream of nitrogen gas to accelerate the process.
    c. If volumes of 1 ml or greater have been evaporated, rinse the residue from the sides of the tube using 0.5 to 1 ml of chloroform and evaporate again.
    d. To each dried tube add 0.2 ml of chloroform and 0.2 ml of acetic anhydride.
    e. Prepare chromogen by mixing 2 parts chloroform with 1 part trifluoroacetic acid (TFA v/v).
    f. Zero the spectrophotometer by mixing 2 ml of the chromogen mixture and 0.2 ml of chloroform (prepared in individual cuvettes).
    g. To obtain readings of the samples, transfer 2 ml of the chromogen (solution in "e" above) into the screw-cap tubes prepared in "d" above. A blue color will develop if vitamin A is present. Transfer the solution to a cuvette and read at exactly 30 seconds after chromogen addition.
    h. Record amounts of ether used as well as dilution schemes and absorbancy readings.

12. In obtaining absorbancy readings at 620 nm, one will encounter, undoubtedly, the problem of varying vitamin A concentration within the ether phase. The following are suggested volumes of ether needed to obtain an absorbancy reacing of 0.500 or less:

    a. For known vitamin A deficient states, transfer 4 ml of ether to a screw-cap test tube and dry under the hood.
    b. For other concentrations, in this case unknown from pre-existing parameters in the diet, evaporate 0.5 ml of ether as previously mentioned. If a reading of 0.500 or more absorbancy units is obtained, this indicates a strong concentration of vitamin A within the ether layer. To solve this problem make dilutions from portion of the remaining ether of 1/2, 1/4 and 1/8. Evaporate 0.5 ml and read again. However, if readings were less than 0.05, larger amounts of the remaining ether should be tested until a reading of 0.100 to 0.500 is reached.

13. Calculate micrograms per gram of wet liver with results obtained.

NOTE: Accurate record keeping is essential. If large concentrations of vitamin A make dilutions for readings at 620 nm impossible, the original volumes of homogenate taken for extracting (4b and 4c) may be decreased.

APPENDIX E

PREPARATION OF WHOLE TISSUES FOR LIQUID SCINTILLATION COUNTING (LSC)

Tissue Standard

1. Obtain a tissue sample (lung) that does not contain radioactivity, weigh and record the net weight.

2. Dissolve tissue using 1 ml Unisol[a] for every 100 mg wet tissue.

3. Label 11 vials from 0 to 1000. Place solute in 100 µl increments into vials 2 through 11. To each vial add 500 µl methanol and 12 ml Unisol-complement. Mix well.

4. Add 100 µl of known disintegrations per minute (DPM) to each vial (tritiated toluene).

5. Add 200 µl H_2O_2 to each vial. Let stand overnight. Loosen tops and place in an ultrasonic cleaner bath and let stand until most of the bubbles have disappeared. Count in a LSC machine[b] for 10 minutes.

6. Determine percent efficiency (CPM/known DPM) and external standard counts (read off printout sheet).

7. Make graphs for both sets of data (tissue and tissue + H_2O_2). Use the graphs to determine DPM in the tissue samples.

Tissue Sample

1. Obtain whole tissue (lung) sample and record the net weight.

2. Place tissue in a scintillator vial and add 1 ml Unisol for every 100 mg of wet tissue. Record amount used.

3. Cap the vial and let stand overnight at room temperature (preferred method) or, for a faster method, let stand in a hot water bath (55 C) until tissue is dissolved.

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[a] Isolab, Inc., Akron, OH.

[b] Packard Instrument Co., Inc., Downers Grove, IL.

4. For samples needing more than 2 ml Unisol, transfer 2 duplicate 1-ml samples into clean vials and proceed with workup.

5. Add 0.5 ml methanol (water free) to each sample. (Methanol is used so that a difficult to dissolve curd does not form when the complement is added.)

6. Add 12 ml of Unisol-complement to each vial and agitate until the solution appears clear (about 5 seconds).

7. A yellow color may appear. The addition of 200 µl of hydrogen peroxide (H_2O_2 30%) will remove the color. This amount should be added to all samples. Let stand overnight or until color disappears. Heating the samples in a water bath (55 C) quickens the color removal.

8. Hydrogen peroxide alone acts as a quencher in LSC; therefore, excess H_2O_2 must be removed. One method is to loosen the caps and set the vials in an ultrasonic cleaner.

Red Channel	Gain 45% Window A-B 50-1000
Green Channel	External Standard Channel Gain 1% Window C ∞ -300- ∞
Blue Channel	Gain 45% Window E-F 50-1000

9. Set time counter for 10-minute counts. Record those counts per minute (CPM) which are for 3- to 10-minute counts.

10. Before removing the samples from the machine, crosscheck the sample number on the sheet with counting vial number.

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VITA

The author was born December 8, 1946, in Richmond, Virginia. She spent her early life in Philadelphia, Pennsylvania, where she received her high school diploma in 1964.

In 1964 she entered Tuskegee Institute, receiving a Bachelor in Animal Science in 1968 and the Doctor of Veterinary Medicine degree in 1970.

After a year as instructor and research associate in the Department of Microbiology, College of Veterinary Medicine, Tuskegee Institute, she served as a captain in the United States Army Veterinary Corps. She was awarded the Army Commendation Medal in 1974.

In 1969 she married Captain Eddie L. Carlisle of the United States Army Veterinary Corps.