EFFECT OF DIETARY VITAMIN A ON NITROGEN DIOXIDE INDUCED LUNG INJURY IN HAMSTERS

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MICHIGAN STATE UNIVERSITY

MARTIN GEOFFREY JOURDEN

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THESIS

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ABSTRACT

EFFECT OF DIETARY VITAMIN A ON NITROGEN DIOXIDE INDUCED LUNG INJURY IN HAMSTERS

By

Martin Geoffrey Jourden

The effect of dietary vitamin A on the repair of respiratory epithelium following a single five-hour exposure to 10 ppm NO_2 was evaluated by ^3H-thymidine scintillation counting and light microscopic autoradiography.

Group 1 hamsters were maintained on regular hamster chow, whereas groups 2 and 3 received vitamin A-free pellet diets purchased from a commercial source. During the fifth through seventh weeks after weaning, group 3 hamsters were fed approximately 7080 µg of retinyl acetate in cottonseed oil.

Hamsters fed vitamin A deficient diets exhibited clinical and morphologic changes characteristic of vitamin A deficiency within five weeks from the time of weaning. Those maintained on regular diets were not similarly affected. Reversal of vitamin A deficiency was noted for most group 3 animals following an initial feeding of 3600 µg of retinyl acetate during the fifth week after weaning.

Animals were exposed for five hours to 10 ppm NO_2 during the ninth week (11th week for group 2 shipment A hamsters). Following this exposure, hamsters were chosen randomly and killed at postexposure times of 12, 24, 48 and 72 hours.

Peak cellular regeneration, as observed by light microscopic autoradiography, occurred at 12 hours postexposure for bronchial and bronchiolar epithelial counts of each dietary group. However, there was a marked reduction in the labeling indices of vitamin A deficient and hypervitaminosis A hamsters in comparison to regular fed animals (bronchial - $p<.05$, bronchiolar - $p<.01$).

For respiratory bronchiole-alveolar duct regions, peak cellular labeling occurred 48 hours after exposure in regular fed and vitamin A deficient hamsters, whereas hypervitaminosis A animals had similar labeling indices at 24 and 48 hours. Counts at 48 hours postexposure were considerably lower for vitamin A deficient in comparison to regular fed hamsters ($p<.05$).

Similar reductions of ^{3}H-TdR activity for vitamin A deficient and hypervitaminosis A animals from that of regular fed animals were noted in liquid scintillation counts.

From these observations, it appears that vitamin A may be an important nutritional factor or element in epithelial repair following NO_2 induced lung injury.

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By

Martin Geoffrey Jourden

A THESIS

Submitted to
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To my wife Renée

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INTRODUCTION

Recent public awareness has directed considerable attention toward the importance of environmental contaminants in relation to health and continued survival of man and animals. The scope of adverse effects produced by these man-made contaminants has not been fully established and is therefore highly controversial. However, evidence suggests that environmental pollutants may play an important role in the development of chronic bronchitis and inhalation carcinogenesis (Goldstein, 1975). In spite of strenuous governmental attempts to alleviate increasing levels of air pollution, many parts of the United States continue to exceed Federally designated ambient standards (Environmental Quality, 1975).

Nitrogen dioxide (NO_2) is an important gaseous component of pollutant air. Not only is it found as a by-product of combustion in automobiles, industrial processes, welding, and natural gas and coal in the provision of heat, but also in cigarette smoke (Haagen-Smit et al., 1959; Stoker and Seager, 1972; Council on Environmental Quality, 1975). Effective means of control for nitrogen oxides have not been available. As a result, estimated nation-wide emissions of these gases increased 10% from 1970 to 1974 (Environmental Quality, 1975).

Major efforts for limiting the adverse health effects related to air pollution have centered around the removal of primary sources of emission, whereas limited effort has been directed toward manipulation of host factors. Nutritional influences have been shown to be

important, not only in the defense of microbial agents, but against subsequent injury from environmental chemicals and other toxic substances (Lieber, 1976). Prevention of lipid peroxidation by vitamin E as antioxidant was demonstrated in rats and rabbits (Ramazzotto and Engstrom, 1975; Roehm et al., 1971). Such nutritional manipulation of host factors in the future may offer some hope for resistance to anticipated increase in pollution-related health problems.

Although vitamin A has been known for many years to be an important element in epithelial maintenance and differentiation (Wolbach and Howe, 1925, 1928), critical evaluations of its effect on gas-induced lung injury and repair have not been made. Dietary levels of vitamin A have been effectively manipulated in laboratory animal studies of chemical carcinogens (Shamberger, 1971; Chopra and Wilkoff, 1976; Sporn et al., 1977) and wound healing (Hirschi, 1950).

The objective of this study was to determine the effect of acute vitamin A deficiency, as well as hypervitaminosis A, on the repair of respiratory epithelium following a single five-hour exposure to ten parts per million (ppm) nitrogen dioxide gas. The optimal level of NO_2 (10 ppm) injurious to the hamster lung was previously determined (Kim, 1977).

The Golden Syrian hamster was chosen for this experiment because of its known resistance to spontaneously occurring tumors and respiratory infections (Nettesheim, 1972). Previous induction of vitamin A deficiency in this species also provided further impetus for its use (Hirschi, 1950; Rowe and Gorlin, 1959; Kaufman et al., 1972; Clamon et al., 1974).

REVIEW OF LITERATURE

Chemistry and Physiology of Vitamin A

Vitamin A is a fat soluble compound found only in animal tissues. Its structure consists of a 6-membered cyclic ring with a carbon side chain formed from 2 isoprene units (Lehninger, 1970). It exists in several physiological forms. These are: vitamin A alcohol (retinol), vitamin A aldehyde (retinal), vitamin A acid (retinoic acid), and vitamin A acetate (retinyl acetate).

To date, there are no known mechanisms of endogenous formation of this vitamin. Plants, although devoid of vitamin A, provide the major source of provitamins, α, β, and γ carotene. Central division of the side chain in carotene leads to formation of 2 molecules of vitamin A. Because of non-symmetrical chemical structure, there is some question as to whether or not α and γ carotenes form 1 or 2 molecules of this vitamin. Ingestion of synthetic vitamin A, as well as that found in animal reserves, provide other means of dietary supplement.

Once ingested, vitamin A follows similar intestinal absorption mechanisms as fatty acids (Ullrey, 1972). Mahadevan et al. (1963a,b) suggested that hydrolysis of all forms of this vitamin to retinol occurs within the lumen of the intestine prior to absorption. Re-esterification takes place within the mucosal cells.

Transport of vitamin A from the intestine occurs via chylomicron formation and lymphatic flow. Most of the vitamin is filtered from

the blood and stored within the liver. Small amounts have also been observed in the eye, kidney, adrenal gland, lungs and body fat (Davies and Moore, 1934; Popper, 1941; Popper and Greenberg, 1941; Sherman, 1969; Wallace et al., 1975; Pourcho et al., 1975).

Several physiological functions have been linked with vitamin A. Among these are: maintenance and development of epithelial tissues, bone growth and remodeling, vision, and reproduction (DeLuca, 1975). The role of retinal in vision has been demonstrated by Wald and Hubbard (1950). Its involvement in growth and reproduction have not been worked out as thoroughly. Basal cell differentiation occurs as a function of varying levels of dietary vitamin A (Wolbach and Howe, 1925, 1928, 1933). The presence of this vitamin supports mucous secreting epithelia, whereas keratinization of epithelial populations occurs in states of vitamin A deficiency.

This vitamin has not only been demonstrated to affect the direction of epithelial differentiation, but also to play an important role in the level of mitosis occurring in epithelial populations. Sherman (1961) quantitated the effects of vitamin A deficiency, as well as oral and topical administration of high concentrations of vitamin A, on tracheal, epidermal and corneal epithelia. Results of this study indicated considerable decreases in mitotic activity with vitamin A deficiency. Significant increases in mitotic counts were found with both forms of supplementation. Similar increases in mitosis have been noted by Marchok et al. (1975) upon addition of retinol to rat tracheal cultures *in vitro*.

Several theories have been proposed with respect to the action of vitamin A. Among these are the stability of membranes (Dingle and Lucy, 1962; Lucy and Dingle, 1964; Sheldon and Zetterquist, 1955) and

as a carrier of sugars in production of various glycoproteins (DeLuca and Wolf, 1970). Goubern et al. (1976) reported evidence of vitamin A interaction with metabolic pathways involved with nucleic acid synthesis. Its involvement with RNA synthesis has also been reported (Kaufman et al., 1972).

Vitamin A Deficiency

Vitamin A deficiency experiments on laboratory animals were first reported by Shinnosuki (1922). In his work with rats, he described cornification of the conjunctivae with similar changes in pharyngeal, tracheal and salivary duct linings. Yudkin and Lambert (1923) reported similar ocular lesions in their studies. However, they noted that keratomalacia was secondary to bacterial infection by vitamin A deficiency.

Later studies of Wolbach and Howe (1925, 1928) expanded the findings of Shinnosuki to various lining epithelia of the body. They reported keratinization of the nares, sinuses, larynx, trachea and bronchi with deprivation of "fat soluble A vitamin." These changes were often focal in nature. This histological transformation was described as a squamous metaplasia. Changes were more rapid in young rats. Similar findings have been demonstrated in a number of studies since then.

Perhaps the most striking clinical symptom observed with vitamin A deficiency is the impairment of growth. Wolbach and Howe (1925, 1928) reported a leveling and subsequent decrease in body weight with the progressive development of vitamin A deficiency in rats. Similar findings have been reported by investigators working with various animal models (McCarthy and Cerecedo, 1952; Salley and Bryson, 1957;

Rowe and Gorlin, 1959; Moore and Holmes, 1971; Kaufman et al., 1972; Rogers et al., 1975).

Because of the ease in nutritional manipulations, *in vitro* techniques for vitamin A deficiency have been employed by several investigators. In the study of Clamon et al. (1974), squamous metaplasia was induced in hamster tracheal rings. Reversal of keratinized epithelium was achieved by addition of α and β retinyl acetate. Similar findings were reported by Marchok et al. (1975) with rat trachea. In this study, retinol caused reversal of induced squamous metaplasia. With the use of tritiated thymidine (^{3}H-TdR), they were able to demonstrate an increase in the number of labeled cells in the early stages of deficiency. It was suggested that this represented a stage of dedifferentiation with increased utilization of remaining vitamin A stores by dividing cells. In *in vivo* studies, reversal of vitamin A deficient symptoms was demonstrated by Wolbach and Howe (1933), McCarthy and Cerecedo (1952), and Carlisle (1977). The extent of reversal was dependent upon the degree and duration of deficiency.

Hypervitaminosis A

Toxicity upon ingestion of polar bear and seal liver has been a known phenomenon among arctic travelers for some time (Rodahl and Moore, 1943). Rodahl and Moore found extremely high levels of hepatic vitamin A in these animals. From experimental feedings of this liver to rats, they postulated that acute illness following ingestion by travelers was a result of excessive vitamin A.

One of the earliest laboratory studies on the toxicity of vitamin A was that of Davies and Moore (1934). High levels of this vitamin (40 mg/10 grams of diet) were administered daily to 2 rats. Feedings

of this concentration proved to be fatal for both animals. Each rat showed extreme loss of body weight, hunched profile, and problems with hemorrhagic rhinitis. Upon postmortem examination, 1 animal exhibited severe lung disease. Radiographic examination of the other rat exhibited spontaneous fractures of long bones accompanied by irregular healing and callus formation. Death was accredited to severe emaciation in each case. Since then, similar findings have been reported (Moore and Wang, 1943, 1945; Seawright and English, 1967; Cho et al., 1975).

The most common histologic feature with hypervitaminosis A is premature closure of the epiphyseal plate. Lipid infiltration of the liver, lungs, spleen, and tubular epithelium of the kidney has also been described in cases of hypervitaminosis A. Pale green autofluorescence of vitamin A was associated with these organs (Popper, 1941; Popper and Greenberg, 1941; Seawright and English, 1967). Similar measurable increases of vitamin A in the kidney and lung were reported by Davies and Moore (1934). Following large increases in dietary supplement of vitamin A, the concentration of the vitamin in these organs superseded levels normally found in the livers of rats maintained on regular diets.

Sherman (1961) looked at the aspect of epithelial mitosis in rats fed high, as well as toxic, levels of vitamin A. Results from his experiments indicated an elevation of mitotic activity with increased levels of vitamin A. However, extreme concentrations sometimes caused a decrease in activity.

In vitro studies have demonstrated induction of mucous cell metaplasia after supplementation of the culture medium with vitamin A. Fell and Mellanby (1953) were first to demonstrate a complete

transformation of chick ectoderm into mucous-secreting epithelium. This effect has been further utilized by Elias and Friend (1976) in developing a gap-junction model, and by Marchok et al. (1975) on rat tracheal organ cultures.

Membrane instability is yet another *in vitro* effect which has been attributed to increased levels of vitamin A in culture media. Fell et al. (1962) demonstrated a release of lysosomal enzymes with chick-limb cartilage as well as with isolated liver lysosomes. This evidence has been interpreted as one possible mechanism of premature closure of the epiphyseal plate.

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 combusion and exhaust fumes, and it is a serious health hazard since it is found not only in air pollution but also in cigarette smoke (Haagen-Smit et al., 1959; Freeman et al., 1968a; Environmental Protection Agency, 1971; U.S. Department of Health, Education, and Welfare, 1972; Johnson et al., 1973; Goldstein, 1975).

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, microscopically resembling emphysema (Freeman and Haydon, 1964). Microscopically, moderate hypertrophy and hyperplasia of the bronchial and bronchiolar epithelium and increased goblet cells were 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.

Tachypnea occurred at an exposure level of 0.8 ppm (Freeman et al., 1966). However, NO_2 related lesions were not observed. 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 indicated 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 caused 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 1-hour exposure at 40 to 50 ppm resulted in acute death. The effect on monkeys was the same as that on rats in an 8-hour experiment at 65 ppm. Rabbits and dogs were more resistant.

Various animal models (rats, guinea pigs, primates, rabbits and dogs) have been shown to survive continuous exposure of 1 year or more for NO_2 concentrations (generally less than 1 ppm) above ambient levels (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.

Because of morphologic similarity of distal airways of primates to those of man, 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 with characteristics of 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). However, few studies have been made with this species.

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 subsequently returned to normal breathing at the end of each period. Microscopic study revealed marked hypertrophy and hyperplasia for cells of the terminal bronchial area, as well as a 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 in morphology to the NO_2 lesions.

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 was related to inhibition of alveolar macrophage function (Goldstein et al., 1973, 1974). The mucous ciliary transport system did not appear to be affected following 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 (dogs, rats, mice, and men) (Kleinerman, 1972; Breeze et al., 1976). Ciliated and nonciliated cells make up the bulk of the tracheobronchial epithelium. A nonciliated cell (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 attenuated 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 lamellar osmiophilic inclusion bodies, and many organelles. They are believed to be responsible for the production of surfactant (Sorokin, 1967). The alveolar macrophages lie on the surface active film which lines the pulmonary alveoli and epithelium (Evans et al., 1973).

The alveolar surface, which contains squamous endothelial cells, is thicker and more membranous, whereas alveolar epithelial and capillary endothelial cells are closely associated with the basement membranes, which join to form an apparent single membrane in areas of close proximity between the 2 cell types. The septal interstitium contains collagen fibers.

Electron microscopic studies 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., 1976; 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 ^{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 epithelial 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 of 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) suggested that NO_2 in the respiratory tract 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 that rats supplemented with adequate and deficient diets of vitamin E and exposed to NO_2 showed 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 ozone. The surfaces and walls of alveolar ducts 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 studied, experiments were begun to compare the response

of 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 terminal bronchiolar epithelium and loss of cilia. Lung tissues obtained from NO_2 exposed hamsters, fed a vitamin A deficient diet for 4 weeks, showed a critical morphologic difference. Thickening of the basement membranes, collagen proliferation and edema were characteristic. Alveolar necrosis was observed. Variable-sized lipid droplets were noted 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 (*Mesocricetus auratus*) were obtained in 2 separate shipments through the courtesy of the National Institutes of Health Mammalian Genetic Division. Upon arrival, the animals were placed in individual cages with wood chip bedding and paper nesting.

The first shipment of mothers (Shipment A) received equal amounts of commercially purchased vitamin A free diet (Appendix A) and regular hamster chow (Appendix B). Mothers of the second shipment (Shipment B) received the same diet as Shipment A through the second week of lactation, at which time they were converted to a strictly vitamin A free diet. All animals had free access to water. With the exception of food and water maintenance, they were left undisturbed through gestation and the following 3-week postnatal period.

At 21 days, pups from 20 mothers were weaned, sexed, and randomly placed 5 animals per cage (Figure 1). Each group received approximately equal numbers of males and females.

Group 2 hamsters were placed on a vitamin A free pellet diet and water *ad libitum* for the duration of the experiment. Animals of this group were obtained from both shipments of mothers.

Group 3 pups, derived from Shipment B mothers, were also placed on vitamin A free diet. In addition, animals of this group received specially prepared oral supplements of retinyl acetate in cottonseed

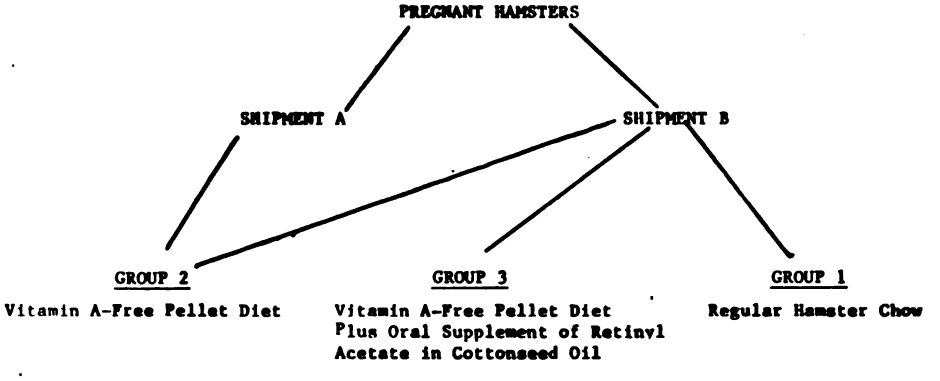


Figure 1. Origin of weanling hamsters.

oil (Appendix C). At 5 weeks after weaning, using round-tipped feeding needles and a 1-cc tuberculin syringe, Group 2 animals received feedings of 3600 µg of retinyl acetate in 0.5 cc cottonseed oil. Four subsequent feedings of 870 µg in 0.1 cc oil were given during the sixth and seventh weeks.

Group 1 hamsters obtained from Shipment B mothers were placed on regular hamster chow and water *ad libitum*.

Weighing

Hamsters were weighed at the time of weekly cage changes. Weights were recorded to the nearest gram for each animal. Clinical observations were also recorded at this time.

NO_2 Exposure

A plastic exposure chamber, measuring 52 x 33 x 18 cm and equipped with a sampling outlet, was used (Figure 2). Hamsters received a single 5-hour exposure of 10 ppm NO_2 during the ninth week after weaning (eleventh week for Group 1 animals obtained from Shipment A mothers).

Because of limited space in the gas chamber, 2 separate exposures were conducted. Although a large number of animals were initially involved in the experiment, 21 hamsters (9 in group 1, 8 in group 2, 4 in group 3) were included in the first exposure and 18 (8 in group 1, 6 in group 2, 4 in group 3) in the other, for a total of 39 utilized for autoradiography and liquid scintillation techniques for this study (Figure 1).

Nitrogen dioxide gas, at a concentration of 2,040 ppm, was purchased from a commercial source (Matheson Gas Products, Joliet, IL). Dilution to 10 ppm was achieved with an additional air flow generated

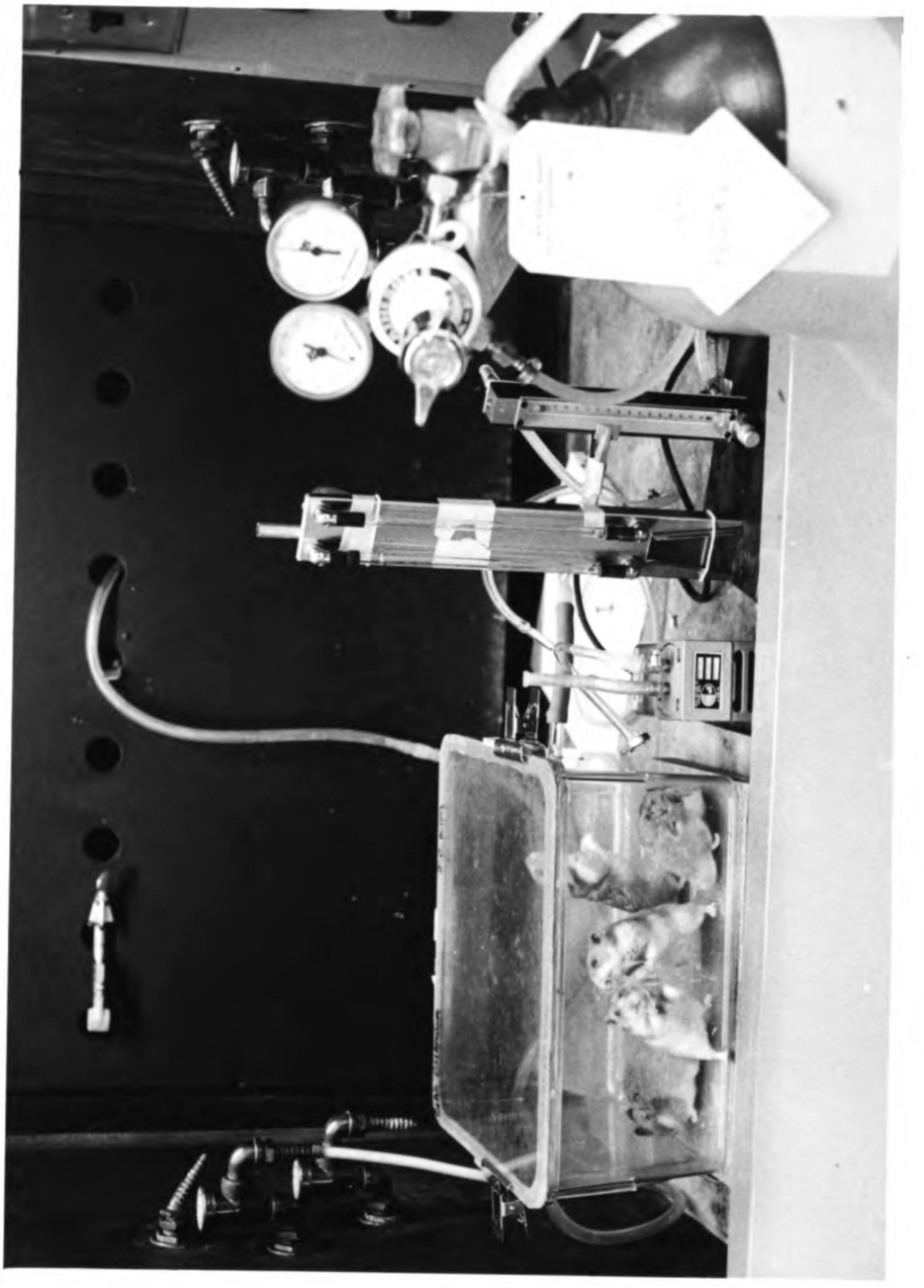


Figure 2. NO_2 gas exposure system used for this study.

by a Gast air compressor (Perkin-Elmer, Norwalk, CT) with a reserve tank of 38 liters prior to entering the exposure chamber.

Flow levels were adjusted to allow approximately 14 air changes per hour. The humidity and temperature were 50% and 72°F, respectively. Concentration of the final diluted mixture was monitored at 90-minute intervals by colorimetric procedures of the Griess-Saltzman Reaction (Intersociety Committee, 1965) (Appendix D), and with a commercial gas detection kit (Matheson Gas Products, Joliet, IL).

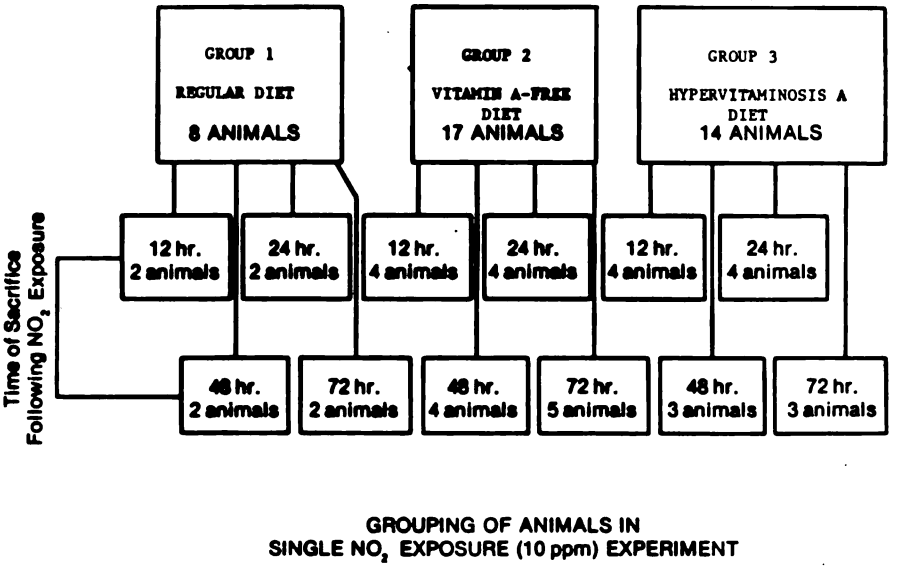
Necropsy Procedure

Following NO_2 exposure, hamsters were chosen randomly and killed at postexposure times of 12, 24, 48, and 72 hours (Figure 3). Intraperitoneal injections of tritiated thymidine (^{3}H-TdR) (Schwartzman, Division of Beckman, Orangeburg, NY), 2 microcuries (μCi) per gram body weight, were given 55 minutes prior to killing with an overdose of ip injection of sodium pentobarbital.

The trachea was exposed prior to opening of the thorax, and perfusion of the lungs *in situ* was accomplished by intratracheal administration of 2.0 cc "Universal Fixative" (McDowell and Trump, 1976). After ligating the trachea with suture, the thorax was opened, and the lungs were removed and placed in a specimen cup containing an additional 25 ml of the same fixative.

Blood smears were prepared in duplicate for each animal and stained with Wright's stain. Differential leukocyte counts were made from 2 slides for each hamster.

Other tissues collected and placed in "Universal Fixative" included skin, spleen, stomach, ileum, urinary bladder, kidney, adrenal gland, cartilage, striated muscle, heart, and eyes. Testicular



GROUPING OF ANIMALS IN SINGLE NO_2 EXPOSURE (10 ppm) EXPERIMENT

Figure 3. Grouping of animals for NO_2 exposure.

tissue and occasionally portions of ovarian ducts were also collected. Specimens for light microscopy were routinely processed, cut at 6 μ, and stained with hematoxylin and eosin.

Only a small portion of each liver was saved for light microscopy. The remainder was assayed for vitamin A content by procedures outlined by Neeld (1963) (Appendix D).

Liquid Scintillation Counting

Uptake of ^{3}H-TdR in the lung was quantitated by liquid scintillation counting (LSC) (Packard Instrument). Wet fixed lung samples of NO_2 exposed hamsters, with weights of 0.65-1.2 gm, were digested with Unisol (Isolab, Inc., Akron, OH) (1 ml Unisol/100 mg wet tissue). Two 1-ml aliquots of this solution were transferred to individual counting vials and processed for LSC (Appendix E). Mean values of ^{3}H-TdR activity were reported in counts per minute (cpm) per 100 mg of wet fixed lung.

Light Microscopic Autoradiography

Autoradiographs were prepared according to methods described in Appendix G. Three longitudinal sections from each animal, cut along the plane of the main bronchus, were examined. Labeled nuclei for 800 bronchial and bronchiolar cells, as well as respiratory bronchioles and alveolar ducts (RBr-Alv) of four longitudinally sectioned terminal bronchioles, were counted for each section.

In order to eliminate bias in selection of bronchioles and RBr-Alv regions to be counted, a systematic scan of lung tissue was adapted from methods described by Tang and Ebesson (1972). A Nikon binocular microscope with a 10 x 10 grid square mounted in the right ocular (10X) was used for counting autoradiographs. Using a 1.2X

objective and an upper condenser with a numerical aperture of 0.32, the left-most sections of each slide were positioned and accurately drawn as shown in Figure 4.

Beginning in the upper left corner of each diagram, and proceeding from left to right, every third grid square was examined in a serpentine pattern for the presence of bronchiole and RBr-Alv structures. Only the grid squares overlying the lung section were used in the scan. If possible, two RBr-Alv regions were selected from each half of the tissue. Epithelium of the four longitudinal terminal bronchioles selected for RBr-Alv counts was always included in the bronchiolar cell count. Following selection, structures were centered beneath the grid with a 40X objective and counted. The labeling index was defined as the number of labeled nuclei divided by the total nuclei counted in a given region.

Bronchial counts were started from the point of entry of the main bronchus into the lung lobe. After selection of a starting point, 800 epithelial cells were counted.

In the remaining two sections only labeled cell counts were made for the areas originally selected in section one by the procedures described.

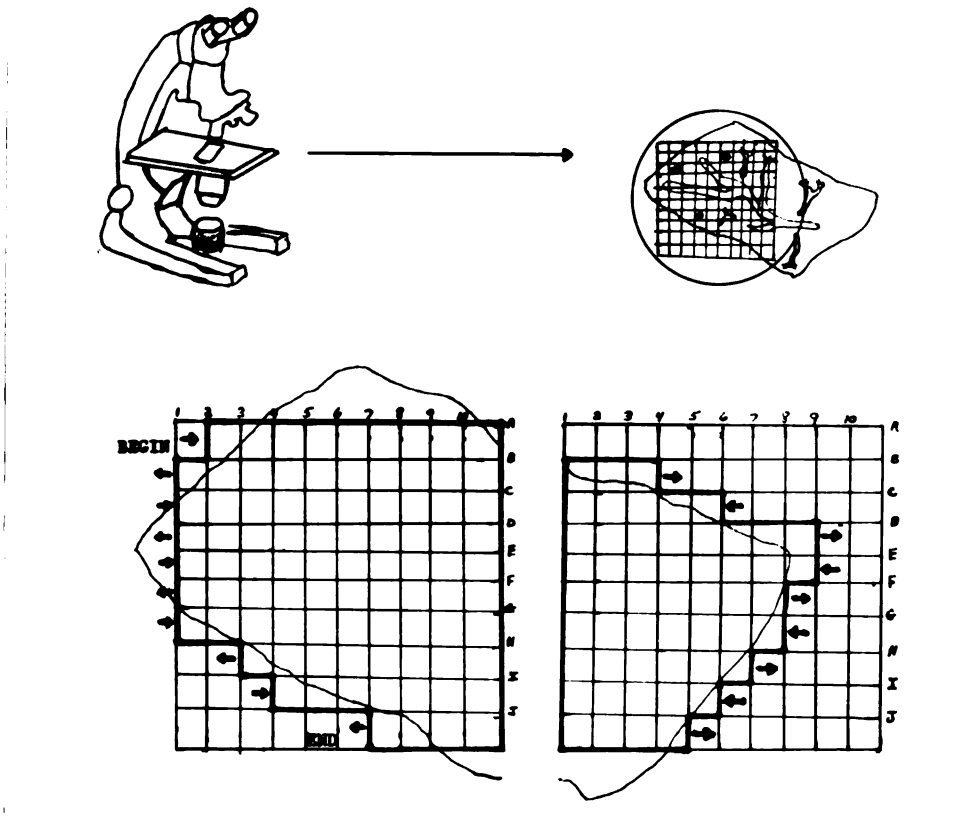


Figure 4. Systematic sampling of lung sections for autoradiography counting.

RESULTS

Group 1 - Hamsters Fed Regular Diets

General Observations

Hamsters fed regular diets were clinically normal. Appetite and appearance were good over the entire duration of the experiment. For the first 5 weeks following weaning, average weight gains of 10 gm per week occurred (Figure 5). Thereafter, weights of 100 to 106 gm were noted for weeks 6 through 9. The mean group weight at the time of NO_2 exposure was 106 gm. No gross lesions or abnormalities were observed.

Vitamin A assay. Liver vitamin A concentrations appeared to increase with the continued maintenance of regular hamster chow as shown in Figures 6 and 7. Hepatic levels of vitamin A from weekly assays increased from 16 µg per gram wet liver (gwl), 1 week after weaning, to 90 µg/gwl by the time of NO_2 exposure conducted during the ninth week. Pathologic changes related to either vitamin A deficiency or hypervitaminosis A were not observed in animals from this group.

NO_2 exposure. Hamsters were exposed during daylight hours, 9 weeks after weaning. Normal breathing and activity were observed for all group 1 hamsters during the first 20 to 30 minutes. Mild stress, as evidenced by rigid, hunched positions, as well as shallow, rapid

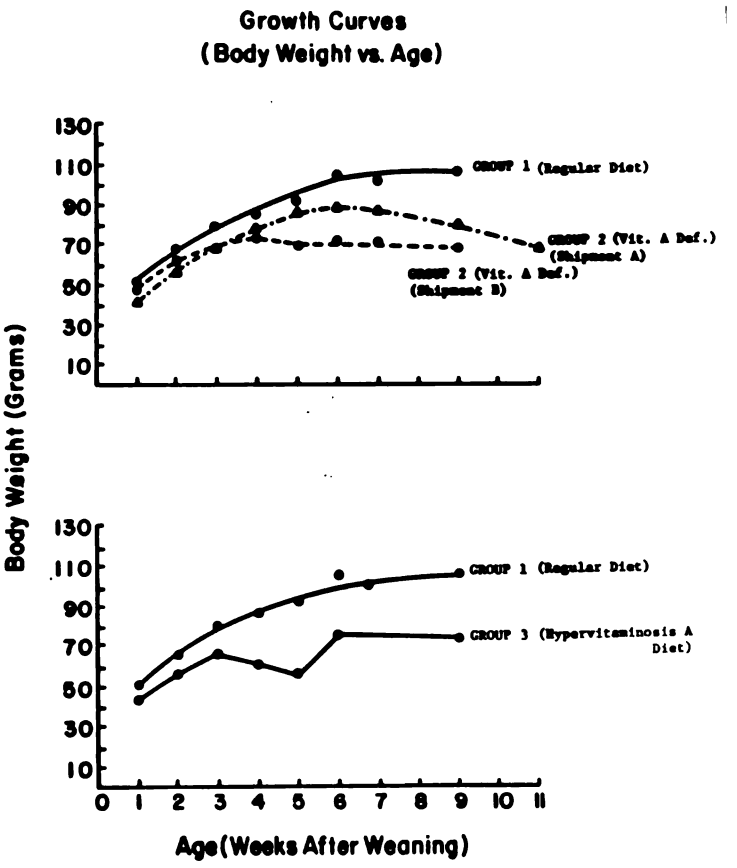


Figure 5. Growth curves for group 1, 2 and 3 hamsters.

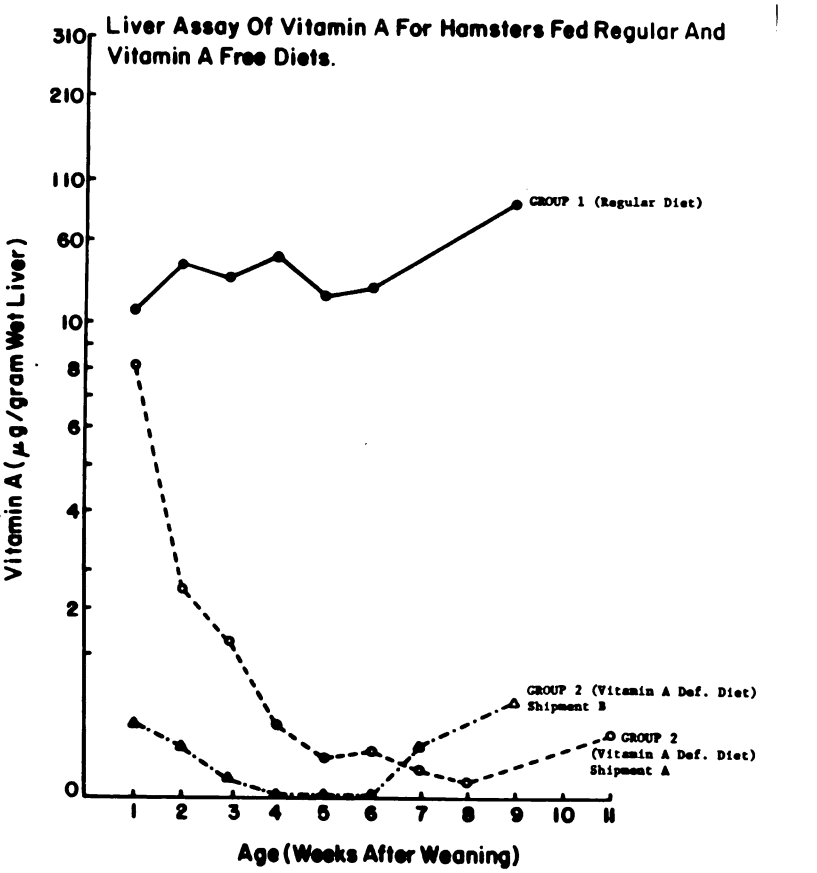


Figure 6. Liver vitamin A assays for group 1 and 2 hamsters.

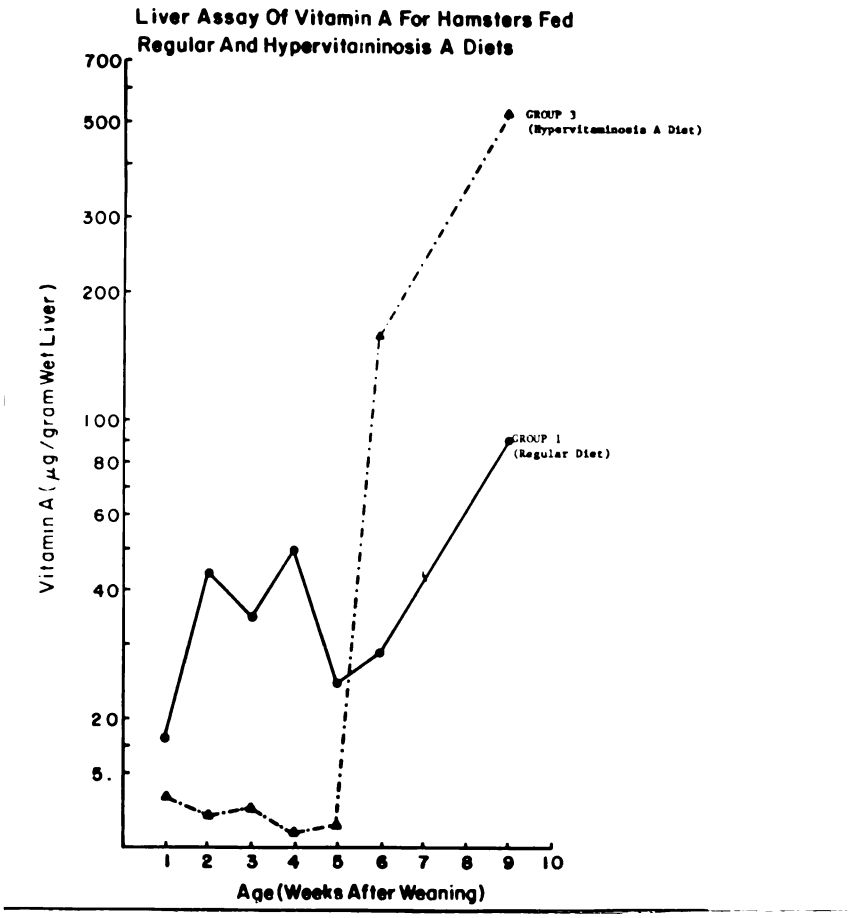


Figure 7. Liver vitamin A assay for group 1 and 3 hamsters.

breathing, were exhibited over the remainder of the exposure. Normal respirations resumed shortly after termination of the exposure.

Necropsy findings. Group 1 hamsters were in good condition. No gross lesions were observed at the time of necropsy. Microscopic examination of lungs indicated mild to moderate degrees of hyperplasia for airway epithelium in animals killed 12 hours after exposure. Marked hyperplasia and hypertrophy of RBr-Alv ducts were noted in animals killed at 24 and 48 hours (Figure 8). The intensity of this lesion diminished by 72 hours. This pattern of lesion development was consistent with 10 ppm NO_2 5-hour exposures in hamsters (Kim, 1977).

Hematology. Non-exposed, regular-fed hamsters demonstrated a slight increase of lymphocyte-to-neutrophil ratio. Following NO_2 exposure, a moderate depression of lymphocyte, and an increase of monocyte, ratios occurred as shown in Figure 9. This is a relative value and does not represent an absolute increase.

Liquid Scintillation Counting

Measurement of ^{3}H-TdR uptake in the lung of NO_2 exposed hamsters was measured by the standard liquid scintillation counting technique. At 12 hours postexposure, a radioactivity of 5,200 cpm/100 mg wet fixed lung was recorded (Figure 10). A maximum radioactivity of 11,000 cpm found at 24 hours was followed by a decrease at 48 hours. This declined further to 2,200 cpm by 72 hours.

This type of cellular repair pattern of ^{3}H-TdR activity exhibited by group 1 animals was similar to autoradiographic findings previously reported by Evans et al. (1973, 1974).

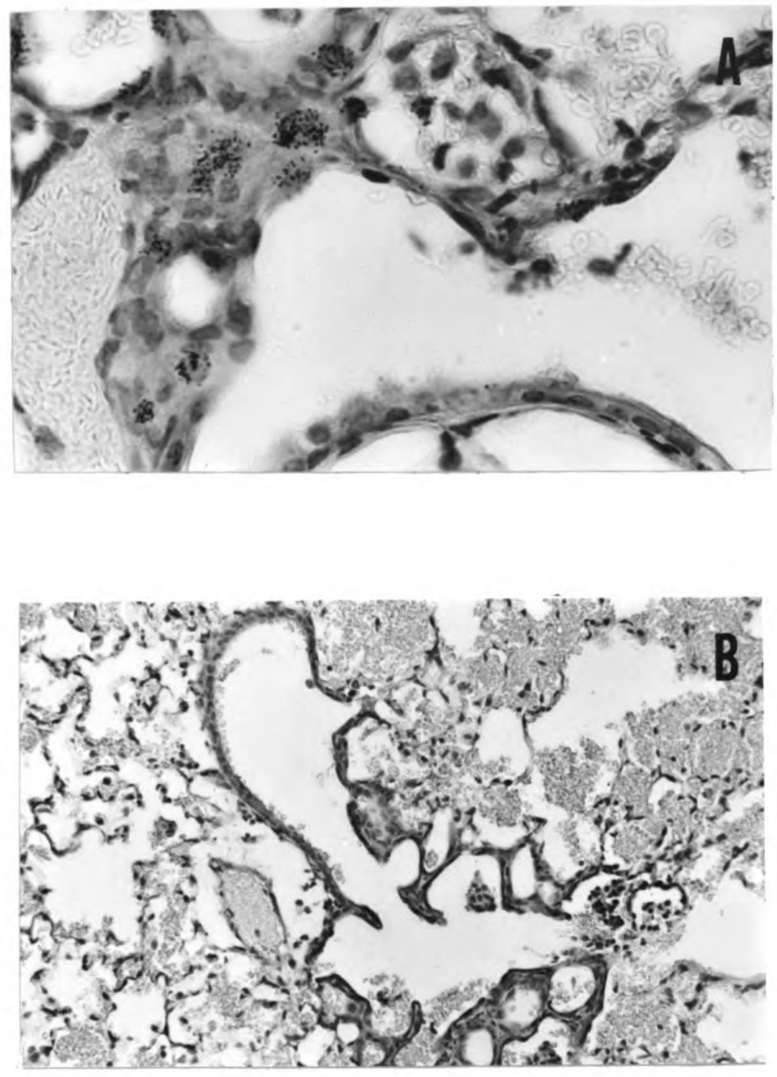


Figure 8. Hypertrophy and hyperplasia seen in the terminal and respiratory bronchioles in group 3 (A) and group 1 (B) hamster lungs at 24 and 48 hours postexposure, respectively. 8A-X125, 8B-X50, H&E stained.

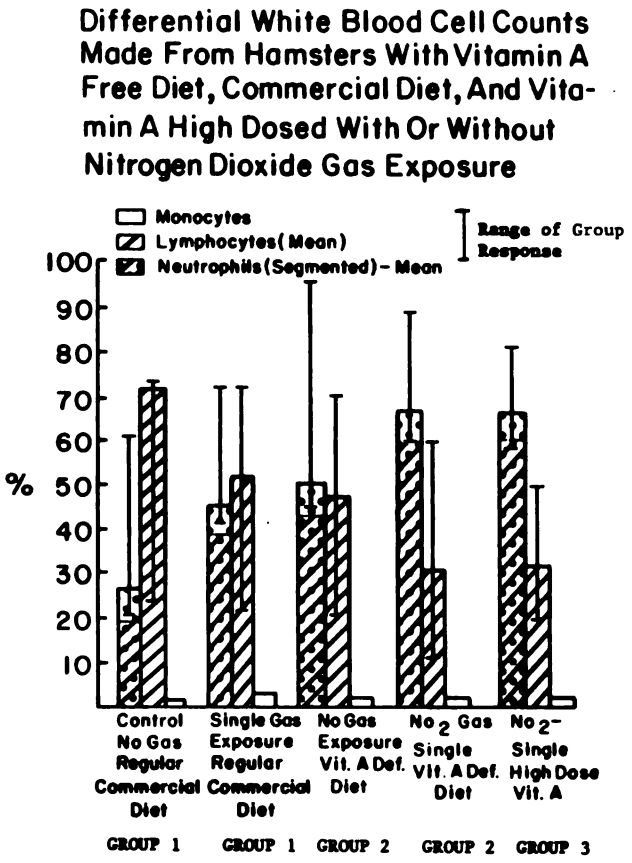


Figure 9. Pre- and postexposure differential leukocyte counts for group 1, 2 and 3 hamsters.

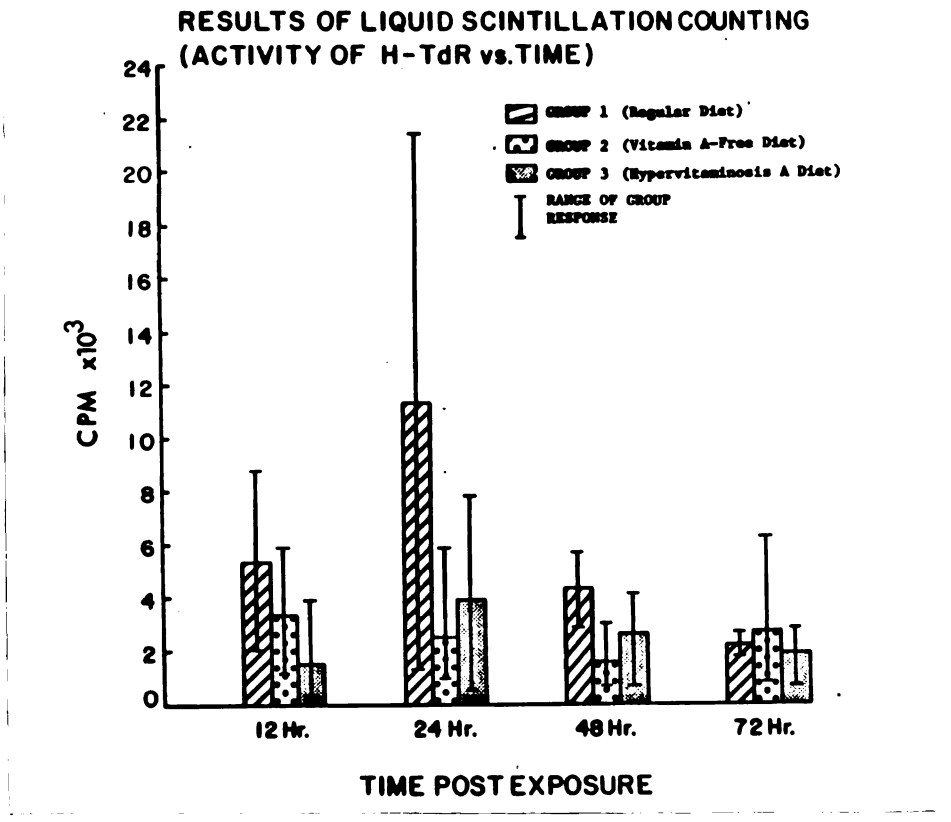


Figure 10. Liquid scintillation counts for hamsters of groups 1, 2 and 3.

Light Microscopic Autoradiography

The results of mean labeled indices tabulated from 3 serial sections of each animal are given in Figures 11, 12 and 13 consecutively. The cell counts were made from 3 major airway structures, namely bronchi, bronchioles, and respiratory bronchiole-alveolar ducts. As shown in Figure 11, a peak labeling occurred in bronchial epithelium at 12 hours postexposure. At 24 hours, the labeled cell index decreased 6-fold from 30×10^{-2} to 5×10^{-2}. This declined further at 48 and 72 hours postexposure. This corresponded with epithelial hyperplasia as described earlier.

A similar pattern of labeling occurred in bronchiolar epithelium as shown in Figure 12. The peak labeling index, which occurred in RBr-Alv regions, showed much contrast to those for bronchiolar and bronchial epithelium (Figure 13). At 12 hours postexposure, the labeled index was slightly greater than 1×10^{-2}. It reached a value of more than 5×10^{-2} at 24 hours and increased to 11×10^{-2} at 48 hours. The labeled index then declined to less than 2×10^{-2} at 72 hours postexposure. The peak occurred, therefore, at 48 hours postexposure. This result goes along with time responses for type 2 cell hyperplasia reported by Evans et al. (1974, 1975). However, type 2 cell counts per se were not made in this study.

Group 2 - Acute Vitamin A Deficient Hamsters

General Observations

Growth patterns of hamsters derived from pregnant mothers shipped at different times were similar, even though the onset of vitamin A deficiency, characterized by weight loss and xerophthalmia, was more rapid in weanling hamsters placed on vitamin A-free diets 1 week prior

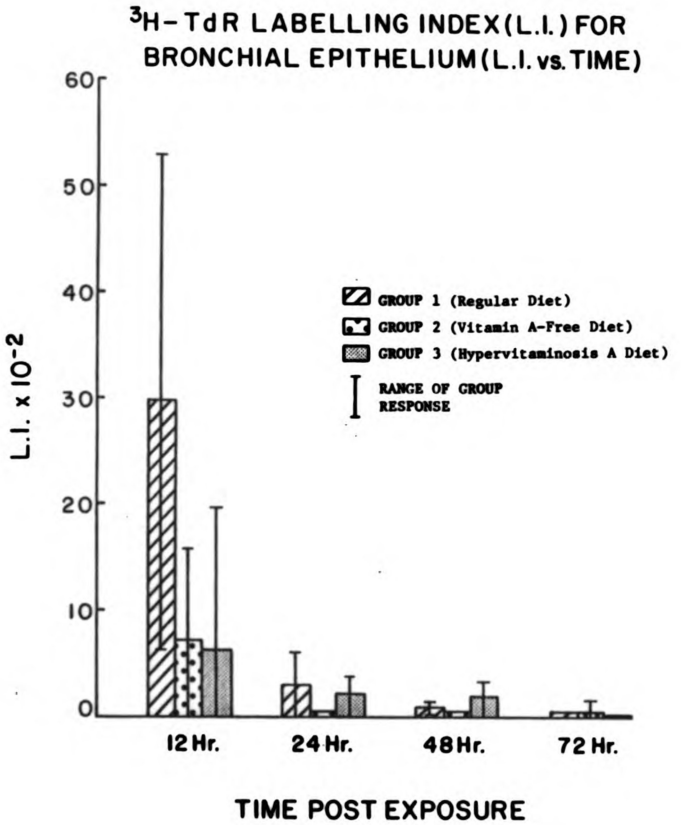


Figure 11. ^{3}H-TdR labeling index for bronchial epithelium of group 1, 2 and 3 hamsters.

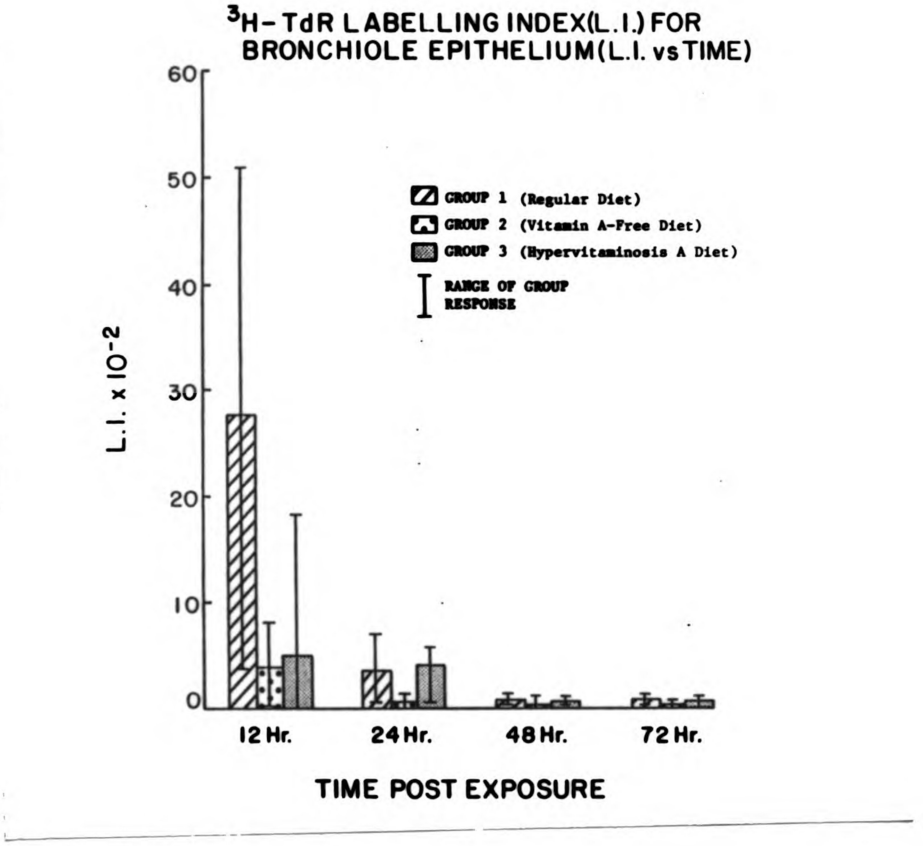


Figure 12. ^{3}H-TdR labeling index for bronchiole epithelium of group 1, 2 and 3 hamsters.

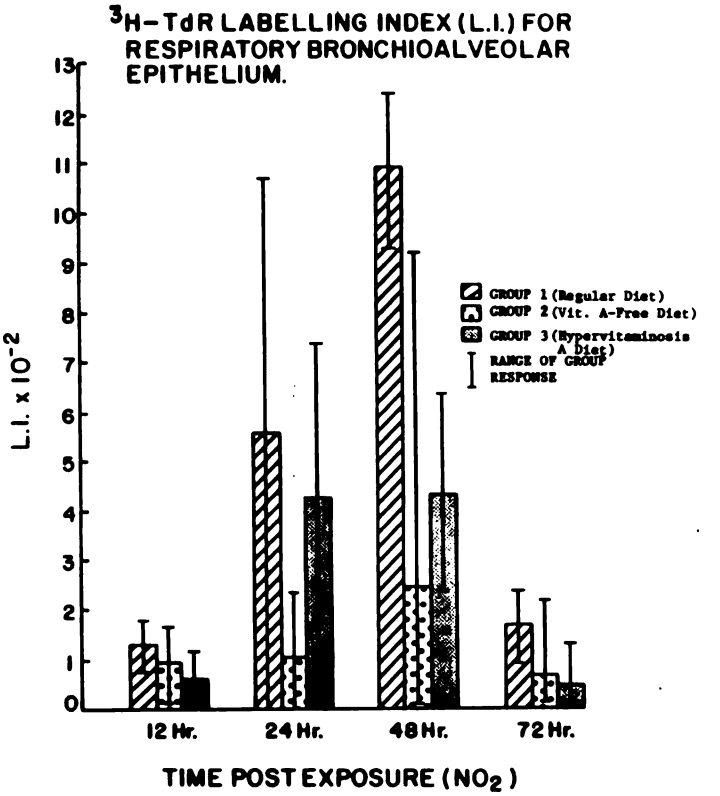


Figure 13. ^{3}H-TdR labeling index for respiratory bronchiole-alveolar regions of group 1, 2 and 3 hamsters.

to weaning. Appetite and physical appearance were good for the first 3 weeks following weaning, as reflected by average weight gains of 10 gm per week (Figure 5). Increase in weight continued until 6 weeks for animals derived from shipment A mothers, whereas the other group of hamsters showed a decline in weight as early as 4 weeks. Weight losses were noted for all group 2 hamsters during the remaining 5 weeks of the experiment. At the time of NO_2 exposure, a mean body weight of 66 gm was noted for all group 2 hamsters. This represented an approximate difference of more than 30 gm when compared with regular-fed group 1 animals. One group of hamsters was 9 weeks old and the other 11 weeks at the time of postexposure killing.

As early as 4 weeks, generalized unthriftiness demonstrated by lethargy, anorexia, rough hair coat, and focal alopecia, as well as xerophthalmia were first noticed. One or both eyes were often sealed by an accumulation of dried purulent exudate. Occasionally the nose was also crusty. The testicles were invariably shrunken by the eighth week (tenth week for animals from shipment A mothers) after weaning. At this point, body weights decreased and spontaneous deaths occurred more often.

Vitamin A assay. Chemical vitamin A assays were made weekly from the beginning of the experiment. Vitamin A assays made 1 week after weaning showed a marked difference between pups from shipment A and B mothers. Animals from shipment A mothers had vitamin A concentrations of 8 µg/gwl, whereas weanling hamsters from shipment B had less than 1 µg/gwl (Figure 6). Thereafter, hepatic concentration of vitamin A decreased weekly for all group 2 animals. Vitamin A concentrations were found to be less than 1 µg/gwl for animals from both groups of mothers at the time of gas exposure.

NO_2 exposure. Animals (group 2) exposed during the ninth week after weaning showed varying degrees of emaciation, dehydration, and xerophthalmia. Breathing of all animals appeared normal over the first 20 to 30 minutes of NO_2 exposure, with a mild degree of distress exhibited thereafter. Most hamsters assumed rigid, hunched positions, as well as shallow, rapid breathing over the remainder of the 5-hour period. Upon termination of the exposure, normal respirations were resumed quickly.

Necropsy findings. At the time of necropsy, poor hair coat and purulent ocular and nasal discharge were evident. The testicles were occasionally abscessed. Gastric content was minimal. The colon was often distended and ballooned as a result of gas formation. The liver was invariably shrunken and fibrotic, and the kidneys were pale and swollen. Microscopically, various stages of squamous metaplasia were observed in the salivary gland, kidney, eye, testicle, and in some portions of gastrointestinal and respiratory epithelium. Hyperplasia of urinary bladder transitional epithelium was observed in several different hamsters.

Hematology. Relative differential leukocyte counts for group 2 are presented in Figure 9. The normal differential leukocyte counts for hamsters consisted of a high lymphocyte-to-neutrophil ratio (Figure 9). Group 2 animals showed a reversal in the ratio of lymphocytes to neutrophils following NO_2 exposure. This was expected in view of the gross and histologic changes associated with vitamin A deficiency and infection.

Liquid Scintillation Counting

Uptake of ^{3}H-TdR during DNA synthesis was measured by standard liquid scintillation counting technique. Group 2 observations for ^{3}H-TdR activity per 100 mg of wet fixed NO_2 exposed lung are given in Figure 10. At 12 hours postexposure, observed activity was 3,318 cpm. This decreased to 2,482 and 1,250 cpm at 24 and 48 hours, respectively. Seventy-two hour counts increased slightly. Group 1 animals, fed regular diets, responded in a much different fashion. Peak activity occurred at 24 hours after exposure and declined thereafter (Figure 10).

Light Microscopic Autoradiography

Labeling indices for group 2 hamsters are shown in Figures 11, 12 and 13. For each anatomical area, labeled cell indices expressed were much lower for vitamin A deficient animals in comparison to regular-fed group 1 hamsters which were exposed to the same NO_2 gas concentration (10 ppm). However, general patterns in labeling were similar. A peak labeling index occurred at 12 hours postexposure in both bronchiolar and bronchial regions. At 24, 48 and 72 hours following exposure, indices declined as shown in Figures 11 and 12. Peak labeling occurred at 48 hours postexposure in RBr-Alv areas. This finding agrees with group 1, although the overall labeling indices were greatly reduced in vitamin A deficient animals.

Group 3 - Acute Hypervitaminosis A Animals

General Observations

Animals of this group received vitamin A-free diets for the first 5 weeks following weaning. Clinical observations during this period

were identical to those discussed for group 2 hamsters from shipment B mothers.

Weight gains of 10 gm per week occurred through the third week following weaning. Thereafter, loss in weight was noted during weeks 4 and 5 as shown in Figure 5. During the sixth week, upon feeding of vitamin A, a marked increase in physical activity and appetite, as well as an improvement in hair coat and testicular size, were noted for most group 3 animals. From 6 weeks onward, no changes were noted in regard to body weight.

Vitamin A assay. Levels of less than 1 µg/gwl liver were observed in group 3 animals 1 week after weaning (Figure 7). After an initial feeding of 3,600 µg retinyl acetate, liver concentrations increased to 158 µg/gwl. Four subsequent feedings of 870 µg each, during the sixth and seventh weeks, increased this concentration to 540 µg/gwl, at the time of NO_2 exposure.

NO_2 exposure. Although most group 3 animals appeared to be healthy and active at the time of NO_2 exposure, several showed varying degrees of emaciation and dehydration. Patterns of respiration noted were similar to those reported for group 2 animals.

Necropsy findings. Upon necropsy, most notable gross lesions were found in the liver. These were invariably shrunken and often fatty and degenerated. The testicles appeared to be normal.

Hyperplasia and hypertrophy observed in terminal bronchioles of lung sections were minimal in comparison to group 1. Microscopically, fatty degeneration and cloudy swelling were observed in examined liver sections. A mild degree of mucoid hyperplasia occurred in

transitional epithelium of urinary bladder. No microscopic changes were observed in the testicles.

Hematology. Differential cell counts made from group 3 were similar to those found in group 2 (Figure 9).

Liquid Scintillation Counting

A peak in radioactivity occurred at 24 hours, as shown in Figure 10. Counts of less than 2,000 recorded at 12 hours increased to more than 4,000 by 24 hours. Thereafter, it gradually declined to less than 2,000 at 72 hours. The general pattern of radioactivity observed was much lower than group 1, but was slightly higher than group 2 at 24 and 48 hours.

Light Microscopic Autoradiography

As shown in Figures 11 and 12, a much similar pattern of labeling indices occurred in bronchial and bronchiolar epithelium when compared to responses for groups 1 and 2. A peak labeling was noted at 12 hours and declined thereafter. The cellular response of RBr-Alv regions was somewhat different. In this group, peak labeling occurred at 24 and 48 hours and declined at 72 hours, as shown in Figure 13.

Statistical Analysis of Liquid Scintillation Counting and Light Microscopic Autoradiography

Liquid Scintillation Counting

Normalization of the data by log transformation was done prior to statistical analysis. Use of a two-way analysis of variance indicated an influence of dietary vitamin A on subsequent uptake and activity of ^{3}H-TdR of the lung ($p \tilde{=} .2$) (Table I-4). A two-sided Dunnett's T-distribution was used for comparison of group 2 and 3

animals with group 1 regular diet hamsters (Table I-5). Although values never reached the level needed for .05 significance ($T>2.35$), relative differences were apparent (Tables for .1 and .2 significance are not available for Dunnett's T-distribution).

Light Microscopic Autoradiography

Normalization of the data by angular transformation was performed prior to statistical analysis. Results from a two-way analysis indicated an influence of vitamin A on the labeling indices of bronchial ($p\tilde{=}.25$), bronchiolar ($p\tilde{=}.1$), and RBr-Alv ($p\tilde{=}.15$) regions (Table I-4). Labeling indices of group 2 and 3 hamsters were significantly lower than those of group 1 animals for bronchial ($p<.05$) and bronchiolar ($p<.01$) cell counts made 12 hours after NO_2 exposure (Table I-5). An overall difference of RBr-Alv labeling was noted for counts from group 1 and 2 animals ($p<.05$). The labeled index of vitamin A deficient hamsters was significantly smaller ($p<.05$) than that of regular fed animals 48 hours following exposure (Table I-5).

DISCUSSION

The effect of NO_2 on the lungs of various laboratory animals is well documented (Freeman, 1968; Kleinerman, 1969; Stephens, 1972; Evans et al., 1973, 1975). However, very little is known about modification of its injurious effect by host factors other than vitamin E (Roehm et al., 1971; Ramazzoto and Engstrom, 1975). Although the modifying role of vitamin A in connection with lung cancer has been actively pursued in recent years (Chopra and Wilkoff, 1976; Sporn et al., 1977), no data were available for the effects of this vitamin on the repair of lung cells following injury by gaseous air pollutants. Because of the known effects of vitamin A on epithelial differentiation (Wolbach and Howe, 1925, 1928) and mitotic activity (Sherman, 1961), it was thought that optimal repair of respiratory epithelium may be influenced by dietary vitamin A. Therefore, this experimental study, although drastic in design, was undertaken to determine the repair of respiratory epithelium, following a single 5-hour exposure to NO_2 (10 ppm), of hamsters maintained on acute vitamin A deficient and hypervitaminosis A diets.

Previous studies of hamsters fed regular balanced diets reported optimal lung injury, characterized by visual microscopic epithelial and basal cell hyperplasia of terminal bronchioles, with 10 ppm of NO_2 for 5 hours exposure (Kim, 1977). Results from this pilot experiment strongly suggested the importance of dietary vitamin A in lung repair following NO_2 gas injury. Vitamin A deficient hamsters, with

typical clinical symptoms, and assayed liver concentrations of less than 1 µg/gwl, exhibited reduced cell repair when measured by both liquid scintillation techniques and light microscopic autoradiography.

Vitamin A deficiency, resembling those seen in man, were readily created in hamsters maintained on commercially purchased (Appendix A) vitamin A-free diets. Placement of pups on this diet prior to weaning was essential for successful induction of deficient states. The delayed onset of deficiency for group 2 hamsters obtained from shipment A mothers in comparison to those from shipment B pregnancies corresponded with prolonged administration of regular hamster chow prior to weaning. McCarthy and Cerecedo (1952) reported similar findings in mice.

The shortcomings of biochemical assays, pointed out by Ullrey (1972), are many. The color formed in the reaction of vitamin A with trifluoroacetic acid, as described by Neeld (1963), is only linear over a limited range of vitamin A concentration and is transient, requiring spectrophotometric readings after 30 seconds. As a result, some problems with selection of initial sample size and subsequent dilutions for livers of hamsters maintained at different dietary levels of vitamin A were encountered. In addition, one must be careful in interpreting the results of chemical assays for hepatic vitamin A and its biopotency. The release of this vitamin from the liver is dependent upon other factors, such as dietary protein, fatty acid, and the presence of vitamin E. Thus, liver concentrations may not provide an accurate reflection of the overall utilization of this vitamin in a given animal.

Deficiency of vitamin A is common in the United States and elsewhere in spite of abundant food supply. Selective deactivation of

hepatic vitamin A by chronic ingestion of alcohol has been firmly established in man and experimental animals (Lieber, 1976). Other hepatotoxic agents in the environment may alter storage of vitamin A by disturbing liver metabolism or by producing irreparable damage to hepatic cells.

Evans et al. (1973, 1976) indicated a progenitor function for nonciliated terminal bronchiole and type 2 alveolar epithelium in the replacement of injured ciliated bronchiolar and type 1 epithelium following injury to NO_2. In this study, an overall decrease in labeling indices for RBr-Alv ducts of vitamin A deficient and hypervitaminosis A hamsters suggested a possible generalized impairment of overall metabolic activity. Whether this decrease reflects a specific lack of regeneration of type 2 epithelial cells in the lung is not known and is therefore receiving further consideration in this laboratory.

Chronic hypervitaminosis A in man from long-term intake of moderate to high doses of this vitamin is a common phenomenon which recently aroused world-wide discussion concerning the safety of retinoic acetate (Korner, 1975). Vitamin A toxicity following ingestion of polar bear and seal liver has been reported frequently among arctic travelers (Rodahl and Moore, 1943). The reduced cell labeling occurring in various respiratory passages of hamsters given toxic ranges of retinyl acetate may reflect an overall toxicity similar to that described by Sherman (1961) in studies with the skin and trachea. Approximately 7,080 µg of retinyl acetate were given to each group 3 animal. Toxicity, manifested by hepatic degeneration, may have reduced the production of albumin, which serves as an important carrier of vitamin A in blood circulation.

Equivalent labeling indices occurred at 24 and 48 hours post-exposure for autoradiographic counts of RBr-Alv regions in hamsters fed large amounts of vitamin A. This pattern differed from those of group 1 and 2 animals.

Non-exposed ^{3}H-TdR injected controls were not incorporated into this study because of the minimal cell labeling encountered in non-exposed hamster lungs (Kleinerman, 1970). In general, lung cell populations are stable; however, they may be stimulated to divide following insult. The marked reduction of ^{3}H-TdR uptake for group 2 vitamin A deficient hamsters appeared to be consistent with additional studies made by this laboratory. The dose level of vitamin A has been shown in subsequent experiments to be critically important in the repair of hamster lungs following injury by 10 ppm NO_2. In animals with liver concentrations greater than 40 µg/gwl, uptake of ^{3}H-TdR increased more than 2-fold, whereas hamsters with concentrations greater than 100 µg/gwl appeared to be protected against NO_2 injury. Therefore, further dose response studies are warranted to delineate the role of dietary vitamin A in the role of respiratory epithelium.

Liquid scintillation techniques and light microscopic autoradiography provided 2 different, but equally effective, means of assessing the repair of lung cells following injury by NO_2 (10 ppm). Liquid scintillation counting produced quick results for uptake of ^{3}H-TdR by lung tissue. Use of entire left lung lobes for liquid scintillation and a single right lung lobe for autoradiographic studies allowed better sampling and determination of cell repair. Although the preparation of autoradiographs was time consuming and often presented technical difficulties, such as humidity control and development of fog, it provided a means of pinpointing cellular

regeneration occurring at specific anatomical regions of the lung. Thus, the combined use of both techniques provided a much better qualitative and quantitative assessment of lung injury and repair.

The results of this experiment provided needed guidlines necessary for formulating further studies of more realistic dose levels of vitamin A and its effects on lung cell repair in hamsters (Carlisle, 1977).

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 acute vitamin A deficient, hypervitaminosis A, and regular diets following a single five-hour exposure to 10 ppm NO_2.

The experimental observations were as follows:

1. Clinical symptoms related to vitamin A deficiency such as xerophthalmia, roughened hair coat and loss in body weight were noted within five weeks from the time of weaning in group 2 hamsters maintained on a commercial vitamin A-free pellet diet.

2. Time of administration of vitamin A-free diets was a critical factor in the subsequent development of vitamin A deficiency in pups.

3. A marked reduction in ^{3}H-thymidine labeled epithelial cells of vitamin A deficient hamsters occurred for various respiratory structures as assayed with both liquid scintillation and autoradiography when compared with regular fed animals.

4. A peak labeling response was noted at 12 hours postexposure for bronchial-bronchiolar epithelium, and at 48 hours for respiratory bronchial alveolar regions of vitamin A deficient animals.

5. Weekly liver vitamin A assays corresponded with a reduction in body weight and development of clinical symptoms, thus indicating

successful use of the hamster as a model for selective states of vitamin A deficiency.

6. Hypervitaminosis A was readily produced in group 3 hamsters by oral feeding of approximately 7,080 µg retinyl acetate in cottonseed oil.

7. Vitamin A toxicity of group 3 animals was evidenced by hepatic degeneration and reduced body weight in comparison to that of regular fed hamsters.

8. Group 3 animals exposed to 10 ppm NO_2 showed a marked reduction in labeling indices of bronchial, bronchiolar, and respiratory bronchial alveolar cell counts, and ^{3}H-TdR activity measured by liquid scintillation counting, when compared with group 1 animals, fed regular diets.

9. A different pattern of respiratory bronchial alveolar labeling, as evidenced by similar indices at 24 and 48 hours, occurred in hypervitaminosis A (group 3) hamsters when compared with group 1 and 2 animals.

From these observations, it appears that absence or excess of vitamin A in the diet can cause overall reduction of respiratory epithelial regeneration of hamsters following induced lung injury by a single exposure to 10 ppm NO_2.

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

REGULAR HAMSTER CHOW*

Protein, %	24.52
Fat, %	4.15
Fiber, %	3.20
Ash, %	8.45
Nitrogen-Free Extract, %	49.70
Metabolizable energy, kcal/lb	1,250.00
Amino Acids, % (of diet)	
Threonine, %	1.03
Valine, %	1.35
Cystine, %	0.40
Methionine, %	0.46
Isoleucine, %	1.38
Leucine, %	1.85
Tyrosine, %	0.83
Phenylalanine, %	1.17
Lysine, %	1.64
Histidine, %	0.62
Arginine, %	1.44
Tryptophan, %	0.31
Minerals	
Calcium, %	1.20
Phosphorus, %	0.99
Potassium, %	0.80
Magnesium, %	0.20
Sodium, %	0.30
Chlorine, %	0.50
Iron, ppm	340.00
Zinc, ppm	50.00
Manganese, ppm	170.00
Copper, ppm	11.00
Cobalt, ppm	0.60
Iodine, ppm	1.40

Vitamins	
Carotene, ppm	2.00
Vitamin A, IU/gm	15.00
Vitamin D, IU/gm	4.41
Alpha-tocopherol, ppm	35.00
Thiamine, ppm	14.00
Riboflavin, ppm	6.50
Niacin, ppm	60.00
Pantothenic acid, ppm	18.00
Choline, ppm	1,780.00
Pyridoxine, ppm	8.13
Folic acid, ppm	1.60
Biotin, ppm	0.15
Vitamin B-12, mcg/lb	13.33
Menadione sodium (Bisulfite, ppm)	2.80
Vitamin C, ppm	---

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*Wayne Lab-Blox, Wayne Laboratory Animal Diets, Allied Mills, Inc., Specialty Feeds Department, 110 North Wacker Drive, Chicago, IL.

APPENDIX C

RETINYL ACETATE PREPARATION FOR ORAL FEEDING

The following equations were used to calculate the amount of vitamin A and cottonseed oil needed for preparation of predetermined concentrations of retinyl acetate (µg/0.1 ml cottonseed oil).

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

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

PROCEDURE

1. Tare a 50 ml Erlenmeyer flask to 4 decimal places.
2. Weigh out the amount of retinyl acetate determined by equation 1.
3. Dissolve vitamin A crystals in 0.1 ml chloroform.
4. Add the amount of cottonseed oil as determined in Equation 2.
5. Using a 1.0 ml tuberculin syringe, feed each animal 0.1 ml of this mixture.

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 should be stored at -20°C (or -80°C) under vacuum in a nitrogen atmosphere.
3. Protect the retinyl acetate preparations from light and air after mixing.

APPENDIX D

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 E

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 group 2 animals use as much of the homogenate as possible.
   b. For group 3 animals use 0.5 ml of homogenate.
   c. For group 1 use 2 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 scre-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 reading 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 F

PREPARATION OF LUNG TISSUES FOR LIQUID SCINTILLATION COUNTING (LSC)

Tissue Sample

1. Obtain lung tissue sample and record the wet fixed 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.

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.

9. Set channels of LSC machine **

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

10. Place vials in LSC machine and count each for 3 10-minute periods.

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

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*Isolab, Inc., Akron, OH.

**Packard Instrument Co., Inc., Downers Grove, IL.

APPENDIX G

PREPARATION OF LIGHT MICROSCOPIC AUTORADIOGRAPHS

Autoradiographs were prepared with a standard liquid emulsion dipping technique. The following sequence of preparation was followed:

1. Three serial lung sections with approximately 30 μ spacings in between were cut at 6 μ and placed on a single glass slide.

2. Slides were deparaffinized in xylene and rehydrated in 100% ethyl alcohol (EtOH), followed by 90% EtOH, and finally water.

3. After rehydration, slides were placed in a 38°C oven overnight to dry.

4. Dipping of slides:

   a. Kodak NTB_3 emulsion was taken into the darkroom and placed in a 45°C water bath for 45 minutes. The cap was removed from the emulsion to allow release of air bubbles during melting.

   b. All darkroom work was done in total darkness. The room temperature was approximately 70°C.

   c. After dipping, slides were placed upright in a test-tube rack which was set inside a foil-covered box. Drierite was placed generously in the bottom of the box to aid in the drying of slides. These were left for 1 1/2 to 2 hours.

   d. Dried slides were placed, sections up, in plastic slide boxes. Small perforated screw-cap vials containing Drierite were placed in the bottom of each box.

   e. Slide boxes were then wrapped in aluminum foil and taken from the darkroom and placed in a refrigerator at 4°C.

5. After an exposure period of 2 weeks, slides were processed in freshly prepared solutions of Microdol-X and Kodak Rapid Fixer. Development and fixation times used were as follows:

Microdol-X	5 minutes
Distilled water	3 dips
Kodak Rapid Fixer	5 minutes
Distilled water	5-10 minutes

Slides were agitated frequently during development and fixation.

6. Following development, slides were stained with hematoxylin and eosin according to the following schedule:

1.	Hematoxylin	2 min
2.	Tap water rinse	2 min
3.	HCl and water (2:300)	5 sec
4.	Tap water rinse	2 min
5.	70% EtOH	3 min
6.	Eosin	20 sec
7.	95% EtOH	several dips
8.	95% EtOH	5 min
9.	100% EtOH	10 min
10.	100% EtOH	15 min
11.	Xylene	5 min

Slides were then coverslipped with Permount.

APPENDIX H

RAW DATA TABLES FOR: BODY WEIGHT, VITAMIN A LIVER ASSAYS, LIQUID SCINTILLATION COUNTING, AND AUTORADIOGRAPHY

Table H-1. Record of weekly mean group body weight (grams)

Age (wks post-weaning)	Group 1	Shipment A Group 2	Shipment B Group 2	Group 3
1	51.1	40.9	48.7	43.2
2	66.9	56.6	62.5	55.8
3	79.2	67.0	68.2	65.3
4	86.2	77.2	73.0	62.9
5	92.5	80.5	65.1	56.7
6	105.0	83.5	66.0	75.0
7	101.3	81.5	66.1	
8				
9	105.9	74.0	64.9	74.1
10				
11		67.0		

Table H-2. Mean group concentration for weekly liver vitamin A assays (µg/gm wet liver)

Age (wks post-weaning)	Group 1	Shipment A Group 2	Shipment B Group 2	Group 3
1	16.24	8.12	0.44	0.34
2	43.67	2.50	0.28	0.20
3	34.83	1.25	0.10	0.25
4	49.12	0.42	0.02	0.08
5	24.30	0.22	0.03	0.14
6	28.40	0.26	0.00	158.15
7		0.15	0.29	
8		0.09		
9	89.56		0.58	540.18
10				
11		0.37		

Table H-3. Liquid scintillation counting mean ^{3}H-TdR activity (cpm/100 mg wet fixed lung) for duplicate 10-minute counts - Group 1

Animal No.	Postexposure time (hrs)	cpm/100 mg wet fixed lung
7674	12	8703
7690	12	2027
7679	24	21394
76101	24	1262
7684	48	2964
76106	48	5781
7695	72	1868
76110	72	2616

Table H-4. Liquid scintillation counting mean ^{3}H-TdR activity (cpm/100 mg wet fixed lung) for duplicate 10-minute counts - Group 2

Animal No.	Postexposure time (hrs)	cpm/100 mg wet fixed lung
7675	12	5837
7676	12	4186
7691	12	2098
7692	12	1153
7680	24	5863
7681	24	976
76102	24	2265
76103	24	826
7685	48	770
7686	48	1455
76107	48	1526
76108	48	2003
7696	72	809
7697	72	6288
7698	72	1194
76111	72	2305
76112	72	3292

Table H-5. Liquid scintillation counting mean ^{3}H-TdR activity (cpm/100 mg wet fixed lung) for duplicate 10-minute counts - Group 3

Animal No.	Postexposure time (hrs)	cpm/100 mg wet fixed lung
7677	12	1531
7678	12	3894
7693	12	378
7694	12	370
7682	24	7785
7683	24	6289
76104	24	899
76105	24	429
7687	48	3152
7688	48	4183
76109	48	686
7699	72	2498
76100	72	654
76113	72	2842

Table H-6. Autoradiographic counts for bronchial epithelium*

Animal No.	Group	Postexposure time (hrs)	#1	#2	#3	Average
7674**	1	12	406/800	453	412	423.7
7690**	1	12	62/800	59	34	51.7
7679**	1	24	57/800	42	35	44.7
76101**	1	24	0/800	0	0	0
7684**	1	48	5/800	0	3	2.7
76106**	1	48	14/800	14	9	12.3
7695**	1	72	1/800	1	2	1.3
76110	1	72	1/800	0	0	0.3
7675	2	12	120/800	138	67	108.3
7676	2	12	134/800	145	97	125.3
7691	2	12	0/800	0	0	0
7692	2	12	0/800	1	0	0.3
7680	2	24	16/715	6	1	4.3
7681	2	24	3/800	4	0	2.3
76102	2	24	0/800			0
76103	2	24	0/800	0	0	0
7685	2	48	0/800	1	1	0.7
7686	2	48	0/800	0	0	0
76107	2	48	0/800	0	0	0
76108	2	48	6/800	4		5.0
7696	2	72	0/800	0	0	0
7697	2	72				
7698	2	72	0/800	0	1	0.3
76111	2	72	14/800	19	0	11.0

Table H-6 (continued)

Animal No.	Group	Postexposure time (hrs)	#1	#2	#3	Average
76112	2	72	7/800	3	0	3.3
7677	3	12	47/800	38	39	41.3
7698	3	12	174/800	165	134	157.7
7693	3	12	0/800	0	0	0
7694	3	12	2/800	2	0	1.3
7682	3	24	37/800	22	9	22.7
7683	3	24	34/800	39	16	29.7
76104	3	24	0/800	1	1	0.7
76105	3	24				
7687	3	48	0/800	0	0	0
7688	3	48	27/800	23		25.0
76109	3	48	0/800	0	0	0
7699	3	72	0/800	0	0	0
76100	3	72	0/800	0	0	0
76113	3	72	4/800	2	0	2.0

*Raw data were obtained from autoradiographic counts of 3 serial sections. The total number of cells counted for a given area is the denominator reported in the column marked #1. The numerator of this column, as well as the numbers in columns 2 and 3, represents the number of labeled cells observed. An average has been calculated for labeled cells.

**Data obtained from counts of left lung diaphragmatic lobe preparations.

Table H-7. Autoradiographic counts for bronchiolar epithelium*

Animal No.	Group	Postexposure time (hrs)	#1	#2	#3	Average
7674**	1	12	341/726	361	404	368.7
7690**	1	12	15/802	43	33	30.3
7679**	1	24	60/801	44	58	54.0
76101**	1	24	2/854	0	4	2.0
7684**	1	48	4/805	8	1	4.3
76106**	1	48	7/796	4	2	4.3
7695**	1	72	0/807	0	2	0.7
76110	1	72	3/813	1	0	1.3
7675	2	12	74/826	83	38	65.0
7676	2	12	48/845	49	59	52.0
7691	2	12	1/806	0	0	0.3
7692	2	12	5/901	0	0	1.7
7680	2	24	16/715	13	1	10.0
7681	2	24	3/819	5	2	3.3
76102	2	24	0/811			0
76103	2	24	0/696	0	0	0
7685	2	48	0/801	0	0	0
7686	2	48	0/653	0	0	0
76107	2	48	0/916	0	0	0
76108	2	48	7/809	7		7.0
7696	2	72	0/754	0	0	0
7697	2	72	1/803	0	0	0.3
7698	2	72	1/711	1	0	0.7
76111	2	72	5/814	0	2	2.3

Table H-7 (continued)

Animal No.	Group	Postexposure time (hrs)	#1	#2	#3	Average
76112	2	72	6/756	2	0	2.7
7677	3	12	17/813	15	14	15.3
7698	3	12	146/814	154	129	143.0
7693	3	12	0/801	0	0	0
7694	3	12	1/801	0	0	0.3
7682	3	24	56/884	47	46	49.7
7683	3	24	47/740	43	29	39.7
76104	3	24	11/803	1	0	4.0
76105	3	24				
7687	3	48	0/767	4	0	1.3
7688	3	48	7/822	4		5.5
76109	3	48	1	2/807	1	1.3
7699	3	72	0/810	0	0	0
76100	3	72	0/844	0	0	0
76113	3	72	17/838	6	1	8.0

*Raw data were obtained from autoradiographic counts of 3 serial sections. The total number of cells counted for a given area is the denominator reported in the column marked #1. The numerator of this column, as well as the numbers in columns 2 and 3, represents the number of labeled cells observed. An average has been calculated for labeled cells.

**Data obtained from counts of left lung diaphragmatic lobe preparations.

Table H-8. Autoradiographic counts for respiratory bronchiole-alveolar areas*

Animal No.	Group	Postexposure time (hrs)	#1	#2	#3	Average
7674**	1	12	8/869	5	7	6.6
7690**	1	12	13/560	10	7	10.0
7679**	1	24	174/1398	165	108	149.0
76101**	1	24	4/832	1	4	3.0
7684**	1	48	103/1063	87	106	98.6
76106**	1	48	208/1398	173	137	172.6
7695**	1	72	76/2089	27	45	49.3
76110	1	72	16/1044	10	2	9.3
7675	2(Shipment B)	12	33/1318	23	11	22.3
7676	2(Shipment B)	12	25/1094	15	13	17.7
7691	2(Shipment A)	12	4/770	1	0	1.7
7692	2(Shipment A)	12	1/544	1	0	0.7
7680	2(Shipment B)	24	15/680	11	1	9.0
7681	2(Shipment B)	24	23/766	19	11	17.7
76102	2(Shipment A)	24	4/671			4.0
76103	2(Shipment A)	24	0/725	0	0	0
7685	2(Shipment B)	48	2/827	0	0	0.7
7686	2(Shipment B)	48	0/798	0	2	0.7
76107	2(Shipment A)	48	10/1768	9	0	6.3
76108	2(Shipment A)	48	129/1287	107		118.0
7696	2(Shipment B)	72	5/752	0	0	1.7
7697	2(Shipment B)	72	9/859	10	10	9.6
7698	2(Shipment B)	72	1/910	1		1.0

Table H-8 (continued)

Animal No.	Group	Postexposure time (hrs)	#1	#2	#3	Average
76111	2(Shipment A)	72	1/971	2	0	1.0
76112	2(Shipment A)	72	15/702			15.0
7677	3	12	10/822	11	8	9.7
7678	3	12	11/959	6	14	10.3
7693	3	12	0/682	0	0	0
7694	3	12	0/643	1	0	0.3
7682	3	24	67/844	62	57	62.0
7683	3	24	55/847	39	28	40.7
76104	3	24	7/901	7	0	4.7
76105	3	24				
7687	3	48	58/1107	19	0	25.7
7688	3	48	69/1007	57		63.0
76109	3	48	12/658	26	8	15.3
7699	3	72	0/612	0	0	0
76100	3	72	0/1014	0	0	0
76113	3	72	30/907	14	1	11.7

*Raw data were obtained from autoradiographic counts of 3 serial sections. The total number of cells counted for a given area is the denominator reported in the column marked #1. The numerator of this column, as well as the numbers in columns 2 and 3, represents the number of labeled cells observed. An average has been calculated for labeled cells.

**Data obtained from counts of left lung diaphragmatic lobe preparations.

APPENDIX I

CALCULATED DATA: MEAN LABELED INDICES FOR AUTORADIOGRAPHS, RESULTS OF TWO-WAY ANALYSIS OF VARIANCE, AND DUNNETT'S T-DISTRIBUTION

Table I-1. Group 1 mean labeling indices for light microscopic autoradiography

Animal No.	Postexposure time (hrs)	RBr-Alv	Bronchiolar	Bronchial
7674*	12	.00767	.5078	.52958
7690*	12	.01785	.03782	.06458
7679*	24	.01658	.06742	.0558
76101*	24	.0036	.00234	.0000
7684*	48	.0928	.00538	.0033
76106*	48	.1235	.00544	.0154
7695*	72	.0236	.0008	.0017
76110	72	.0089	.0016	.0004

*Data obtained from counts of left lung middle lobe preparations.

Table I-2. Group 2 mean labeling indices for light microscopic autoradiography

Animal No.	Postexposure time (hrs)	RBr-Alv	Bronchiolar	Bronchial
7675	12	.0169	.0787	.1354
7676	12	.0162	.0165	.1566
7691	12	.0022	.0004	.0000
7692	12	.0013	.0019	.0004
7680	24	.0132	.0140	.0054
7681	24	.0231	.0040	.0029
76102	24	.0060	.0000	.0000
76103	24	.0000	.0000	.0000
7685	48	.0008	.0000	.0009
7686	48	.0009	.0000	.0000
76107	48	.0036	.0000	.0000
76108	48	.0917	.0087	.0063
7696	72	.0023	.0000	.0000
7698	72	.0011	.0010	.0004
76111	72	.0010	.0028	.0138
76112	72	.0214	.0038	.0041
7697*	72	.0113	.0004	not available (section could not be counted)

*Data obtained from counts of left lung middle lobe preparations.

Table I-3. Group 3 mean labeling indices for light microscopic autoradiography

Animal No.	Postexposure time (hrs)	RBr-Alv	Bronchiolar	Bronchial
7677	12	.0118	.0188	.0516
7678	12	.0107	.1757	.1971
7693	12	.0000	.0000	.0000
7694	12	.0005	.0004	.0016
7682	24	.0735	.0562	.0284
7683	24	.0481	.0536	.0371
76104	24	.0052	.0050	.0009
76105	24		not available**	
7687	48	.0232	.0017	.0000
7688	48	.0626	.0067	.0313
76109*	48	.0232	.0018	.0000
7699	72	.0000	.0000	.0000
76100	72	.0000	.0000	.0000
76113	72	.0129	.0095	.0025

*Data obtained from counts of left lung middle lobe preparations.

**Technical problems occurred in processing of autoradiographic slide.

Table I-4. Results for two-way analysis of variance

Source of variation	df	SS	MS	F
		RBr-Alv		
Subgroups	11	616.04		
Diet	2	199.09	99.54	2.18 ($p \approx .15$)
Time	3	263.09	87.70	1.92 ($p \approx .25$)
Diet x Time	6	153.85	25.64	0.56
Within subgroups (error)	26	1188.76	45.72	
Total	37	1804.79		
		Bronchial		
Subgroups	11	1753.21		
Diet	2	241.50	120.75	1.77 ($p \approx .25$)
Time	3	1041.81	347.27	5.11 ($p < .01$)
Diet x Time	6	469.90	78.32	1.15
Within subgroups (error)	25	1698.58	67.94	
Total	36	3451.79		
		Bronchiole		
Subgroups	11	1458.46		
Diet	2	290.62	145.31	2.74 ($p \approx .10$)
Time	3	703.09	234.36	4.42 ($p < .025$)
Diet x Time	6	464.75	77.46	1.46 ($p \approx .25$)
Within subgroups (error)	26	1379.10	53.04	
Total	37	2837.56		
		Scintillation		
Subgroups	11	1.388		
Diet	2	0.736	0.37	2.04 ($p \approx .20$)
Time	3	0.048	0.16	0.09
Diet x Time	6	0.604	0.10	0.56
Within subgroups (error)	27	4.863	0.18	
Total	38	6.251		

Table I-5. Values for Dunnett's T-distribution*

Comparisons	Group 2 with Group 3	Group 1 with Group 3
RBr-Alv		
Overall	1.98	2.77 (p<.05)
12 hour	0.68	0.34
24 hour	0.09	1.58
48 hour	1.78	2.98 (p<.05)
72 hour	1.08	0.71
Bronchial		
Overall	1.39	1.87
12 hour	2.82 (p<.05)	2.69 (p<.05)
24 hour	-0.08	0.70
48 hour	0.24	0.50
72 hour	0.11	-0.16
Bronchiole		
Overall	1.44	1.51
12 hour	3.16 (p<.01)	3.11 (p<.01)
24 hour	-0.23	0.99
48 hour	0.15	0.46
72 hour	0.02	0.01
Scintillation		
Overall	1.50	2.06
12 hour	1.75	0.49
24 hour	1.08	1.25
48 hour	0.75	1.32
72 hour	0.31	0.03

*Results from calculations of a two-sided Dunnett's distribution (Dunnett, 1964) for m=2 and df=24. ($p<.05$ for $T_D>2.37$ and $p<.01$ for $T_D>3.07$)

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