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VITAMIN A STATUS, POLYBROMINATED BIPHENYL TOXICOSIS AND COMMON BILE DUCT HYPERPLASIA IN RATS

By

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ABSTRACT

VITAMIN A STATUS, POLYBROMINATED BIPHENYL TOXICOSIS AND COMMON BILE DUCT HYPERPLASIA IN RATS

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Two experiments were conducted to evaluate the interaction between vitamin A status and PBB toxicosis. In the first experiment 24 male weanling rats were allocated in a 2 x 3 factorial experiment. Diets were either deficient or adequate in vitamin A and contained 0, 10 or 100 ppm PBB. In the second experiment 36 male weanling rats were used in a similar 2 x 3 factorial design. Diets were either adequate, excessive or deficient in vitamin A and contained 0 or 100 ppm PBB.

Mortality and early appearance of clinical signs of vitamin A deficiency were seen in rats fed diets deficient in vitamin A and containing high doses of PBB. Vitamin A provided partial protection against decreased weight gain associated with PBB. The negative effect of PBB toxicosis on the thymic weight was prevented by the addition of vitamin A to the diet.

The most conspicuous gross lesion observed in these experiments was the massive enlargement of the common bile duct of rats fed a diet deficient in vitamin A and containing high doses of PBB. Histologically, this lesion consisted of extensive hyperplasia and mimicked a preneoplastic condition.

Combined effects of high doses of PBB and low vitamin A in the diet produced a significant decrease in retinol values in the sera. The interaction between a deficiency of vitamin A and PBB toxicosis affected vitamin A metabolism in the liver as manifested by the appearance of significant amounts of retinyl acetate in the profile. Vitamin A status did not influence the concentration of PBB either in the liver or the fat tissue.

Results of these experiments emphasize the importance of nutritional factors such as vitamin A in assessment of PBB toxicosis. Interaction between vitamin A deficiency and PBB toxicosis produced markedly different changes than that produced by either alone.

DEDICATED WITH LOVE TO

MY WIFE, JULIJANTI, AND MY SONS

ARIYONO AND KARYONO

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INTRODUCTION

Routine use of agricultural and industrial chemicals is now an essential ingredient in human life to improve productivity. Of the numerous chemical substances to which man is exposed, the group of chemicals loosely classified as environmental contaminants probably presents the greatest potential threat to human health. Although there are large groups of substances of diverse chemical structure that are hazardous to humans, aromatic halogenated hydrocarbons are the most significant environmental contaminants in recent years (Munro and Charbonneau, 1978). Among the more prominent of those chemicals are polychlorinated biphenyls (PCB) used in electrical transformers and capacitors, polybrominated biphenyls (PBB) used in fire retardant applications, and organochlorine insecticides such as DDT, heptachlor, aldrin, lindane, and kepone (Baker, 1977). Excessive usage and improper handling of some of those chemicals can cause a harmful effect to the environment. Those harmful effects are due to their stability to the ultraviolet rays of sunlight, stability to heat and acids, insolubility in water and because they are poorly biodegradable. These characteristics facilitate the compounds in entering the human food chain (Ware, 1978; Duffus, 1980).

Recent research in several laboratories demonstrates that nutritional status has an important influence on the pharmacotoxicological activities of chemicals which are foreign to the living system (xenobiotics)(Campbell and Hayes, 1974). The positive effect of excessive vitamin A in the diet has already been demonstrated on the toxicity of PCB, and researchers have

even found that vitamin A has chemopreventive and chemotherapeutic effects on certain carcinogens (Innami et al., 1974; Sporn, 1977). Several data also show that some xenobiotics can reduce the concentration of certain nutrients. DDT, PCB, PBB, TCDD (tetrachloro-dibenzo-p-dioxin) and chlorinated naphthalene have been demonstrated to reduce either serum or liver vitamin A (Villeneuve et al., 1971; Mangkoewidjojo, 1979; Thunberg et al., 1979; Olafson, 1947).

Vitamin A is one of the essential micronutrients usually classified as a fat soluble vitamin. Deficiency of this vitamin has been reported to have a variety of effects including disturbed normal skeletal growth, disturbed division and differentiation of epithelial cells and impairment of the visual process (Wolbach and Howe, 1925). Despite its ample sources and inexpensive price, cases of deficiency are still prevalent in impoverished areas of the world (Oomen, 1976). Even in a well developed country, the status of vitamin A varies widely among individuals. A recent study in New York City showed that the mean liver vitamin A content was 126 μg/g wet tissue with a range of 7 to 668 μg/g and a median of 66 μg/g. Average values of vitamin A reserve vary in different countries ranging from 24 μg/g for China to 191 μg/g for Scotland (Roels and Lui, 1980).

Realizing that nutritional imbalance and nutritional deficiency are still a major problem for most people, and because of the constant increase in the danger of environmental contamination, there is a growing need for research in many aspects related to the negative effect of the interaction between those two elements on human health. Although there are available data describing such interaction, those data are still very minimal compared to the importance of the issue. This experiment was conducted to provide further information concerning those interactions,

and was done by using PBB as a model for the environmental contaminant and vitamin A as a class of nutrient. The first objective of this study was to evaluate the interaction between deficiency of vitamin A and PBB toxicity. The second objective was to explore the positive effect of vitamin A on PBB toxicity, and the third objective was to supply additional information concerning the pathologic effects of PBB toxicity.

LITERATURE REVIEW

Vitamin A

Introduction

In 1913, McCollum and Davis reported the existence of an essential lipid soluble substance in food capable of promoting growth in rats. This substance was called Fat Soluble A to distinguish it from another essential nutrient they called Water Soluble B. Drummond (1920) suggested later that the Fat Soluble Factor A should be named Vitamin A.

Vitamin A is a nearly colorless compound, even though vitamin A activity was associated initially with certain yellow colored fats. The problem was resolved by Steenbock (1919) by showing that carotene (plant material) fed to rats possessed vitamin A activity. The carotenes became known as provitamin A. The provitamin role of carotene became clear after Karrer and his associates determined the chemical structure of β-carotene in 1930 and retinol in 1931 (Goodman, 1979). In 1937, Holmes and Corbert were able to crystallize pure vitamin A from fish liver. Arens and Van Dorp (1946a) succeeded in synthesizing pure vitamin A, whereas the synthesis of β-carotene was done by Milas et al. in 1950.

In the body, there are 3 active forms of vitamin A; retinol, retinal and retinoic acid. Retinol is the circulating form, which in certain tissues such as the retina, is converted to retinal by alcohol dehydrogenase (Arens and Van Dorp, 1946b; Wald and Hubbard, 1950). This reaction is reversible. Emerick et al. (1967) showed in vivo that retinol could be

oxidized further to retinoic acid. Retinoic acid could not be reconverted to retinol, but could perform some of the functions of retinol. Retinoic acid can not replace retinol as a visual pigment precursor (Dowling and Wald, 1960), and is not able to support reproduction (Thompson et al., 1964).

As mentioned earlier, a decrease of growth rate is one of the earliest and the most sensitive index of vitamin A deficiency (Corey and Hayes, 1972). Zile et al. (1977) investigated the effect of vitamin A deficiency on intestinal cell proliferation and suggested that vitamin A may play a role in the regulation of cell division. In rats under synchronous vitamin A deficiency (rats given only retinoic acid such as described by Lamb et al., 1974), growth is depressed within 1 to 2 days after the withdrawal of retinoic acid (Anzano et al., 1979).

Besides growth, vitamin A is known to affect almost every tissue in the body and to have a key role in a great variety of body functions and processes. Vitamin A is necessary for the normal function of the eyes and vision. Vitamin A deficiency was shown to be responsible for xerophthalmia (McCollum and Simmonds, 1917) and certain forms of night blindness (Fridericia and Holm, 1925). The physiologic function of vitamin A in the mechanism of the visual cycle was described in detail by Wald in 1968. It is the energy release from the stereochemical conversion of cis-retinal to trans-retinal that produces the nerve excitation resulting in visual sensation.

The other function of vitamin A is its role in maintaining the normal growth of epithelial tissues. Keratinization or atrophy of epithelial tissues is one of the more characteristic features of vitamin A deficiency (Olson, 1972). Hayes (1971) speculated that deficiency of vitamin A

produces a defect in differentiation of epithelial and mesenchymal cells, which is manifested as different pathological entities depending on the tissues or organs involved. Bernard and Halpern (1968)observed that in vitamin A-deficient rats, the loss of appetite is associated with the infiltration of keratin into the pores of the taste buds.

The minimum requirement of vitamin A in the diet for optimal growth, reproduction, tissue levels and liver storage is 4,000 IU/kg for retinyl acetate or palmitate stabilized in gelatin-coated beadlets. If β-carotene is fed in gelatin beadlets, 4-6 mg/kg is recommended (Anonymous, 1978). The maximum serum level of vitamin A in rats is about 60 µg/100 ml and is reached when liver deposition is moderate, that is about 250 µg/g liver (Muto et al., 1972). Since 1969, the daily requirement and food content of vitamin A have been based on "µg retinol equivalent", that is, retinol is expressed by weight and the carotenoids by the weight of retinol to which they would be converted in the body (Worthington-Roberts, 1981). Therefore, the following figures apply: 1 retinol equivalent = 1 µg retinol = 6 µg β-carotene = 12 µg other provitamin A carotenoids = 3.33 IU vitamin A activity from retinol = 10 IU vitamin A activity from β-carotene.

Metabolism

Dietary carotene is cleaved at the central double bond by a soluble mucosal enzyme to yield two molecules of retinaldehyde (Goodman and Olson, 1969). This retinaldehyde is then reduced to retinol by an enzyme tentatively called retinaldehyde reductase (Smith and Goodman, 1976). The enzyme has been purified from the soluble fraction of intestinal mucosa (Fidge and Goodman, 1968).

The dietary retinyl esters are hydrolyzed while in the lumen of the intestine by the action of a pancreatic retinyl ester hydrolase (Ganguly, 1969). The retinol which is absorbed or newly synthesized from carotene inside the intestinal mucosal cells is rapidly esterified with long chain saturated fatty acid. Irrespective of the type of ester fed (acetate, stearate, laurate, palmitate, linoleate), palmitate was the most abundant in the mucosa (Mahadevan et al., 1963). The retinyl ester formed is then transported into the body in association with lymph chylomicrons (Goodman et al., 1965). Small amounts of retinal formed from β-carotene are oxidized to retinoic acid, which is then transported through the portal vein in the form of its carboxylate anion bound to serum albumin (Smith and Goodman, 1976; Ganguly, 1969).

The chylomicron is cleared from triglyceride, and the chylomicron remnant that contains virtually all of the retinyl ester absorbed is removed from the circulation by the liver (Goodman et al., 1965). During the process of uptake and storage of vitamin A by the liver, some evidence suggests that hydrolysis and reesterification also occurs (Lawrence et al., 1966). In the normal liver, most of the vitamin A is stored in the parenchymal cells (Hori and Kitamura, 1972). Kupffer cells store less than 4% of the vitamin (Linder et al., 1971). Another cell type called the lipocyte (fat-storing cells) stores the vitamin in the liver in an abnormal conditions, such as administration of massive doses of the vitamin (Wake, 1974). In the liver, vitamin A is stored primarily as retinyl palmitate. The liver contains a microsomal enzyme for the formation of those esters, but the mechanism of the esterification and the source of the fatty acid used is still unknown (Futterman and Andrews, 1964). Vitamin A is mobilized from the liver to the target tissue in the form of the lipid alcohol retinol.

Accordingly, prior to the mobilization, the stored retinyl esters must be hydrolyzed to form retinol. Mahadevan et al. (1966) reported that retinyl palmitate hydrolyzing activity (RPHA) was found in the nuclear and mitochondrial-lysosome-rich fractions of rat liver homogenate, but Harrison et al. (1979) stated this enzymatic activity was not localized in any single, characterized subcellular structure. Prystowsky et al. (1979) showed that the greatest RPHA was seen in the washed nuclear fraction, and the least activity in the microsomal fraction. Retinyl acetate was not actively hydrolyzed by retinyl palmitate hydrolase. The RPHA is stimulated by both cholate and taurocholate (Harrison et al., 1979; Prystowsky et al., 1979). The retinol formed is bound to retinol binding protein (RBP), and transported via the plasma to the target organs (Kanai et al., 1968).

With the belief that the mechanism of action of retinol at the cellular level might be similar to that of steroid hormones, researchers investigated whether tissues known to be influenced by retinol contained a protein that could bind retinol specifically. Bashor et al. (1973) discovered such an intracellular binding protein (cellular retinol binding protein) in vitro. This protein does not bind retinoic acid. A protein which bound retinoic acid with high specificity was discovered in rats by Ong and Chytil (1975). The protein was called cellular retinoic acid binding protein. The hypothesis that retinol and retinoic acid act by some mechanism similar to steroid hormones by the specific interaction of these compounds with the nucleus is based on the fact that alteration in RNA synthesis is observed in vitamin A deficient animals (Tsai and Chytil, 1978). Another possibility is that the intracellular binding proteins mainly serve as intracellular transport proteins and act to transport specific retinoids in a directed

way from one locus to another within the cell (Goodman, 1980). Besides its similarity in function to steroid hormones, vitamin A also influences the synthesis of DNA, but the mechanism is still unknown (Zile et al., 1979). Some studies also show that retinol and retinoic acid have a direct role in the incorporation of mannose into glycoproteins of mammalian membranes (De Luca et al., 1979; Wolf et al., 1979).

Vitamin A (retinol and retinoic acid) is gradually catabolized by a variety of metabolic reactions that include oxidation, isomerization, esterification, decarboxylation, metabolism of the side chain and conjugation with glucuronic acid (Dunagin et al., 1976; Lippel and Olson, 1968; De Luca, 1977; Sundaresan, 1977). The metabolites are excreted either through the feces (via the bile) or through the urine. The retinoyl-β-glucuronide is partially reabsorbed from the intestine and transported back to the liver, thereby producing an enterohepatic circulation (Olson, 1967).

Pathology

Gross lesions: The most conspicuous gross lesions and clinical signs of vitamin A deficiency are the changes in the eye. Tyson and Smith (1929) described those changes as: first, a slight swelling of the eyelids accompanied by photophobia; second, a clear discharge from the eyes which sometimes becomes blood tinged; third, drying of the eyes, the lids becoming glued together and the eyeball appearing to sink into its socket. Wolback and Howe (1925) summarized the gross changes in vitamin A deficiency as humped posture, rough coat, emaciation, complete absence of body fat, encrusted eyelids and atrophy of certain organs. In germ free rats, Beaver (1961) noticed that the lymphoid tissues and pituitary gland were

hypoplastic, the liver appeared smaller than normal and varied in color and the spleen was smaller and somewhat browner than normal.

Histopathology: As mentioned earlier, the tissue changes caused by vitamin A deficiency are mainly due to the defect in differentiation of epithelial and mesenchymal cells.

Tyson and Smith (1929) stated that the earliest histological changes were seen in the salivary glands. These changes consisted of dilatation of the main duct with metaplasia of the duct epithelium to the squamous keratinizing type. Infection was always present. Beaver (1961) mentioned that such an inflammatory reaction was almost totally absent in germ free animals. Acinary atrophy and a great reduction in the number of convoluted granular tubules were prominent in this gland (Trowbridge, 1969). Anzano et al. (1980) observed the increase of interlobular space, fibrosis and the appearance of dense granules in the convoluted granular tubules.

Tyson and Smith (1929) reported that changes in the trachea and bronchi followed those in the submaxillary glands. The first change was an atrophy of the surface epithelium and was followed by a dilatation of the ducts of the glands which were filled with polymorphonuclear leukocytes. Later, there were patchy areas of squamous metaplasia and keratinization of the larynx and trachea, whereas the bronchi and bronchioles were uninvolved (Beaver, 1961). In rats under synchronous vitamin A deficiency, hyperplasia in the basal region of the tracheal epithelium was observed within 4 days of the withdrawal of the retinoic acid, and metaplasia from ciliated columnar cells to keratinized cells was observed at day 6 (Anzano et al., 1980). Wolbach and Howe (1925) and Tvedten et al. (1973) noticed squamous metaplasia and keratinization of parts of the respiratory tract such as in the scrolls of the turbinates, nasal septum and sinuses communicating with the nares.

In the reproductive organs, significant changes were seen in the testes. Tubular degeneration involving Sertoli cells and germinal epithelium were noticed. Spermatozoa were absent and the tubular epithelium was separated from the basement membrane by protein rich fluid (Beaver, 1961). The atrophy of the tubules when complete, left apparently only cells derived from sustentacular cells which frequently have two to four nuclei (Wolbach and Howe, 1925). In 1942, Wolbach and Bessey speculated that atrophy of the seminiferous tubules in vitamin A deficiency as in other epithelial organs, spared the undifferentiated cells, and so recovery was possible with replacement therapy. Injection of retinol, but not retinoic acid into the testis produced a distinct improvement in spermatogenesis in the tubules near the site of injection, but not in those away from the injection site (Anonymous, 1972). In the female reproductive tract, there was an apparent absence of the developing follicles as well as actual atresia of those already present (Beaver, 1961). The mucosa of the uterus and later that of the oviduct were replaced by stratified, keratinizing epithelium (Wolbach and Howe, 1925; Beaver, 1961). Keratinization also occurs at the epithelium of the alimentary tract, urinary tract and the eye and its glands (Wolbach and Howe, 1925).

Wolbach and Howe (1925) stated that there are no peculiar changes in the liver due to the avitaminosis A. Beaver (1961) working with germ-free rats noticed a remarkable change in the liver which varied from the presence of cytoplasmic hyaline droplets in occasional hepatic cells to actual liver necrosis. Tvedten et al. (1973) did not agree with this description and stated that this hepatic necrosis was probably due to vitamin E deficiency instead of avitaminosis A. Bieri et al. (1981) postulated that dietary retinoic acid reduces the intestinal absorption

of α-tocopherol and may also promote its oxidation; elevated intake of vitamin A (retinol ester) reduces liver storage of α-tocopherol (Pudelkiewiez et al., 1964).

Changes in the bile duct consisted of superficial squamous metaplasia and slight keratinization with associated parakeratosis. The duct system was apparently unobstructed with no evidence of bile stasis (Beaver, 1961).

In the thyroid gland, vitamin A deficiency produces keratinization of the preexisting ultimobranchial follicle (Van Dyke, 1955; Krupp, 1972). Strum (1979) suggested that this keratinization process may develop to squamous cell carcinoma. Some of the acinar cells were enlarged and contained many large cytoplasmic vacuoles (Strum, 1979).

The thymus is extremely reduced in size due to the almost complete loss of small thymic cells in prolonged vitamin A deficiency (Wolbach and Howe, 1925); other lymphoid tissues are mostly hypoplastic except Peyer's patches (Beaver, 1961).

Wolbach and Bessey (1942) observed retarded skeletal growth in an established vitamin A deficiency even though there was a normal growth of central nervous system and other soft tissues. This disproportionate growth will produce mechanical damage to the nerves and nerve fibers in various tracts of the spinal cord and in the brain. Cerebrospinal fluid pressure is also increased in this condition (Corey and Hayes, 1972).

Polybrominated Biphenyls

Introduction

Polybrominated biphenyls are organic chemicals that were widely used as fire retardants in several plastic products, especially in the electrical

industry. Their stability and flame retardant properties are characteristic for many widely used halogenated aromatic compounds (Hutzinger et al., 1976). Firemaster BP-6 is a commercial formulation of PBB containing 2% tetrabromobiphenyl, 10.6% pentabromobiphenyl, 62.8% hexabromobiphenyl, 13.8% heptabromobiphenyl and 11.4% other biphenyls (Kay, 1977).

In 1974, it was discovered that Michigan cattle were contaminated with PBB due to a chemical mix-up in the production of cattle feed. Instead of using Nutrimaster that contained magnesium oxide (feed additive), the mill accidently used Firemaster (Carter, 1976). The cows that had been fed with this heavily contaminated feed were reported to have anorexia, loss of milk production, infertility, hematocysts, elongated hooves, delayed parturition, lameness and thickening of the skin (Jackson and Halbert, 1974). As a consequence of this accident, millions of dollars worth of farm animals and products were destroyed to protect humans against exposure to PBB (Sleight, 1979).

The biological properties of PBB show a marked similarity to those reported for the structurally related PCB (Dent et al., 1976). PCB induce hepatic microsomal enzyme activities and cause hepatic porphyria (Alvares et al., 1973; Goldstein et al., 1974). PBB also have been shown to have a porphyrinogenic action (Strik, 1978) and induce microsomal enzyme activity (Dent et al., 1976). Both PCB and PBB increase liver size (Kimbrough, 1974; Sleight, 1979) and decrease liver vitamin A content (Cecil et al., 1973; Mangkoewidjojo, 1979). There is no solid evidence of mutagenicity and teratogenicity for PCB and PBB (Kay, 1977; Sleight, 1979).

Since PBB are not used as liquids, they are much less likely to contaminate the environment than PCB. PBB are also not easily leached

from thermoplastics, therefore the potential for contamination of the aquatic environment appears primarily to be related to production and formulation rather than to usage (Hesse and Powers, 1978).

Stross et al. (1981) studied the effects on human health resulting from exposure to PBB on chemical workers and farmers from the heavily contaminated areas. They concluded that the farmers had a high frequency of constitutional symptoms, hepatomegaly and skin rashes that were not commonly found in the chemical workers. Despite extensive biochemical testing, very few abnormalities were found.

Metabolism

Analysis of gut contents showed that approximately 90% of ingested PBB are absorbed (Tuey and Matthews, 1980). The mechanism of absorption has not been elucidated, but studies indicate that PBB with less bromine atoms are more readily absorbed (Fries et al., 1976). After absorption, PBB are distributed to various tissues in the body with the largest amount to the fat of liver, muscle, kidney, and adipose tissue (Fries et al., 1978).

The body attempts to modify or metabolize these xenobiotic compounds by converting the substances into more polar and less lipophilic metabolites to facilitate excretion. According to Moore et al. (1980), metabolism of PBB is facilitated when the number of parasubstitutions decrease, the number of orthosubstitutions increase, and the total number of substitutions decrease. Therefore, some of the PBB congeners are readily metabolized and excreted while others are not (Moore et al., 1980; Sipes, 1980).

As already mentioned, PBB are potent inducers of liver microsomal drug-metabolizing enzymes. The induction is classified as phenobarbital (PB)-type, 3-methylcholanthrene (MC)-type, or as mixed (PB and MC)-type

(Dent et al., 1976). The model for the MC-type induction (Cytochrome P_1-450/P-448/aryl hydrocarbon hydroxylase/AHH) mechanism is proposed by Poland et al. (1979) as follows. The inducer enters the cell (hepatocyte) and then binds to the cytosolic receptor. The complex of receptor and compound then translocates to the nucleus and initiates the transcription of the genes which code for the cytochrome P_1-450. The resulting RNA moves to the rough endoplasmic reticulum, and is translated to new enzyme proteins.

The major function of these hepatic drug metabolizing enzymes is to modify the lipid soluble compounds into more polar and thus water soluble metabolites which are readily excreted by the urine or bile (Milburn et al., 1967). Besides xenobiotic compounds, these enzymes also metabolize endogenous compounds such as steroid hormones. This enzyme complex is comprised of cytochrome P-450, phosphatidyl choline, a flavoprotein reductase, closely invested into the structural membrane (microsomal membrane), and requires oxygen and NADPH (Gillette et al., 1972). The proposed scheme of the action of this enzyme (usually also called cytochrome P-450-monooxygenase-catalyzed reaction) is illustrated by Neal (1980) as follows. The substrate combines with the oxidized form of cytochrome P-450 (Fe^{3+}) to form a substrate-cytochrome P-450 complex. Two electrons are then transferred to the substrate-cytochrome P-450 complex as it oxidizes NADPH to NADP. The reduced (Fe^{2+}) substrate-cytochrome P-450 complex combines with the molecular oxygen. In a series of steps that are not well understood, one atom of the molecular oxygen in the presence of two protons is reduced to water and the other oxygen atom is introduced into the substrate. The oxygenated substrate then dissociates, regenerating the oxidized form of cytochrome P-450. The resulting metabolite is then conjugated with UDP glucuronic acid by the action of enzyme UDP glucuronyl transferase, producing a water soluble compound (Lucier et al., 1975).

The mode of excretion of PBB was investigated by Matthews et al. (1977) using ^{14}C-hexabromobiphenyl in rats. Following intravenous administration of a single dose of this compound, only 0.6% was excreted in the feces and 0.1% in the urine. Willett and Irving (1976) reported that PBB are excreted in feces, urine, and milk, with feces as the major route. Boylan et al. (1979) demonstrated that besides biliary excretion, intestinal wall excretion also exists for hexachlorobenzene and Kepone. Evaluation for the existence of such excretion for PBB is necessary, since such nonbiliary excretion varies for different compounds (Aust and Kimbrough, 1980).

Pathology

Gross lesions: The most conspicuous and consistent finding resulting from PBB toxicosis in laboratory animals is hepatomegaly (Sleight and Sanger, 1976; Akoso, 1977; Pratt, 1979). Thyroid enlargement has also been reported in rats by Sleight et al. (1978) and Akoso (1977). Gupta and Moore (1979) observed thymic involution in rats fed FM FF-1 at high doses. Other organ weights which are reported to not change as a result of PBB administration in rats are the seminal vesicles, adrenal, testes, ovary, and brain (Harris et al., 1978; Mc Cormack and Hook, 1979; Pratt, 1979).

Histopathology: In the liver, Sleight and Sanger (1976) reported cellular swelling and vacuolation in rats ingesting feed containing 10 or 100 ppm of FM BP-6 for 30 and 60 days. The vacuoles contained lipid. Gupta and Moore (1979) and Kimbrough et al. (1978) noticed similar changes in the liver of rats given FM FF-1. Neoplastic nodules which were composed of enlarged cells with clear cytoplasm were noticed by Kimbrough et al. (1978). Kimbrough et al. (1980) also reported steatosis, megalohepatocytes, necrosis and interstitial fibrosis in the liver. Intraparenchymal bile

duct hyperplasia was observed by Akoso (1977) in rats fed iodine deficient diets containing 100 ppm FM BP-6 for 60 days. Pratt (1979) reported bile duct hyperplasia in 2 of 5 rats which were fed diets containing 1 ppm FM BP-6 for 18 months. Such focal bile duct hyperplasia was also noticed by Gupta and Moore (1979) in rats fed 22 doses of 100 mg/kg FM FF-1 at 100 days.

In the thyroid gland, Sleight et al. (1978) reported mild follicular epithelial hyperplasia with absent or poorly staining colloid in rats fed diets containing 100 ppm FM BP-6. Such changes were also observed by Gupta and Moore (1979) in rats given 22 doses of 30 mg/kg FM FF-1 at 6 months.

Gupta and Moore (1979) observed alterations of normal architecture of the thymus with marked atrophy and loss of demarcation between the cortical and medullary regions; the cortical thymocytes had disappeared, leaving only a framework of supporting connective tissue.

Interaction Between Vitamin A and Xenobiotic/Carcinogen

Influence of Xenobiotic/Carcinogen on Vitamin A

As early as 1939, Goerner and Goerner reported that some carcinogens such as dibenz(a,h)anthracene and 2-amino-6-azotoluene considerably decreased hepatic vitamin A content. Vitamin A concentrations were depressed in cattle with hyperkeratosis (x-disease) (Olafson, 1947). This was later shown to be caused by chlorinated napthhalene (Sikes and Bridges, 1956). Phillips (1963) demonstrated that DDT (1,1,1-trichloro-2,2-bis[p-chlorophenyl]ethane) decreased liver vitamin A storage in rats. In multigeneration studies with rabbits, Villeneuve et al. (1971) demonstrated that vitamin A storage in the fetal liver was decreased by the administration of high doses of Arochlor

1254 (polychlorinated biphenyls). Cecil et al. (1973) reported that Arochlor 1242 also reduced liver vitamin A storage in male and female rats or male Japanese quail. Significant reduction of hepatic vitamin A concentration has also been produced by the administration of 3-methylcholanthrene, 2 acetyl aminofluorene and phenobarbital. Benzo(a) pyrene and 4-dimethyl amino azobenzene had no influence on total hepatic vitamin A (Hauswirth and Brizuela, 1976). Thunberg et al. (1979) after exposing rats to TCDD (tetrachloro-dibenzo-p-dioxin) for 8 weeks demonstrated that the liver vitamin A storage of these rats was only 30% of the control. Mangkoewidjojo (1979) reported a dose-dependent depression of hepatic vitamin A content in rats fed 1 or more ppm of PBB. This finding was confirmed by Pratt (1979) who showed that liver vitamin A content of rats given 10 ppm PBB was decreased to one-half of the control in a month, one-third after 6 months and one-tenth after a year. Recently, Reddy and Weisberger (1980) reported that the hepatocarcinogen, 2-amino-anthraquinone, significantly decreased total liver vitamin A in both male and female rats when fed a 2% level in the diet.

Hauswirth and Brizuela (1976) and Kato et al. (1978) stated that the decline in hepatic concentration of vitamin A could be due to the increase of microsomal metabolism of this vitamin. This speculation is based on the fact that some carcinogens/xenobiotics are good inducers of microsomal enzymes, and the assumption that vitamin A might be metabolized by these enzymes. Another possibility is the alteration of glucuronidation of retinoic acid, one of the steps in the catabolism of vitamin A (Hauswirth and Brizuela, 1976; Thunberg et al., 1980). Besides the fact that some carcinogens/xenobiotics can induce the glucuronidation process (Convey, 1967; Lippel and Olson, 1968), Thunberg et al. (1980) also mentioned that

the cell will lose the inhibitory control on UDP-glucuronosyltransverase activity when vitamin A levels are decreased. Reddy and Weisburger (1980) suggested that the decrease in liver vitamin A may be due to the increased requirement of vitamin A for cellular repair mechanism during chemical carcinogenesis. This suggestion is based on the fact that the presence of atypical membranes is one of the morphological changes noted during hepatocarcinogenesis (Hruban, 1979).

Influence of Vitamin A on Xenobiotic/Carcinogen

The retinoids, a group of natural and synthetic compounds that possess vitamin A-like activity, have been shown in many studies to have chemopreventive activity. Chu and Malmgren (1965) reported that addition of 0.5% vitamin A palmitate to the diet prevented the development of carcinoma of the gastrointestinal tract of Syrian hamsters treated orally with 7,12 dimethylbenzanthracene (DMBA) or benzo(a)pyrene (BP). In 1967, Saffioti et al. found that supplementary feeding of vitamin A inhibited tracheobronchial squamous metaplasia and squamous cell tumors in hamsters given intratracheal injections of BP. Davies (1967) demonstrated that when Rhino mice were fed a diet containing 100 IU of vitamin A per gm of feed, fewer papillomas were produced than when similar mice were fed a diet deficient in vitamin A. Retinyl acetate applied to mice given DMBA as tumor initiator concomitantly with croton resin and croton oil, reduced the tumor incidence by 76% (Shamberger, 1971). In organ cultures of hamster tracheas, vitamin A inhibited squamous metaplasia and proliferative epithelial lesions induced by BP (Crocker and Sanders, 1970). Rogers et al. (1973) reported that a high level of vitamin A in the diet (sufficient to raise the vitamin content in the serum and liver and reduce growth)

did not change the incidence of colon tumors, but decreased the number of tumors per rat at the highest dose of DMH (1,2-dimethylhydrazine). Chronic dietary deficiency of vitamin A increased the incidence of tumors slightly and may have reduced the induction time. Administration of retinoic acid (3480 mg/week) for 6 weeks prior to the intratracheal administration of 3-methylcholanthrene (MC) inhibited the subsequent formation of metaplastic squamous changes and early squamous tumors in the respiratory tract of rats (Cone and Nettesheim, 1973). Nettesheim and Williams (1976) stated that the susceptibility of rats to a pulmonary carcinogen (MC) was increased in the absence of vitamin A intake even when considerable amounts (0.4 nmole/mg) of vitamin A were stored in the liver and deficiency symptoms were absent. Only moderate amounts of all-transretinyl acetate in the diet (7.6 nmole/g) were required to prevent the development of metaplastic lung nodules caused by the administration of MC. Innami et al. (1974) demonstrated that rats fed a 0.1% PCB diet supplemented with 2,400 IU of vitamin A for 6 weeks grew better than those fed a 0.1% PCB diet only. Rats given vitamin A deficient diet with 0.1% PCB showed a significant growth retardation when compared with those given a 0.1% PCB diet only.

The chemopreventive action of vitamin A mentioned previously had been achieved by the administration of the retinoids during the period of preneoplasia. Some experimental results however indicated that certain retinoids were able to suppress or reverse the development of cancer (chemotherapy). Merriman and Bertram (1979) demonstrated that complete inhibition of transformation was observed when MC-treated cell cultures were continuously treated with retinyl acetate (0.1 mg/ml) starting 7 days after MC exposure (2.5 mg/ml; 24 hr). In another experiment,

malignant transformation induced by γ-radiation of $10T^{1}/_{2}$ cells in culture was suppressed by the aromatic trimethyl methoxyphenyl analog of ethyl retinamide (Harisiadis et al., 1978). Matter et al. (1980) demonstrated that treatment with retinoids caused a reversal of skin papillomas induced by DMBA in the mouse and a reversal of hyperplasia and metaplasia induced by MC in prostate organ cultures.

Besides the well-known antitumorigenic activities of retinoids mentioned earlier, there are several isolated reports demonstrating just the opposite, that is, retinoid-induced tumor enhancement. Levij and Polliack (1968), working with DMBA in the hamster cheek pouch, demonstrated that the combination of DMBA and vitamin A induced more anaplastic and larger tumors than DMBA alone. Shamberger (1971) reported that β-carotene applied to mice initiated with DMBA, concomitantly with croton resin and croton oil, increased tumor incidence by 58%. Smith et al. (1972) demonstrated that when tissue cultures treated with MC were transferred to mice, more carcinoma were seen in those fed adequate diet supplemented with vitamin A palmitate than were seen in mice maintained on an adequate diet without supplementary vitamin A. Similar results have been achieved by Smith et al. (1975) in male Syrian hamsters given an intratracheal injection of BP. Hamsters given 2400 mg retinyl acetate had a significantly higher incidence of respiratory tract tumors than those given 100 mg retinyl acetate per week. The chemopreventive activities of retinyl acetate also could not be demonstrated in the study of Welsch et al. (1981) in murine mammary tumorogenesis; instead this vitamin A analog appeared to enhance the oncogenic process in the steroid hormone-treated GR mouse mammary cancer model.

The mechanism of the preventive action of vitamin A on carcinogenesis is still unknown. Saffioti et al. (1967) proposed that vitamin A has a

systemic inhibitory effect on the induction of squamous metaplasia as well as the development of benign and malignant squamous tumors. Vitamin A may inhibit the induction of the tumors by labilizing the lysosomes of premalignant cells (Shamberger, 1971). A somewhat contradictory hypothesis is that premature breaking of lysosomal membranes may release deoxyribonuclease, which may induce tumors by altering the genetic material of the cell (Allison and Paton, 1965). Vitamin A may play a role in the in vivo metabolism of carcinogens. Several vitamin A compounds and analogs were found to inhibit in vitro, the microsomal mixed-function oxidases that metabolize carcinogenic polycyclic hydrocarbons (Hill and Shih, 1974). Genta et al. (1974) reported that vitamin A deficiency enhanced the binding of benzopyrene to DNA of bronchial epithelium. Besides these indications that vitamin A probably influences tumor initiation, present evidence favors the mechanism whereby the vitamin counteracts the carcinogen after the initiation stage. Verma and Boutwell (1977) demonstrated that topical application of retinoic acid to mouse skin led to a dramatic inhibition of phorbol ester (12-O-tetradecanoyl-phorbol-13-acetate)-induced epidermal ornithine decarboxylase activity, an event proposed to be essential for tumor promotion. In a subsequent study with this finding, Verma et al. (1979) reported that in DMBA-initiated mice, retinoic acid treatment of the skin one hour before application of a promoter, reduced the skin tumor incidence per mouse by 75%. On the other hand, if retinoic acid were applied before, during or just after DMBA administration, and the tumor promoter was applied with the usual manner, no reduction in tumor incidence occurred.

Todaro et al. (1978) demonstrated that retinoids are able to block phenotypic cell transformation produced by sarcoma growth factors (SGF),

an ultimate carcinogen. SGF are polypeptides that directly affect cell transformation as shown by morphological changes and altered growth patterns in monolayer cultures (Sporn and Newton, 1979).

MATERIALS AND METHODS
(Exp. I)

Experimental Design

Twenty-four male weanling Sprague-Dawley rats[a], initially weighing 80 g to 95 g were used. All rats were in good general condition at the start of the experiment.

The rats were divided randomly into 2 groups of 12 and fed diets either adequate or deficient in vitamin A. Each group was divided into 3 subgroups of 4 rats each and given either 0, 10 or 100 ppm FM BP-6. The experimental design is illustrated in Table 1.

Table 1. Experimental design of dietary treatment with PBB in rats fed vitamin A adequate or vitamin A deficient diet.

Vitamin A \ PBB	0 ppm	10 ppm	100 ppm
Normal	4	4	4
Deficient	4	4	4

The rats were housed 2 to a cage in metal wire top plastic cages and the bedding was changed once a week. Room temperature was maintained

[a]Spartan Research Animals, Haslett, MI.

at 22.2 C. Lights were controlled automatically to allow 12 hours of darkness.

Feeding Practices

During 3 days of acclimation the rats were fed ground commercial diet[b] and tap water was provided ad libitum.

The basal diet for the rats was a commercial vitamin A deficient diet[c]. The composition of this diet was:

Salt Mixture No. 2	4%
Dried Yeast (Vitamin D)	8%
Starch	65%
Vegetable Oil	5%
Vitamin Free Casein	18%
Viosterol	0.5 gm/45.5 kg

Analysis in our laboratory showed that the basal diet contained 2120 IU/kg feed compared to the commercial diet for rats that contained 7630 IU/kg feed.

The feed for the group with adequate vitamin A was provided by adding 3000 IU vitamin A palmitate[d]/kg feed to the basal diet.

Each subgroup of rats fed either an adequate or deficient vitamin A diet was given either 0, 10 or 100 ppm PBB[e].

Cotton seed oil was used as the vehicle for the addition of vitamin A and PBB to the basal diet. The amount of the cotton seed oil added to the feed was 2% of the whole diet. Diets were mixed at the beginning of the experiment and refrigerated at 4 C.

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[b]Wayne Lab Blox, Allied Mills, Chicago, Illinois.

[c]Vitamin A Test Diet Rat, Nutritional Biochemicals, Cleveland, Ohio.

[d]United States Biochemicals Co., Cleveland, Ohio.

[e]Firemaster BP-6, Michigan Chemical Co., St. Louis, MI.

Feed was provided in porcelain containers with stainless steel tops. Drinking water was available ad libitum in inverted bottles with rubber stoppers and stainless steel sipper tubes. The water was changed every 3 days.

Feed consumption and body weights were recorded every 3 days, and rats were observed daily for clinical signs of disease.

Laboratory Investigation Procedures

Collection and Processing of Tissues

The experiment was terminated on day 82 and the rats were killed after feed was withheld overnight. The final body weights were recorded prior to necropsy and the rats were killed with carbon dioxide. Blood was obtained from the heart while the rat was anesthetized. Serum was removed after coagulation and centrifugation and was stored at 4 C for later chemical analyses.

Necropsy was performed soon after the animal was killed. All tissues were examined grossly and liver and testes were weighed with a toploading balance[f]. The thyroid gland was weighed on an analytical balance[g] after being fixed with 10% neutral buffered formalin for 24 hours.

Tissues for histological examination were preserved in 10% neutral buffered formalin. Tissues collected included trachea, lung, heart, spleen, liver, kidneys, stomach, intestine, skeletal muscle, thyroid, pituitary gland, adrenal gland, salivary gland, eye, skin, urinary bladder,

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[f]Mettler Series P, Model 163 (Readability: 0.01 g), Mettler Instrument Corp., Highstown, New Jersey.

[g]Model H-15 (Readability: 0.0001 g), Mettler Instrument Corp., Highstown, New Jersey.

thymus, pancreas, testes and brain. Liver tissues for PBB and vitamin A analyses were wrapped with aluminum foil and saved at -70 C for later analysis.

Formalin-fixed tissues were trimmed, processed in an Autotechnicon[h] and embedded in paraffin. The tissues were then cut with a microtome at 5 μ and stained with hematoxylin and eosin.

Vitamin A Analysis

The determination of vitamin A in the serum, liver tissues and feed was done in the Clinical Nutrition Laboratory, Department of Large Animal Surgery and Medicine, Michigan State University. Vitamin A was quantitated by the method established by Stowe (1982) as a modification of the high performance liquid chromatography procedure described by Dennison and Kirk (1977).

In preparation for vitamin A quantitation in the serum, 0.5 ml of serum was mixed with 0.5 ml absolute ethanol and vortexed for 5 seconds to form a suspension of the denaturated protein. One milliliter of hexane (68 to 69 C boiling point) was added to the mixture which was then vortexed for 1 minute and centrifuged at 3000 rpm for 10 minutes. The hexane supernatant was removed and passed through a 0.45 μm Millipore filter[i] in a Swinny-type filter holder. One hundred microliters of aliquot was injected into the chromatographic system[j]. Separation was isocratic in a Microporasil column (3.9 mm ID x 30 cm long) with a 60:40 mixture of degassed and

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[h]Lustomatic, Model 166, Fischer Scientific Co., Pittsburgh, Pennsylvania.

[i]Millipore, Corp., Bedford, Massachusetts.

[j]Waters Associates, Inc., Milford, Massachusetts.

filtered hexane[k] and the mixture was pumped through the HPLC unit at 2.5 ml/minute at 63.4 kg/cm^2. Forms of vitamin A were detected with a 35 µl flow cell in a spectrofluorometer[1] set at 330 and 470 nm for the excitation and emission wave lengths, respectively.

Liver vitamin A content was assayed by the following procedure. To determine the dry weight of the liver, 2 grams of liver tissue were placed in an aluminum pan and dried in an oven at 56 C. The dried liver tissues were weighed after 24 hours in the oven. For the quantitation of the vitamin A content, 1 gram of liver tissue was placed in a large tube and distilled water was added up to 5 ml. The mixture was then homogenized[m] and from this homogenate, 0.5 ml was pipetted into a disposable test tube. An equal amount of absolute ethanol was added and then the mixture was vortexed for 5 seconds. For the extraction, 5 ml hexane was used and the subsequent steps were similar to those used for serum samples. Vitamin A content per gram of liver tissue was calculated from the value derived from the chromatogram, and considering the tissue homogenate used during the assay and the dry weight of the liver tissues.

In preparation for vitamin A analysis of the feed, 0.2 grams of feed were homogenized with 0.8 ml of distilled water and 1 gram of ascorbic acid. The homogenate was then saponified with 2 ml fresh 60% KOH and 2 ml absolute ethanol. The mixture was vortexed for 5 seconds and then put into a 75 C water bath for 15 minutes. Extraction of the mixture was done by adding 2 ml hexane after the saponified mixture was cooled at room temperature for

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[k] Burdick and Jackson Laboratories, Inc., Muskegon, Michigan.

[1] Aminco-Bowman Spectrophotofluorometer, Silver Spring, Maryland.

[m] Polytron Homogenizer, Brinkman Instruments, Westburn, New York.

10 minutes. The remaining steps of the assay were similar to those used for serum samples or liver tissues.

Polybrominated Biphenyl Analysis

Analysis for the PBB concentration in the liver was done by the following procedure. A 0.5 g of sample was washed with petroleum ether and ground together with washed ignited sand[n]. The mixture was dehydrated by adding 10 to 20 g of granular anhydrous sodium sulfate[n]. Fifteen milliliters of distilled hexane[o] was added and the mixture was brought to boil over an 80 C water bath. The contents were filtered into a 100 ml volumetric flask. The addition of hexane and filtration was repeated 3 times. Hexane was added to bring the volume up to 100 ml. Two aliquots of 20 ml each were separated and each was condensed to approximately 0.5 ml by evaporation[p]. The first aliquot was dried in a prewashed aluminum pan by evaporation and then weighed to determine the lipid weight.

Acetone-prewashed columns[q] were filled with activated magnesium silicate. The tapered end was plugged with a small amount of glass wool to hold the magnesium silicate. A small amount of granular anhydrous sodium sulfate was added to the top, the contents of the column washed with 5 ml of glass-distilled hexane and the washing discharged. The previously condensed sample was transferred into the column and repeatedly

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[n]Mallinckrodt, Inc., Paris, Kentucky.

[o]J.T. Baker Chemical Co., Phillipsburg, New Jersey.

[p]N-Evap, Model III, Meyer Organomation Assoc., Inc., Shrewsbury, Massachusetts.

[q]Chromaflex, 200 mm x 7 mm ID.

rinsed with hexane. The eluate was condensed to 0.5 ml and then brought up to 2 ml with the addition of iso-octane[r].

Two microliters of the sample eluant was injected into the gas chromatograph[s]. The gas chromatograph was equipped with an electron capture detector[s] and operated with an injector temperature of 280 C. The column temperature was 250 C and the detector temperature was 310 C. The carrier was gaseous nitrogen at a flow rate of 30 ml/min. The result was compared to a standard sample containing a 0.05 or 0.1 µg of PBB. Normal calf liver tissue was used to control the accuracy of the extraction procedure. The result was expressed as ppm of PBB on a fat basis.

Statistical Evaluation

The data were analyzed by analysis of variance. Specific tests for multiple comparison of means such as the Dunnett test or post data contrast Scheffe were used. The predicting interaction of the two factors with unbalanced data was calculated by using the method described by Federer-Zelen (Gill, 1978).

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[r]Burdick and Jackson Laboratories, Inc., Muskegon, Michigan.

[s]G C Model 3700, Varian Instrument Division, Palo Alto, California.

RESULTS
(Exp. I)

Clinical Signs

Clinical signs of vitamin A deficiency were seen by day 69 in rats fed vitamin A deficient diet containing 100 ppm PBB. Those signs included porphyrin accumulation around the eye, accumulation of excessive watery exudate especially at the medial canthus and after 3 to 4 days, xerophthalmia. The eyes were almost completely closed by a week after the appearance of the previous signs. One rat in this subgroup died on day 82. Similar signs of deficiency of vitamin A were seen by day 82 in rats fed the diet adequate in vitamin A and containing 100 ppm PBB.

Feed Intake

The daily feed intake was normal in subgroups fed diets adequate in vitamin A, deficient in vitamin A, adequate in vitamin A plus 10 ppm PBB or deficient in vitamin A plus 10 ppm PBB. Feed consumption increased steadily from the start of the experiment, reached a peak of 20 g per day on day 36 and remained relatively constant until the experiment was terminated. Feed consumption of rats in subgroups fed adequate vitamin A plus 100 ppm PBB or diet deficient in vitamin A plus 100 ppm PBB reached a peak of 16 g per day on day 24, plateaued until day 60 and dropped steadily until intake was below 10 g per day at the end of the experiment (Fig. 1).

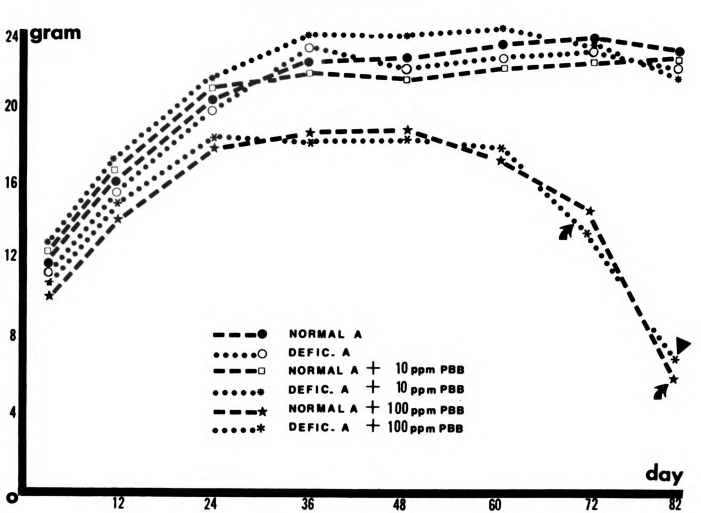

Figure 1. Means of daily feed intake of rats fed diets adequate or deficient in vitamin A and with or without PBB. Arrows indicate the days of appearance of the clinical signs in some subgroups.

Body Weight

Weight gain of rats in all of the subgroups was parallel to the feed intake. The body weight of rats fed diets adequate in vitamin A, deficient in vitamin A, adequate in vitamin A plus 10 ppm PBB or deficient in vitamin A plus 10 ppm PBB steadily increased reaching 450 g at the end of the experiment. The body weight of rats fed adequate vitamin A plus 100 ppm PBB or diet deficient in vitamin A plus 100 ppm PBB reached a peak of 325 g on day 48 and then steadily declined to 275 g when the experiment was terminated (Fig. 2).

Organ Weights

Organ to body weight ratios are shown in Table 2. Analysis of data relating to liver weight to body weight ratios indicated an interaction between vitamin A status and PBB ($p<0.1$). Such interaction was proved not significant in relation to testicle and thyroid weights, although the data show a trend of increasing weight of both organs in relation to the increase of concentration of vitamin A and PBB in the diet. The testicle and thyroid weights of rats fed a vitamin A adequate or vitamin A deficient diet with or without PBB were not significantly different from each other. Dietary exposure to PBB caused a significant increase in the ratio of thyroid weight to body weight of rats given either an adequate or deficient vitamin A diet ($p<0.05$).

Laboratory Investigation

Serum Vitamin A

Results of serum vitamin A determinations are given in Table 3. Analysis of data using Federer-Zelen methods for a factorial experiment

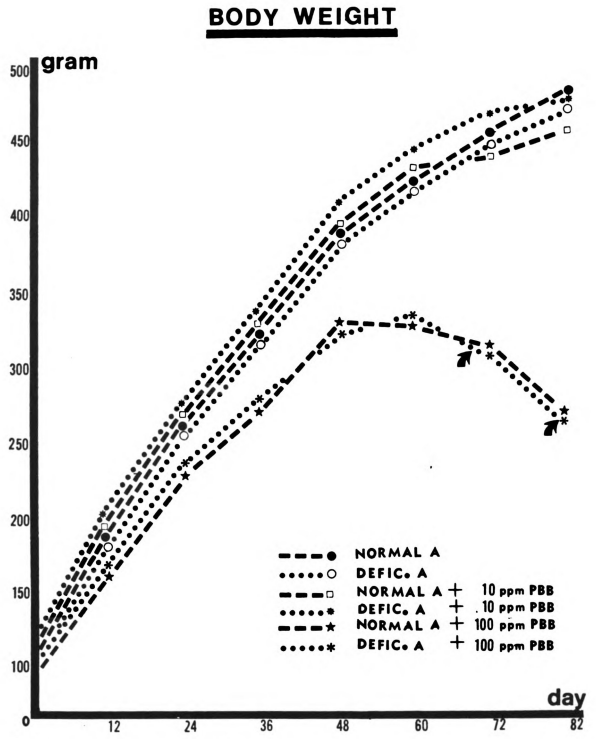

Figure 2. Means of body weight of rats fed diets adequate or deficient in vitamin A and with or without PBB. Arrows indicate the day of appearance of the clinical signs of some subgroups.

Table 2. Organ weight to body weight ratios of rats fed vitamin A adequate or vitamin A deficient diets containing 0, 10 or 100 ppm PBB for 82 days.

Modification of Diet	PBB (ppm)	Liver (g/100 g BW)	Testicle (g/100 g BW)	Thyroid (mg/100 g BW)
Vitamin A Adequate	0	3.10±0.18	1.45±0.06	6.90±1.26
	10	4.73±0.42	1.43±0.05	14.35±3.57
	100	9.98±1.53	2.10±0.35	16.67±1.98
Vitamin A Deficient	0	2.98±0.31	1.40±0.12	7.15±1.90
	10	4.28±0.44	1.45±0.13	12.30±1.76
	100	7.97±0.55	1.96±0.22	13.18±0.90

Data are expressed as mean ± SD.

Table 3. Vitamin A concentrations in sera (ng/ml) from rats fed vitamin A adequate or vitamin A deficient diets containing 0, 10 or 100 ppm PBB for 82 days.

Vitamin A \ PBB	0 ppm	10 ppm	100 ppm
Normal			
Retinyl Palmitate	58.45±19.18	39.15± 7.57	44.58±12.79
Retinol	159.33±66.28	79.98±17.71	8.95± 3.87
Total	217.78±83.87	119.20±22.70	53.53±14.72
Deficient			
Retinyl Palmitate	36.00± 4.29	29.92± 7.78	40.58± 6.49
Retinol	38.89± 8.76	50.05± 5.96	11.68± 3.85
Total	75.66±12.11	79.81±12.60	52.26± 9.18

Data are expressed as mean ± SD.

with two factors and unbalanced data shows that there is a significant interaction between status of vitamin A and PBB toxicity in relation to the total vitamin A concentration in the sera ($p<0.01$). The data also show that the value of retinyl palmitate fraction is almost constant from subgroup to subgroup. The difference in total vitamin A concentration between treatment combinations is caused by the difference in the retinol values. The combined effect of a high concentration of PBB and low concentration of vitamin A in the diet produced a significant decrease in retinol concentration in the sera.

Liver Vitamin A

The profile of vitamin A in the liver is illustrated in Table 4. The data show that there is no interaction between vitamin A status and PBB toxicity in relation to the total vitamin A in the liver. Values for total vitamin A in some subgroups are distorted by the appearance of significant amounts of retinyl acetate in the profile. In normal rat liver, retinyl acetate fraction usually is undetected. The data also show that there are significant differences in the retinyl acetate values between subgroups given PBB and subgroups without PBB ($p<0.05$ to $p<0.001$). The retinol fraction was undetectable in almost all rat livers in this experiment; only 2 rats in the control group had very small amounts of retinol.

Polybrominated Biphenyl Residue in the Liver

The concentration of PBB in the liver is shown in Table 5. Analysis of data indicated that there is no significant interaction between status of vitamin A and PBB toxicity in relation to the residue of the PBB in the liver.

Table 4. Vitamin A concentration in livers (μg/g) from rats fed vitamin A adequate or vitamin A deficient diets containing 0, 10 or 100 ppm PBB for 82 days.

PBB / Vitamin A	0 ppm	10 ppm	100 ppm
Normal			
Retinyl Palmitate	3.19±0.88	2.65±1.81	4.27±1.06
Retinyl Acetate	0.99±0.58	6.82±1.06	2.40±1.44
Total	5.15±1.17	9.53±1.22	6.78±1.98
Deficient			
Retinyl Palmitate	3.02±0.73	2.61±0.75	4.18±1.60
Retinyl Acetate	1.93±0.50	3.99±1.40	4.72±0.73
Total	5.48±1.22	6.55±1.83	8.90±0.72

Data are expressed as mean ± SD.

Table 5. PBB concentration in the fat of liver (ppm) from rats fed vitamin A adequate or vitamin A deficient diets containing 0, 10 or 100 ppm PBB for 82 days.

Vitamin A \ PBB	0 ppm	10 ppm	100 ppm
Normal	6.3±2.9	433.3±81.3	5795.0± 950.5
Deficient	2.5±1.2	244.3±44.2	4799.5±1929.8

Data are expressed as mean ± SD.

Pathology

Gross Lesions

On gross examination, in addition to the consistent lesions in the eyes associated with vitamin A deficiency, significant and noticeable changes were seen in the bile duct. The most severe lesions in the bile duct were seen in the rat that died on the last day of the experiment, but all rats in the two subgroups given high doses of PBB had similar lesions. Changes in the bile duct consisted of severe dilatation and thickening of the wall. In normal rats, the diameter of the duct is about 2 mm, whereas in affected rats the diameter was more than 1 cm or more than 5 times the normal size. Affected ducts were full of fluid and tissue debris (Fig. 3).

Livers of rats from the subgroups that were given PBB in the diet were enlarged and had a slight yellowish discoloration. Significant enlargement of the thyroid glands was also noticed in these subgroups.

Some of the rats, especially in the subgroup fed diet deficient in vitamin A and with high doses of PBB, had inflammatory processes in the lung and epididymis.

Histopathology

Bile duct. Rats in subgroups with adequate or deficient vitamin A without PBB did not have any changes in the bile duct (Figs. 4, 5 and 6). Slight changes were observed in the ducts of rats in the subgroup with adequate vitamin A and 10 ppm PBB, consisting of small areas of metaplasia of the surface epithelium (Figs. 7 and 8). Rats in the subgroup with diet deficient in vitamin A and containing 10 ppm PBB had more metaplasia and slight hyperplasia at the surface epithelium of the duct (Fig. 9). Bile

ducts of the rats in subgroups fed diets adequate or deficient in vitamin A with 100 ppm PBB had extensive metaplasia, keratinization, hyperplasia and dysplasia. Mitotic figures were numerous, and some were found at the surface epithelium far away from the basal membrane. These changes mimicked a preneoplastic condition. In certain areas, the glandular proliferation in the wall of the extraparenchymal bile duct had projected into the hepatic parenchyma. The lumen of the bile duct contained considerable cell debris (Figs. 10 and 11).

Liver. Livers of rats fed adequate or deficient vitamin A without PBB were essentially normal. Rats fed diets adequate or deficient in vitamin A with 10 ppm PBB had swollen hepatocytes and vacuolation of the cytoplasm. Extensive hepatic changes were seen in rats in subgroups given diet adequate or deficient in vitamin A with 100 ppm PBB. In addition to vacuolation of the hepatocytes, prominent and diffuse proliferation of the intraparenchymal bile ducts were noticed, especially at the area close to the trigona. The radial structural arrangement of the hepatic cords was distorted, and some hepatic cells were isolated because of extensive proliferation of the surrounding bile ducts. In some instances, inflammatory cells had diffusely infiltrated into liver parenchyma (Figs. 12 and 13).

Salivary gland. Changes in the salivary glands were prominent in rats in subgroups fed diet adequate or deficient in vitamin A and with high doses of PBB. Metaplasia and keratinization were extensive at the epithelial lining of the large excretory ducts. These ducts were surrounded by proliferation of numerous smaller excretory ducts. Inflammatory cells were also seen in some salivary glands, especially inside the lumen of the large excretory duct (Fig. 14). Salivary glands from rats in the other subgroups were normal.

Thymus. Significant depletion of cortical lymphocytes were noticed in the thymus of rats fed diet either adequate or deficient in vitamin A with 100 ppm PBB (Fig. 15).

Thyroid gland. Thyroid glands of rats fed diet either adequate or deficient in vitamin A were normal. The thyroid follicles of rats in subgroups given PBB were increased in number, the lumina were smaller and the height of the individual cells lining the follicles was increased. The colloid inside the follicles was scanty or nearly absent (Figs. 16 and 17).

Testicle. Rats in the subgroups fed diet with 100 ppm PBB had some atrophy of the seminiferous tubules. This atrophic process was especially prominent in the rat that died at the end of the experiment. Very small numbers of spermatozoa were noticed inside those atrophic seminiferous tubules, and in some tubules spermatozoa were absent. The epididymis contained small numbers of mature spermatozoa and some large rounded cells with karyorhextic nuclei. Those cells were probably germ cells that failed to mature inside the seminiferous tubules (Figs. 18 and 19). An inflammatory process was in the epididymis of certain rats in both of the subgroups which were given high doses of PBB.

Figure 3. Photograph of abdominal organ of a rat fed diet deficient in vitamin A and 100 ppm PBB. Notice the massive enlargement of the common bile duct and the focal thickening of its wall (arrow).

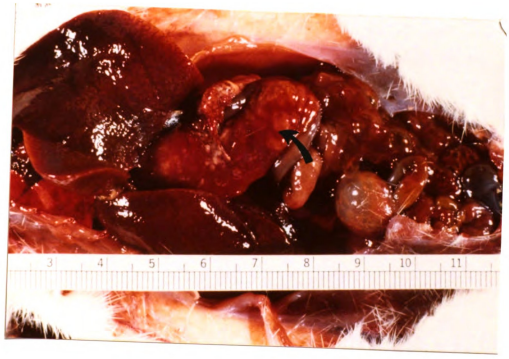

Figure 3

Figure 4. Photomicrograph of an extraparenchymal bile duct of a rat fed diet adequate in vitamin A. Notice the single layer of epithelium lining the duct and the scarcity of the mucous glands. (H & E stain, 160X)

Figure 5. Photomicrograph of an extraparenchymal bile duct of a rat fed diet adequate in vitamin A. Notice the columnar type of epithelium lining the duct. (H & E stain, 400X)

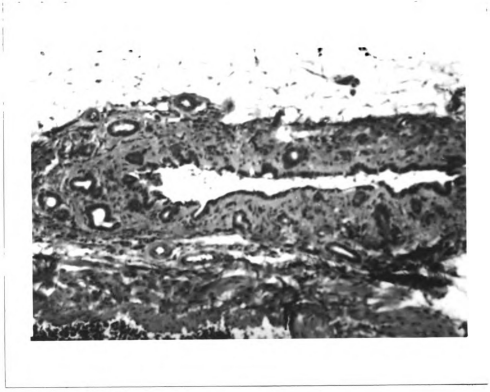

Figure 4

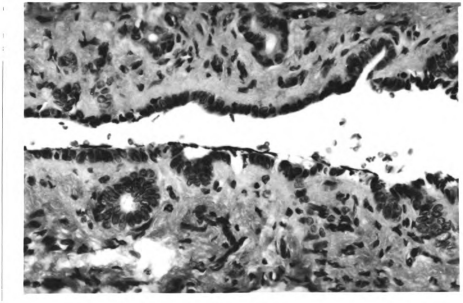

Figure 5

Figure 6. Photomicrograph of an extraparenchymal bile duct of a rat fed diet deficient in vitamin A. Notice the epithelium lining the duct is normal. (H & E stain, 160X)

Figure 7. Photomicrograph of an extraparenchymal bile duct of a rat fed diet adequate in vitamin A and 10 ppm PBB. Notice the metaplastic process in the epithelium lining the duct, but the mucous glands are scarce. (H & E stain, 400X)

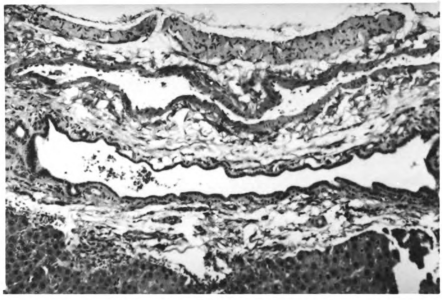

Figure 6

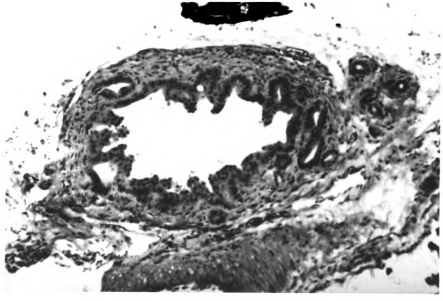

Figure 7

Figure 8. Photomicrograph of an extraparenchymal bile duct of a rat fed diet adequate in vitamin A and 10 ppm PBB. Notice the several layers of epithelium lining the duct and the beginning of keratinization. (H & E stain, 400X)

Figure 9. Photomicrograph of an extraparenchymal bile duct of a rat fed diet deficient in vitamin A and 10 ppm PBB. Notice keratinization of the surface epithelium lining the duct and the significant increase of the mucous glands. (H & E stain, 160X)

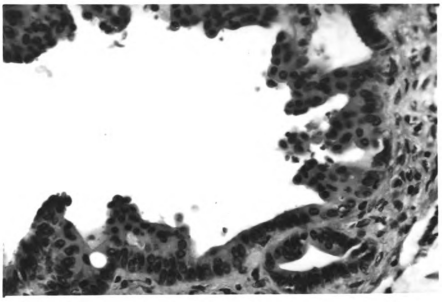

Figure 8

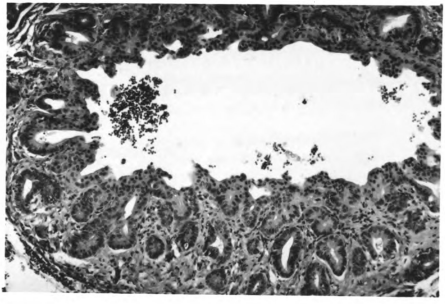

Figure 9

Figure 10. Photomicrograph of an extraparenchymal bile duct of a rat fed a diet deficient in vitamin A with 100 ppm PBB. Notice extensive keratinization and hyperplasia of the surface epithelium and massive increase of the mucous glands. (H & E stain, 64X)

Figure 11. Higher power of Figure 10. Notice the numerous mitotic figures close to the luminal surface of the glands. (H & E stain, 160X)

Figure 10

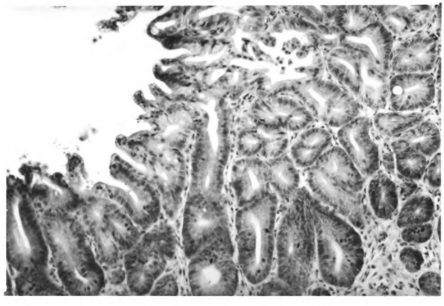

Figure 11

Figure 12. Photomicrograph of a liver of a rat fed diet deficient in vitamin A and 100 ppm PBB. Notice the extensive hyperplasia of the intraparenchymal bile ducts. (H & E stain, 400X)

Figure 13. Photomicrograph of the liver of the rat in the subgroup fed diet deficient in vitamin A and 100 ppm PBB that died at the end of the experiment. Notice the extensive hyperplasia of the intraparenchymal bile ducts which have infiltrated the liver stroma. (H & E stain, 160X)

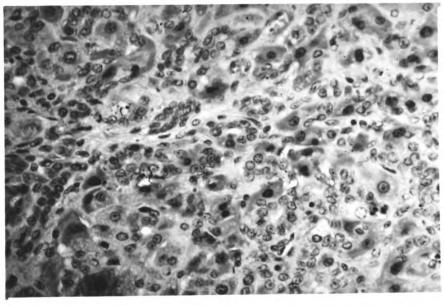

Figure 12

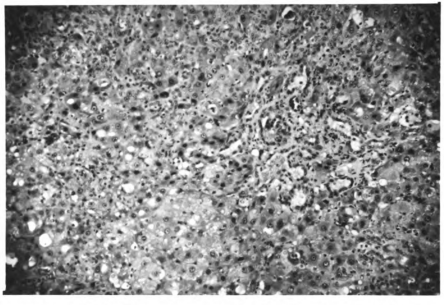

Figure 13

Figure 14. Photomicrograph of a salivary gland of a rat fed diet deficient in vitamin A and 100 ppm PBB. Notice the keratinization of the surface epithelium of the larger duct and extensive proliferation of the smaller ducts. (H & E stain, 160X)

Figure 15. Photomicrograph of a thymus of a rat fed diet deficient in vitamin A and 100 ppm PBB. Notice the marked depletion of the population of cortical lymphocytes. (H & E stain, 64X)

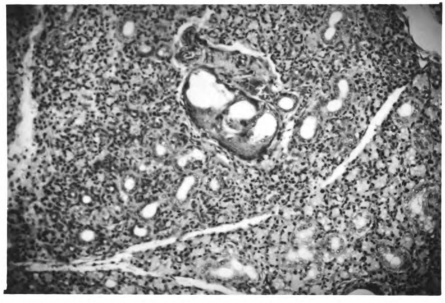

Figure 14

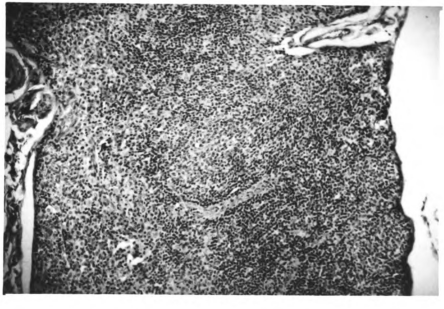

Figure 15

Figure 16. Photomicrograph of a thyroid gland of a rat fed diet deficient in vitamin A and 100 ppm PBB. Notice the hyperplasia of the cells lining the follicles and the absence of colloid inside the follicles. (H & E stain, 160X)

Figure 17. Photomicrograph of a thyroid gland of a rat fed diet deficient in vitamin A and 100 ppm PBB. Notice the keratinization of an ultimobranchial follicle. (H & E stain, 400X)

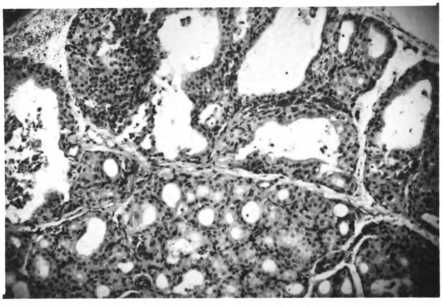

Figure 16

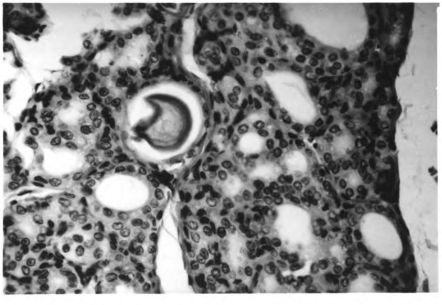

Figure 17

Figure 18. Photomicrograph of a testicle of a rat fed diet deficient in vitamin A and 100 ppm PBB. Notice the atrophy of some of the seminiferous tubules and the absence of spermatogenesis in those tubules. (H & E stain, 160X)

Figure 19. Photomicrograph of an epididymis of a rat fed diet deficient in vitamin A and 100 ppm PBB. Notice the scarcity of mature spermatozoa inside the duct and the appearance of numerous large rounded cells with karyopyknotic and karyorrhexic nuclei. (H & E stain, 160X)

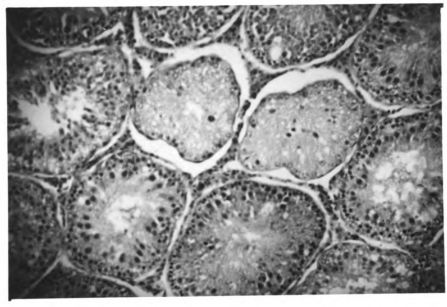

Figure 18

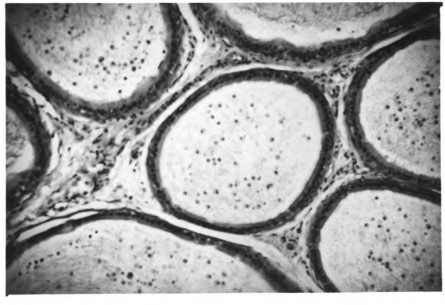

Figure 19

MATERIALS AND METHODS
(Exp. II)

Experimental Design

Thirty-six male weanling Sprague-Dawley rats[a] initially weighing 80 g to 90 g were used. All rats were in good general condition at the start of the experiment.

Rats were divided randomly into 2 groups of 18 and given either 0 ppm or 100 ppm PBB. Each group was divided into 3 subgroups of 6 rats each and given diet either deficient, adequate or excess in vitamin A. The experimental design is illustrated in Table 1.

Table 1. Experimental design of dietary treatment with PBB in rats fed diet deficient, adequate or excess in vitamin A.

PBB \ Vitamin A	Normal	Excess	Deficient
0 ppm	6	6	6
100 ppm	6	6	6

[a]Spartan Research Animals, Haslett, Michigan.

Feeding Practices

The basal vitamin A deficient diet was the same as used in Exp. I[b]. For the subgroup fed adequate vitamin A, the feed was prepared by adding 3000 IU vitamin A palmitate[c]/kg feed to the basal diet. Feed for the subgroups given excessive vitamin A was prepared by adding 30,000 IU vitamin A palmitate/kg feed. The technique and procedure for mixing the basal diet, PBB and vitamin A were basically the same as that in Exp. I. In this experiment the mixing of the feed was done on day 1 and day 40 of the experiment.

Laboratory Investigation Procedures

The experiment was terminated on day 66 and the rats were killed after feed was withheld overnight. The final body weights were recorded prior to necropsy and the rats were killed with ether. The technique and procedure for collecting serum, tissue samples and for analysis of vitamin A and PBB were the same as that in Exp. I.

Small pieces of bile duct were sliced into approximately 2 mm blocks, fixed in Karnovsky's fixative and stored at 4 C prior to further preparation for ultrastructural examination.

Transmission Electron Microscopy

Karnovsky's-fixed pieces of bile duct were cut into approximately 0.5 to 0.1 mm^3 blocks and were then washed into Zetterqvist's solution at pH 7.4. The tissue was then frost-fixed in 1% osmium tetroxide in Zetterqvist's

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[b]United States Biochemical Co., Cleveland, Ohio.

[c]United States Biochemical Co., Cleveland, Ohio.

fixative. The tissue was transferred to propylene oxide after dehydration with graded alcohols and subsequently embedded into a mixture of Epon and Araldite.

The embedded tissue was cut with a glass knife on an ultramicrotome[d]. For tissue-lesion orientation a 1 μ semithin section was stained with toluidine blue and observed by light microscopy. Thin sections, approximately 900 Å thick, were made and stained with uranyl acetate and lead citrate. The sections were observed by using an electron microscope[e].

Isolation of Microsomes

Microsomal isolation was done in the Toxicology Laboratory, Department of Biochemistry, Michigan State University. A portion of liver was placed in cold 1.5% potassium chloride containing 0.2% nicotinamide. The liver was homogenized, and the homogenate was centrifuged at 10,000 xg for 20 minutes. The supernatant was recentrifuged at 105,000 xg for 90 minutes. The microsomes were washed with a 0.3% sucrose solution containing 0.1 M sodium pyrophosphate to remove ribosomes and to absorb protein (Welton and Aust, 1974). The amount of microsomal protein and cytochrome P-450 were then determined.

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[d]LKB Ultratome III[R], Instrument Group 8800, Sweden.

[e]CEM 952, Carl Zeiss, Germany.

RESULTS
(Exp. II)

Clinical Signs

Clinical signs of vitamin A deficiency in the eyes appeared on day 55 in the subgroup of rats fed vitamin A deficient diet and 100 ppm PBB. Two rats from this subgroup died on days 64 and 65, and the experiment was terminated on day 66. Some rats in the subgroup fed an adequate vitamin A diet and 100 ppm PBB started to show signs of deficiency on day 65. Rats in the subgroup fed diet with excess vitamin A and 100 ppm PBB did not have such signs when the experiment was terminated.

Feed Intake

The daily feed intake of rats in the group fed 100 ppm PBB was significantly lower than the group without PBB by day 18 ($p<0.05$). This difference grew wider throughout the course of the experiment. The feed intake of rats in the subgroup fed diet deficient in vitamin A and 100 ppm PBB dropped steadily and was significantly different from that of the subgroup fed vitamin A excess diet and 100 ppm PBB by day 54 ($p<0.05$) (Fig. 1).

Body Weight

The weight gain of the rats in all the subgroups was parallel to the feed intake. The body weight of rats in the group fed 100 ppm PBB was already significantly lower than the group without PBB by day 18

FEED INTAKE

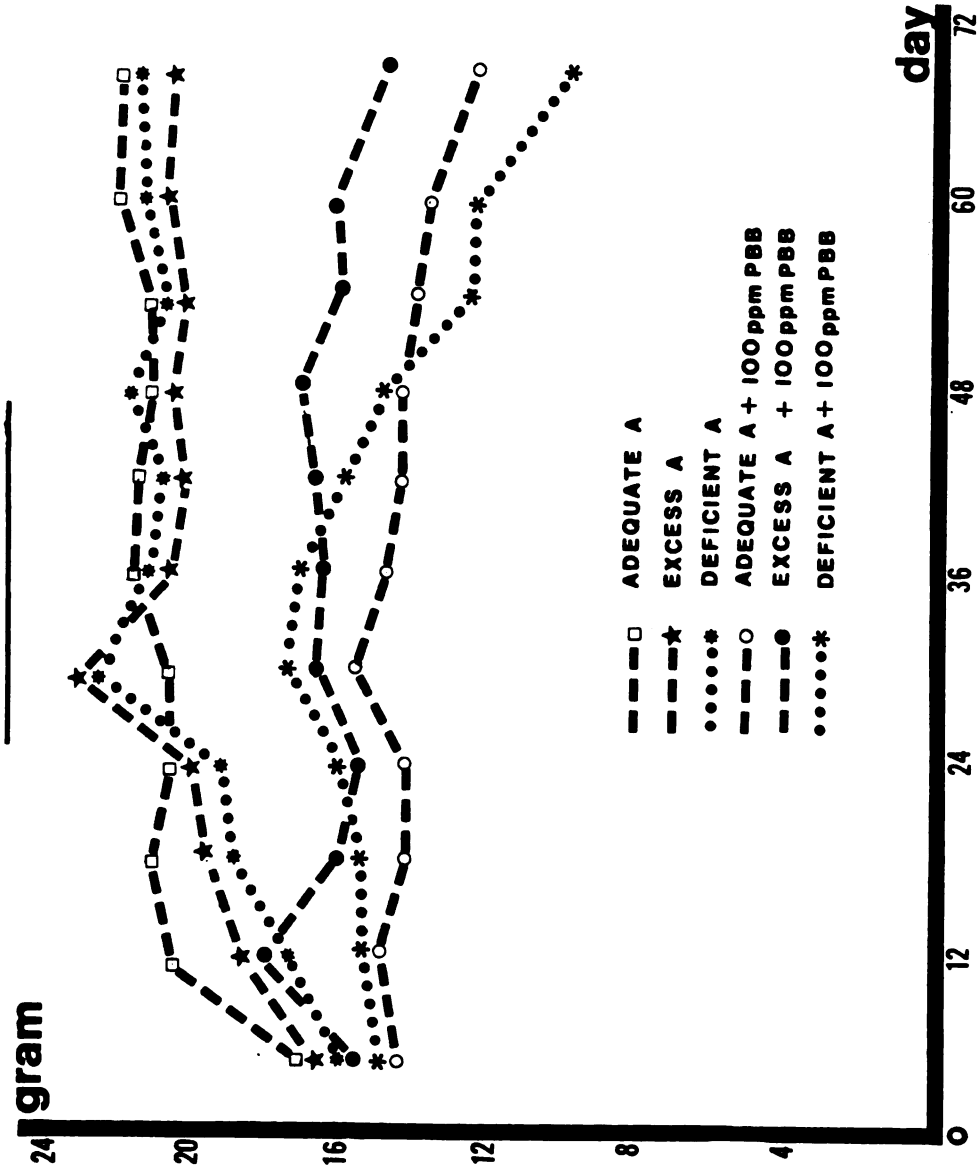

Figure 1. Means of daily feed intake of rats fed an adequate, excess or deficient vitamin A diet with or without PBB.

($p<0.05$). Significant differences in body weight started to be seen on day 57 between the subgroup of rats fed diet with excess vitamin A and 100 ppm PBB and the subgroup of rats fed deficient vitamin A diet and 100 ppm PBB ($p<0.05$) (Fig. 2).

Organ Weights

The data for liver, testicle, thymus, spleen and thyroid weight are shown in Table 2. There was no interaction between vitamin A status and PBB toxicity in relation to the weight of organs under investigation. Significant differences in liver, testicle, thymus and thyroid weight occurred between subgroups fed PBB and subgroups not given PBB ($p<0.05$). There was no meaningful difference in organ weights between subgroups of rats fed adequate, excess or deficient vitamin A diets in the group given no PBB. In the group that was given PBB, only thymus weights were significantly different in relation to the status of vitamin A in the diet fed to each subgroup. Using the Dunnett test and the post data contrast Scheffe test, it was shown that the lowest thymus weight is from the subgroup fed deficient vitamin A diet when compared to the subgroups fed adequate and excess vitamin A ($p<0.05$ and $p<0.01$). Although the mean value of the thymus weight of the subgroup of rats fed excess vitamin A with PBB is lower than the mean value of the subgroup fed excess vitamin A without PBB, statistically there is no significant difference between these two subgroups in relation to the thymus weight.

Laboratory Investigation

Serum Vitamin A

The results of serum vitamin A determinations are given in Table 3. Analysis of data using the Federer-Zelen method for a factorial experiment

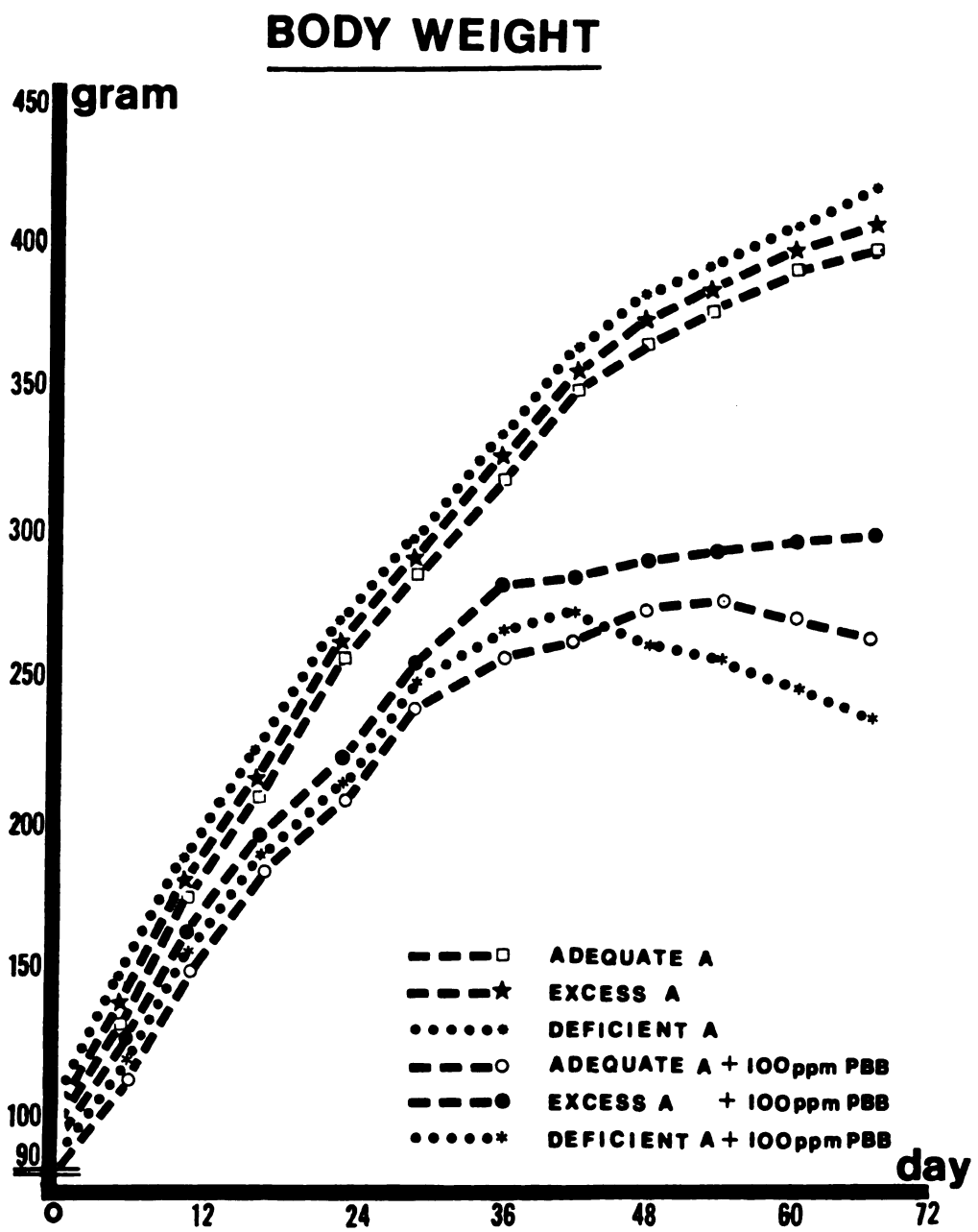

Figure 2. Means of body weight of rats fed diet adequate, excess or deficient in vitamin A with or without PBB.

Table 2. Organ weights in rats fed vitamin A adequate, excess or deficient diets containing 0 and 100 ppm PBB for 66 days.

Organ	Vitamin A \ PBB	0 ppm	100 ppm
Liver (g/100 g BW)	Adequate	4.71±0.28	10.02±0.83
	Excess	4.53±0.69	10.02±0.56
	Deficient	3.98±0.23	9.57±0.66
Testicle (g/100 g BW)	Adequate	1.51±0.10	1.99±0.12
	Excess	1.43±0.09	1.76±0.12
	Deficient	1.50±0.06	1.86±0.25
Thymus (g/100 g BW)	Adequate	0.22±0.04	0.13±0.02
	Excess	0.20±0.03	0.18±0.03
	Deficient	0.20±0.03	0.09±0.03
Spleen (g/100 g BW)	Adequate	0.27±0.07	0.25±0.05
	Excess	0.21±0.03	0.25±0.04
	Deficient	0.18±0.02	0.29±0.05
Thyroid (mg/100 g BW)	Adequate	7.55±0.82	29.49±11.93
	Excess	6.22±0.81	22.27± 7.30
	Deficient	6.36±0.96	30.46± 9.55

Data are expressed as mean ± SD.

Table 3. Vitamin A concentrations in sera (ng/ml) from rats fed vitamin A adequate, excess or deficient diets containing 0 and 100 ppm PBB for 66 days.

Vitamin A \ PBB	0 ppm	100 ppm
Normal		
Retinyl Palmitate	40.04± 0.57	39.67± 7.37
Retinol	234.12±22.70	91.00±19.12
Total	274.40±18.56	130.67±24.58
Excess		
Retinyl Palmitate	44.23± 7.67	47.49± 7.55
Retinol	411.86±18.68	241.48±34.51
Total	456.09±22.16	288.97±33.97
Deficient		
Retinyl Palmitate	41.04±13.45	44.84± 9.64
Retinol	82.67±18.64	5.67± 4.53
Total	123.72±19.65	51.00±11.04

Data are expressed as mean ± SD.

with two factors and unbalanced data shows that there is a significant interaction between status of vitamin A and PBB toxicity in relation to the total vitamin A concentration in the sera ($p<0.01$). As in Exp. I, the difference in total vitamin A between treatment combinations was produced by the difference of the retinol values. Combined effects of PBB toxicity and a low concentration of vitamin A in the diet produced a significant decrease of retinol values in the sera.

Liver Vitamin A

The profile of vitamin A in the liver is illustrated in Table 4. The data show that there is no interaction between vitamin A status and PBB toxicity in relation to the total vitamin A in the liver. As in Exp. I, the value of total vitamin A in the group given PBB is distorted by the appearance of significant amounts of retinyl acetate in the profile. The total vitamin A from the subgroup of rats fed the vitamin A deficient diet with PBB is significantly higher than the subgroup fed the vitamin A deficient diet without PBB ($p<0.01$). This difference is mainly due to the appearance of significant amounts of retinyl acetate in the subgroup given PBB. Analysis of data from the group given PBB by using the Dunnett test and the post data contrast Scheffe test shows that there is no significant difference in total vitamin A in the liver between subgroups fed either adequate, excess or deficient vitamin A in the diet. In the group not given PBB, the mean value of total vitamin A in the liver of the subgroup fed a diet excessive in vitamin A is more than 10 times higher than the other two subgroups. Addition of 100 ppm PBB reduced this total value near that of the other two subgroups. Significant amounts of retinol fraction were only seen in the subgroup fed excess vitamin A without PBB.

Table 4. Vitamin A concentrations in liver (μg/g) from rats fed vitamin A adequate, excess or deficient diets containing 0 and 100 ppm PBB for 66 days.

Vitamin A \ PBB	0 ppm	100 ppm
Normal		
Retinyl Palmitate	6.69± 1.74	3.92±0.84
Retinyl Acetate	0	2.39±1.51
Retinol	0.20± 0.17	0
Total	7.90± 2.98	6.38±1.60
Excess		
Retinyl Palmitate	95.63±32.33	4.81±0.63
Retinyl Acetate	0	4.15±1.84
Retinol	2.27± 0.94	0
Total	97.95±32.03	9.05±1.69
Deficient		
Retinyl Palmitate	3.73± 0.82	4.75±0.39
Retinyl Acetate	0	2.92±0.80
Retinol	0	0
Total	4.00± 0.95	7.73±0.93

Data are expressed as mean ± SD.

Liver Cytocnrome P-450

The values of cytochrome P-450 in the liver of rats of certain subgroups are presented in Table 5. These data are presented without any purpose of making conclusions concerning the possible difference in the amount of cytochrome P-450 in the livers. The data were derived by pooling the liver tissues from just 2 rats from 4 subgroups. Therefore, only 4 of 6 possible interactions were investigated. Results of the analysis showed that there was no conspicuous difference in the amount of cytochrome P-450 between subgroups fed different concentrations of vitamin A in the diet with 100 ppm PBB.

PBB Residue in the Liver and Fat Tissue

The concentration of PBB in the liver and fat tissue are shown in Table 6 and Table 7. Analysis of the data indicates that there is no significant interaction between the status of vitamin A and PBB toxicity in relation to the residue of PBB in the liver and the fat tissue.

Pathology

Gross Lesions

The characteristic dilatation and thickening of the bile duct seen in Exp. I was only seen in the subgroup of rats fed a deficient vitamin A diet and 100 ppm PBB. Slight dilatation without thickening of the wall of the bile duct was noticed in some rats from the subgroup fed adequate vitamin A and 100 ppm PBB. All rats in the subgroup fed excessive vitamin A with 100 ppm PBB had normal bile ducts.

Table 5. Amount of cytochrome P-450 from pooled samples of two rat livers taken from four subgroups.

Modification of Diet	PBB (ppm)	Cytochrome P-450 (nmoles/mg protein)
Adequate vitamin A	0	0.966
Adequate vitamin A	100	4.12
Excess vitamin A	100	3.79
Deficient vitamin A	100	3.64

Table 6. PBB concentration in the fat of liver (ppm) from rats fed vitamin A adequate, excess or deficient diets containing 0 and 100 ppm PBB for 66 days.

PBB \ Vitamin A	Adequate	Excess	Deficient
0 ppm	2.1± 1.5	5.0± 3.3	6.1± 2.4
100 ppm	3692.0±777.1	3011.9±913.3	4683.6±2272.0

Data are expressed as mean ± SD.

Table 7. PBB concentration in the fat tissue (ppm) from rats fed vitamin A adequate, excess or deficient diets containing 0 and 100 ppm PBB for 66 days.

PBB \ Vitamin A	Adequate	Excess	Deficient
0 ppm	0.12± 0.02	0.12± 0.02	0.17± 0.11
100 ppm	1657.73±392.41	2371.93±942.95	3285.30±1175.27

Data are expressed as mean ± SD.

Histopathology

Bile duct. Histologically, bile ducts from the rats fed an adequate vitamin A diet, excess vitamin A diet and excess vitamin A diet with 100 ppm PBB were normal (Figs. 3, 4 and 7). Some metaplasia was seen in the bile ducts of rats fed vitamin A deficient diet (Fig. 5). The bile duct of rats in the subgroup with vitamin A adequate diet and 100 ppm PBB had severe metaplasia and slight hyperplasia (Fig. 6). Severe metaplasia and hyperplasia were seen in the bile duct of rats fed the vitamin A deficient diet with 100 ppm PBB. Inside the lumen of those hyperplastic bile ducts, significant amounts of tissue debris were also present (Figs. 8 and 9). These histologic changes are very similar to the changes observed in the bile duct of the rats that were given similar diets in Exp. I.

Electron Microscopy

The ultrastructural study revealed that the extraparenchymal bile duct of rats fed a diet adequate (marginal) in vitamin A was in the initial stage of metaplasia (Figs. 11, 12 and 13). The bile ducts of rats fed a diet excessive in vitamin A were normal (Fig. 10). The surface epithelium of the bile duct of rats fed a diet deficient in vitamin A and containing 100 ppm PBB was extensively hyperplastic. Groups of "clear bile duct cells" were seen in the hyperplastic surface epithelium (Fig. 14). The significance of the appearance of these clear cells is unknown.

Figure 3. Photomicrograph of an extraparenchymal bile duct of a rat fed diet adequate in vitamin A. Notice the single layer of epithelium lining the duct and the scarcity of the mucous glands. (H & E stain, 160X)

Figure 4. Photomicrograph of an extraparenchymal bile duct of a rat fed diet excessive in vitamin A. Notice the normal columnar type of epithelium lining the duct. (H & E stain, 400X)

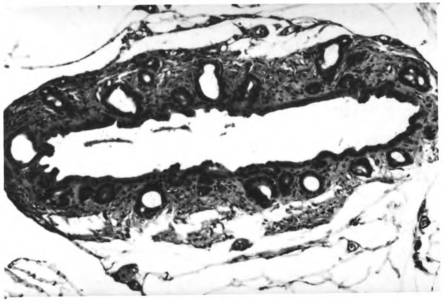

Figure 3

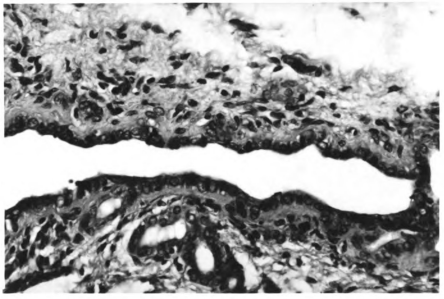

Figure 4

Figure 5. Photomicrograph of an extraparenchymal bile duct of a rat fed diet deficient in vitamin A. Notice the metaplasia in certain areas of epithelium lining the duct. (H & E stain, 160X)

Figure 6. Photomicrograph of an extraparenchymal bile duct of a rat fed diet adequate in vitamin A and 100 ppm PBB. Notice the extensive metaplasia of the surface epithelium and the moderate increase of the mucous glands. (H & E stain, 64X)

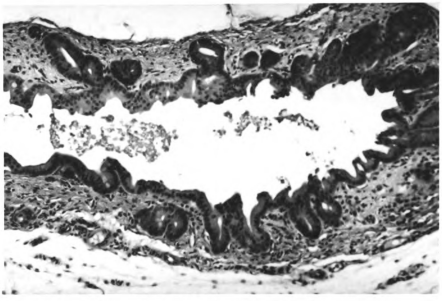

Figure 5

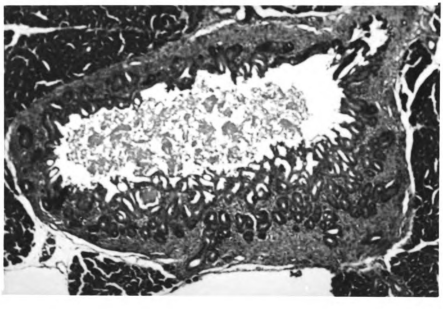

Figure 6

Figure 7. Photomicrograph of an extraparenchymal bile duct of a rat fed diet excessive in vitamin A and 100 ppm PBB. Notice there is only a very small area of metaplasia of the surface epithelium and the overall duct structure is relatively normal. (H & E stain, 160X)

Figure 8. Photomicrograph of an extraparenchymal bile duct of a rat fed diet deficient in vitamin A and 100 ppm PBB. Notice the extensive keratinization and hyperplasia of the surface epithelium and massive increase of the mucous glands. (H & E stain, 64X)

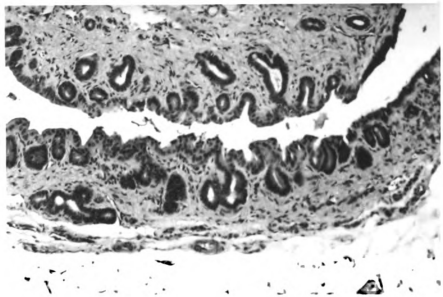

Figure 7

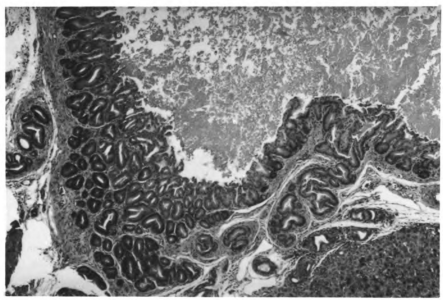

Figure 8

Figure 9. Photomicrograph of an extraparenchymal bile duct of a rat fed diet deficient in vitamin A and 100 ppm PBB. Notice the numerous mitotic figures close to the luminal surface of the gland (arrow). (H & E stain, 160X)

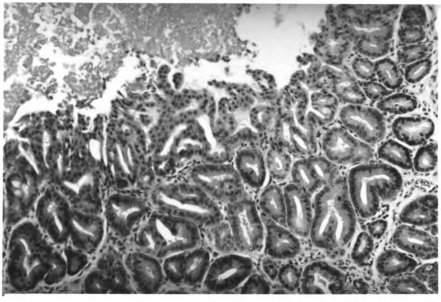

Figure 9

Figure 10. Electron micrograph of surface epithelium of an extra-parenchymal bile duct of a rat fed diet excessive in vitamin A. Notice the high columnar nature of the epithelium. (Lead citrate and uranyl acetate stain, 3,500X)

Figure 11. Electron micrograph of surface epithelium of an extra-parenchymal bile duct of a rat fed diet adequate (marginal) in vitamin A. Notice the appearance of small groups of basal cells which are squamous in nature (arrow). (Lead citrate and uranyl acetate stain, 3,500X)

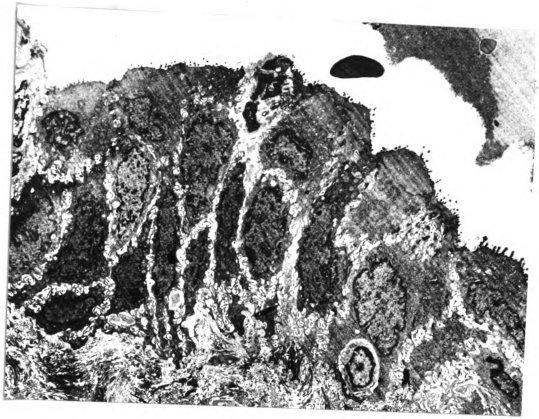

Figure 10

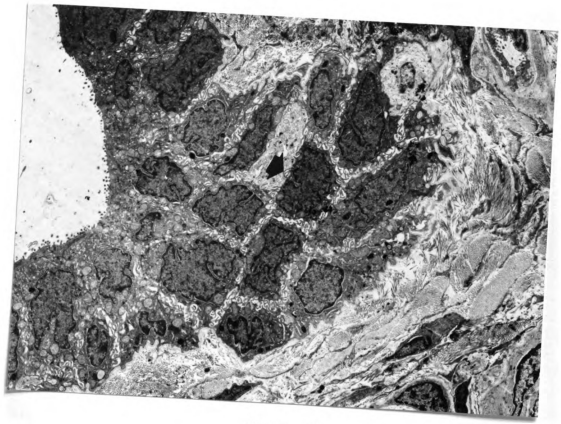

Figure 11

Figure 12. Electron micrograph of surface epithelium of an extraparenchymal bile duct of a rat fed diet adequate (marginal) in vitamin A. Notice the appearance of numerous membrane bound, electron dense granules in one of the epithelial cells (arrow). (Lead citrate and uranyl acetate stain, 3,500X)

Figure 13. Electron micrograph of epithelium of a mucous gland of an extraparenchymal bile duct from a rat fed diet adequate (marginal) in vitamin A. Notice the columnar type of surface epithelium containing secretory granules (black arrow). One of the basal cells is squamous in type and contains membrane bound, electron dense granules (white arrow). (Lead citrate and uranyl acetate stain, 7,200X)

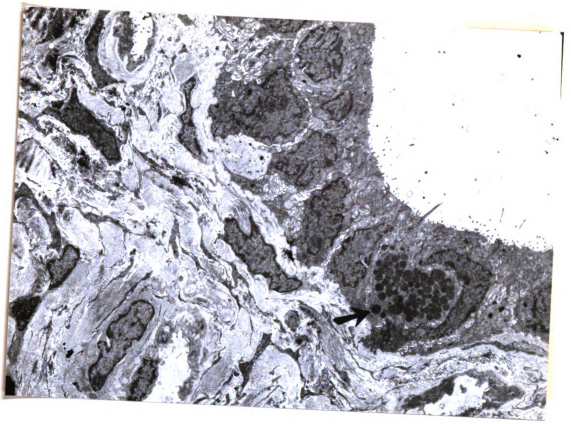

Figure 12

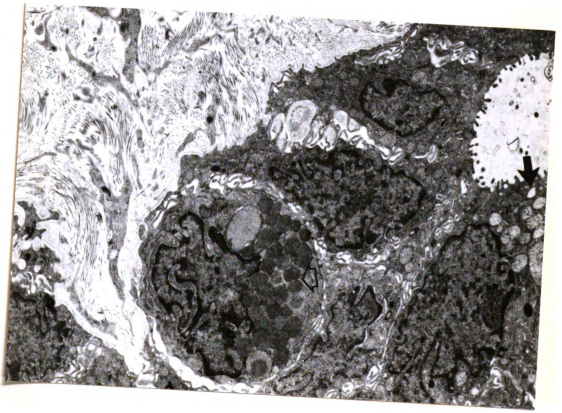

Figure 13

Figure 14. Electron micrograph of surface epithelium of an extra-parenchymal bile duct of a rat fed diet deficient in vitamin A and 100 ppm PBB. Notice the hyperplasia and the appearance of a clear cell (arrow). (Lead citrate and uranyl acetate stain, 3,500X)

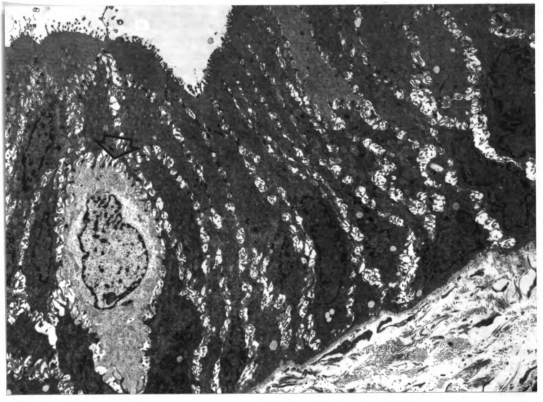

Figure 14

DISCUSSION

The positive effect of vitamin A on the growth rate of rats exposed to PCB was evaluated by Innami et al. (1974). The addition of about ten times as much vitamin A as the amount contained in the basal diet (30,000 IU/kg/diet), produced a partial protective effect against growth retardation caused by PCB toxicity. Results of the present investigation demonstrate the positive effect of vitamin A on the growth rate of rats exposed to PBB. By day 57 of the experiment, rats fed diet with an excess of vitamin A (30,000 IU/kg/diet) and with 100 ppm PBB had a significantly higher body weight than rats fed a vitamin A deficient diet with 100 ppm PBB ($p<0.05$). There was no difference in body weight between rats fed a diet with excess vitamin A and rats fed a diet deficient in vitamin A without PBB in that same period of the experiment (Exp. II). As in the case of PCB, the protective effect of vitamin A on PBB toxicity is also partial, since the body weight of rats fed an excess vitamin A diet with 100 ppm PBB was still significantly lower than the body weight of rats fed an excess vitamin A diet without PBB (Exp. II). Although the mean body weight of rats fed an adequate vitamin A diet without 100 ppm PBB was higher than that of the subgroup of rats fed a vitamin A deficient diet with 100 ppm PBB, statistically there was no difference between the two (Exp. II). These results were similar to those in Exp. I in which there was no difference in body weight between rats fed adequate (marginal) vitamin A and rats fed deficient vitamin A if they were treated with high

doses of PBB. There was also no difference in body weight between control rats and the rats exposed to 10 ppm PBB and fed diets either adequate or deficient in vitamin A (Exp. I).

Differences in organ weights were dominated by the effect of PBB toxicity. Although there was an indication of an interaction between vitamin A status and PBB toxicity in relation to the liver weight in Exp. I, the results of Exp. II do not support this finding. The only significant difference in organ weight in relation to the status of vitamin A and the toxic effect of PBB was the thymus. Rats from the subgroup fed a vitamin A deficient diet with 100 ppm PBB had the lowest thymus weight when compared to those fed diets either adequate or excess in vitamin A with 100 ppm PBB ($p<0.05$ and $p<0.01$). Furthermore, there was no significant difference between the thymus weight of rats fed a diet with excess vitamin A and 100 ppm PBB and rats fed an excess vitamin A diet without PBB (Exp. II). Thymic atrophy might be associated with the early occurrence of death in the subgroups fed a deficient vitamin A diet and high doses of PBB (Exp. I and Exp. II). The results also show that thymic atrophy due to PBB toxicity can be prevented by the addition of excess vitamin A into the diet.

Results of the serum vitamin A analysis show that there was a strong interaction between status of vitamin A in the diet and PBB toxicity in relation to the total vitamin A in the serum ($p<0.01$). Combined effects of high concentrations of PBB and low vitamin A in the diet produced a significant decrease of retinol concentration in the sera (Exp. I and Exp. II). There was no interaction between the status of vitamin A in the diet and PBB toxicity in relation to the total vitamin A in the liver, because the value of total vitamin A in some of the treatment combinations

were distorted by the appearance of significant amounts of retinyl acetate in the profile (Exp. I and Exp. II).

Rats and man, under normal conditions, tend to maintain blood levels of vitamin A within a relatively narrow range peculiar to the individual and influenced only in part by dietary intake of the vitamin, as long as some reserve supply remains in the liver (homeostatic for plasma retinol) (Keilson et al., 1979). Discussion of the vitamin A status in the body should therefore consider the total vitamin A of three elements, that is, total vitamin A in the diet, total vitamin A in the sera and total vitamin A in the liver. The constancy of the total vitamin A in the serum is the most important since this is the closest compartment to the target organs of vitamin A. The total vitamin A in the serum depends on the total value of vitamin A in the diet and in the liver.

Muto et al. (1972) stated that maximal blood levels of vitamin A (60 μg/dl or 600 ng/ml) are reached when liver deposition of vitamin A is moderate (250 μg/g). Plasma levels in rats fed vitamin A deficient diets remained above 30 μg/dl (300 ng/ml) until liver reserves were below 10 μg/g tissue. At this point plasma levels decreased slowly whereas liver vitamin A continued to decline to levels as low as 3 μg/g. Corey and Hayes (1972) reported that in rats fed a vitamin A deficient diet for 43 days, the serum vitamin A concentration was 3.6 μg/100 ml (36 ng/ml) and liver vitamin A was 1.4 μg/g. In rats fed normal vitamin A diet, the serum vitamin A concentration was 63.1 μg/100 ml (631 ng/ml) and the liver vitamin A was 147 μg/g. They also stated that normal serum vitamin A concentrations (43.7 μg/100 ml or 437 ng/ml) were achieved with a minimum of 2,500 IU vitamin A/kg/diet. Because of the instability of vitamin A, Beri recommended that the diet should contain 4,000 IU retinyl

acetate or retinyl palmitate/kg to facilitate normal growth, reproduction, tissue levels and liver storage (Anonymous, 1978). Moore (1957) reported that in order to get a detectable hepatic vitamin A storage, a vitamin A deficient diet of rats should be repleted with 1500 IU vitamin A/kg feed, and to get a "natural" vitamin A storage in the liver, the deficient diet should be repleted with 5000 IU vitamin A/kg feed. In recent experiments, in order to get "a normal vitamin A control diet", Underwood et al. (1979) repleted the deficient vitamin A diet with 3 mg retinol equivalent/kg (10,000 IU vitamin A/kg diet).

In this experiment, in order to get a normal (adequate) vitamin A control diet, the deficient vitamin A diet for rats was repleted with 3000 IU vitamin A palmitate/kg diet. This relatively low repletion was done because a similar experiment was done by Innami et al. (1974) using the same concentration of vitamin A. The liver vitamin A content at the end of these experiments was 5.15 μg/g (control subgroup of Exp. I) and 7.90 μg/g (control subgroup of Exp. II) and were similar to the results of Innami et al. (1974) who reported a value of 8.48 μg/g. Although there was not much difference in the liver vitamin A reserve between subgroups fed a normal vitamin A diet and subgroups fed a deficient diet (5.15 μg/g and 5.48 μg/g in Exp. I, 7.90 μg/g and 4.00 μg/g in Exp. II), the total vitamin A in the serum of the subgroups fed the normal vitamin A diet was almost three times that those of the deficient vitamin A diet (217.78 μg/ml and 75.66 μg/ml in Exp. I, 274.40 μg/ml and 123.72 μg/ml in Exp. II). In conclusion, repletion of the deficient vitamin A diet with 3000 IU vitamin A palmitate/kg diet only kept the total vitamin A in plasma slightly below the lowest point of the homeostatic concentration of vitamin A (300 μg/ml according to Underwood et al., 1979). The liver

vitamin A concentration was still below the critical point of reserve (10 μg/g). Repletion of the deficient vitamin A diet with 30,000 IU vitamin A palmitate increased the total vitamin A in the serum to 456.09 μg/ml with a total vitamin A reserve in the liver of 97.95 μg/g (Exp. II).

As mentioned previously, the combined effects of a high concentration of PBB and low vitamin A in the diet produced a significant decrease in total vitamin A in the sera. The total vitamin A decreasing factor in sera of rats fed diets excessive in vitamin A, adequate in vitamin A or deficient in vitamin A and given 100 ppm PBB were 1.58 (from 456.09 ng/ml to 288.97 ng/ml), 2.09 (from 274.40 ng/ml to 130.67 ng/ml) and 2.43 (from 123.72 ng/ml to 51.00 ng/ml), respectively. Since the value of retinyl palmitate is almost constant from subgroup to subgroup, the difference of total vitamin A concentration among treatment combinations is produced by the difference of retinol fraction. The retinol decreasing factor in sera of rats fed diets excessive in vitamin A, adequate in vitamin A or deficient in vitamin A and given 100 ppm PBB are 1.71 (from 411.86 ng/ml to 241.48 ng/ml), 2.57 (from 234.12 ng/ml to 91.00 ng/ml) and 14.58 (from 82.67 ng/ml to 5 ng/ml), respectively (Exp. II). In conclusion, the rate of decrease of total vitamin A and retinol values in the sera produced by PBB toxicity is faster in rats given a diet with lower concentrations of vitamin A. Increasing the dose of PBB by 10 times (from 10 ppm to 100 ppm) decreases the serum retinol by nearly the same factors. Administration of 10 ppm PBB to rats fed adequate vitamin A diet drops the retinol value by factors of 1.99 (from 159.33 ng/ml to 79.98 ng/ml). Addition of 100 ppm PBB reduces the retinol value by a factor of 17.80 (from 159.33 μg/ml to 8.95). In conclusion, in rats fed marginal vitamin A diet, increasing the dose of exposure of PBB by certain factors will likely reduce the concentration of serum retinol by nearly the same factor (Exp. I).

Mangkoewidjojo (1979) and Pratt (1979) reported a decrease in liver vitamin A content due to the PBB toxicosis. In this experiment, the decrease of liver vitamin A content occurred in the subgroup that was fed excess vitamin A (repletion with 30,000 IU vitamin A palmitate). In the subgroup of rats that had not been given PBB, the total vitamin A reserve was 97.95 μg/g. This value dropped to 9.05 μg/g when the rats were given 100 ppm PBB for 66 days (Exp. II). The other subgroups (Exp. I and Exp. II) had total vitamin A in the liver below the critical reserve (10 μg/g according to Underwood et al., 1979). As has been mentioned previously, although rats fed an adequate vitamin A diet with or without PBB do not have a higher liver vitamin A reserve than rats fed a vitamin A deficient diet, they do have several times higher values for total serum vitamin A.

Normally in the liver there are only 2 fractions of vitamin A in the profile, that is, retinyl palmitate and a small fraction of retinol. In these two experiments, subgroups that consumed PBB (either 10 ppm or 100 ppm), all had a retinyl acetate fraction in the profile. The retinyl acetate fraction in most of those subgroups was as high as the retinyl palmitate fraction. Although in Exp. I, subgroups fed an adequate vitamin A diet and those fed a vitamin A deficient diet without PBB had some retinyl acetate, the amounts were very small compared to the subgroups given PBB. In Exp. II, subgroups fed a vitamin A adequate or vitamin A deficient diet without PBB did not have any retinyl acetate in the profile. Since the subgroup that was fed an excess vitamin A diet and a high dose of PBB also had the retinyl acetate fraction in the liver, the appearance of this fraction does not necessarily relate to the concentration of vitamin A in the diet (Exp. II). The appearance of a retinyl acetate fraction

in the liver vitamin A profile seems related to the disappearance of the retinol fraction from the profile and the amount of vitamin A reserve which was below the critical point. The occurrence of retinyl acetate in the liver is also related to the rapid decrease of retinol concentration in the serum. Prystowsky et al. (1979) demonstrated in vitro that retinyl palmitate hydrolase did not actively hydrolyze retinyl acetate. It is possible that the liver does not have specific enzymes that can hydrolyze retinyl acetate like those in the intestine, since all vitamin A transferred to the liver from the chylomicron remnant is in the form of retinyl palmitate. And although the mechanism or location of esterification and hydrolysis of vitamin A in the liver is not yet known, vitamin A is stored primarily as retinyl palmitate. PBB toxicity may distort the esterification process of vitamin A in the liver and lead to the build up of retinyl acetate which can not be hydrolyzed by retinyl palmitate hydrolase. Excessive amounts of retinyl acetate in the liver might be harmful to the hepatocytes. The possibility that vitamin A metabolism may be dislocated by PBB intoxication is also suggested by the fact that 13-cis-retinoic acid (produced by vitamin A oxidation), has been isolated from the liver of rats given PBB (Kay, 1977).

The decline of hepatic vitamin A reserve was postulated to be due to an increase in microsomal metabolism (Hauswirth and Brizuela, 1976; Kato et al., 1978) and a resultant increase in glucuronidation (Thunberg et al., 1980). Becking (1973) stated that vitamin A deficient animals had a lower microsomal cytochrome P-450 content. However, Hauswirth and Brizuela (1976) reported that MC, phenobarbital and 2-acetylaminofluorene had a greater inductive effect on cytochrome P-450 in vitamin A-deficient rats. Results of the analysis of cytochrome P-450 concentration in some

subgroups in this study showed that there was no conspicuous difference in the amount of microsomal enzymes among subgroups fed adequate, excess or deficient vitamin A with 100 ppm PBB. Analysis of vitamin A content in the liver showed that there were striking differences among subgroups fed diets either excessive in vitamin A (97.95 μg/g), adequate in vitamin A (7.90 μg/g) or deficient in vitamin A (4.00 μg/g). However, the vitamin A content in the liver was the same among subgroups fed a diet either excessive, adequate or deficient in vitamin A and containing 100 ppm PBB; that is below 10 μg/g. These results demonstrate that cytochrome P-450 does not likely play a major role in the reduction of vitamin A reserves in the liver. Measurement of the concentration of vitamin A metabolites in the urine and in the feces might help in solving this problem, but unfortunately such tests were not conducted in this experiment.

The results of Exp. I and Exp. II indicated that there was no interaction between vitamin A status and PBB toxicity in relation to the residue of PBB in liver and fat tissue. Therefore, vitamin A status did not influence the concentration of PBB either in the liver or the fat tissue.

The most striking gross lesion observed in this experiment was the massive enlargement and thickening of the common bile duct of some rats in certain subgroups. Histologically, these changes consisted of severe metaplasia and hyperplasia of the mucosa of the bile duct. In Exp. I, all rats in subgroups fed diets either adequate or deficient in vitamin A and 100 ppm PBB had those characteristic changes. Similar changes were also seen in Exp. II in the subgroup of rats fed a deficient vitamin A diet and 100 ppm PBB. Slight dilatation without thickening of the wall of the

bile duct was noticed in some rats fed adequate vitamin A and 100 ppm PBB, while all rats fed excessive vitamin A and 100 ppm PBB had normal bile ducts. In Exp. I, the subgroup of rats fed adequate vitamin A and 10 ppm PBB had small areas of metaplasia on the surface epitheium of the bile duct, while the subgroup of rats fed deficient vitamin A and 10 ppm PBB had more extensive metaplasia and slight hyperplasia of the glandular epithelium. Beaver (1961) reported superficial metaplasia and slight keratinization in the bile duct of rats fed diets deficient in vitamin A, whereas PBB toxicity only produced intraparenchymal bile duct hyperplasia (Pratt, 1979; Gupta and Moore, 1979). In conclusion, the appearance of the massive enlargement and thickening of the common bile duct of some rats in certain subgroups was due to the interaction between low levels of vitamin A in the diet and exposure to high doses of PBB. Excessive vitamin A in the diet protected rats from the appearance of these changes.

In 1947, Olafson noticed marked hyperplasia and dilatation of the common bile duct of cows later shown to be caused by chlorinated naphthalene (Sikes and Bridges, 1952). Similar changes were also observed in nonhuman primates as a result of TCDD toxicity (McConnell et al., 1978) and in cattle due to pentachlorophenol toxicity (McConnell et al., 1980). McConnell et al. (1980) speculated that those changes might be the result of a direct toxic action related to the enterohepatic circulation of the toxicant. Since both chlorinated naphthalene and TCDD have been demonstrated to reduce serum vitamin A or liver vitamin A content, an interaction between status of vitamin A and those two xenobiotic compounds might exist as in the case of our experiment with PBB.

The characteristic enlargement of the common bile duct observed in this experiment mimics preneoplastic change and provides additional evidence

that vitamin A and PBB can influence carcinogenesis. The protective effect of vitamin A on the development of such characteristic lesions may demonstrate that vitamin A has protective effects against carcinogenesis. These results also indicate the importance of nutritional status on the assessment of carcinogenicity of certain elements in laboratory animals. Deficiency, excess or imbalance of certain nutrients might stimulate or depress carcinogenesis.

The ultrastructural study revealed that the extraparenchymal bile duct of rats fed diet adequate (marginal) in vitamin A was in the initial stage of metaplasia. Although this group of rats had normal concentration of total vitamin A in the sera (274.40 ng/ml) and the observation using light microscopy did not reveal any evidence of vitamin A deficiency, the presence of small groups of squamous basal cells and membrane bound, electron dense granules representing the keratohyalin in those cells, were characteristic for the initial stage of vitamin A deficiency (Wong and Buck, 1971; Harris et al., 1972). These results demonstrate that the common bile duct might be the most sensitive organ and the best indicator for early changes produced by a low vitamin A content in the sera.

SUMMARY

Two experiments to evaluate the interaction between vitamin A status and PBB toxicity were completed. In Exp. I, twenty-four male Sprague-Dawley rats were fed dietary levels of 0, 10 or 100 ppm PBB in a vitamin A deficient or vitamin A-adequate diet. In Exp. II, thirty-six male Sprague-Dawley rats were fed a diet either deficient, adequate or excessive in vitamin A and were given either 0 or 100 ppm PBB. Experiments were terminated on day 82 in Exp. I and on day 66 in Exp. II.

One rat from Exp. I and two rats from Exp. II died towards the end of the experiments. All these rats were fed a diet deficient in vitamin A and containing 100 ppm PBB. Rats from these subgroups also had clinical signs of vitamin A deficiency at least 10 days earlier than a subgroup fed a diet adequate in vitamin A and containing 100 ppm PBB.

Weight gain and feed intake were noticeably decreased in subgroups of rats fed 100 ppm PBB. Rats fed a diet excessive in vitamin A and with 100 ppm PBB had significantly higher body weights than rats fed a diet deficient in vitamin A and with 100 ppm PBB. There were no significant differences in body weight between subgroups of rats fed a diet either adequate or deficient in vitamin A if they were given 100 ppm PBB. Rats from the subgroup fed a diet deficient in vitamin A had the lowest thymus weight when compared to the subgroups of rats fed a diet either excessive or adequate in vitamin A if they were exposed to high doses of PBB. There was no significant difference between thymus weight of rats fed diet

excessive in vitamin A and 100 ppm PBB and rats fed diet excessive in vitamin A alone. These results demonstrated the partial protective effect of vitamin A on PBB toxicity.

The most conspicuous gross lesion observed in this experiment was the massive enlargement and thickening of the common bile duct of rats fed a diet deficient in vitamin A and containing 100 ppm PBB. Slight dilatation without thickening of the wall of the bile duct was noticed in some rats fed a diet adequate in vitamin A and 100 ppm PBB, whereas all rats fed a diet excessive in vitamin A and with 100 ppm PBB had a normal bile duct.

Histologic examination of the bile duct of rats in subgroups fed a diet deficient in vitamin A and with 100 ppm PBB revealed a very severe hyperplastic change which mimicked a preneoplastic condition. These results indicate that interaction between deficiency of a certain nutrient (vitamin A) and an environmental contaminant (PBB) will produce markedly different changes than those produced by either alone.

These two experiments demonstrated the existence of a strong interaction between the concentration of vitamin A in the diet and PBB toxicity in relation to the total vitamin A in the sera. Combined effects of high doses of PBB and low vitamin A in the diet produced a significant decrease in retinol concentration in the sera. The interaction between a deficiency of vitamin A and PBB toxicity produced an abnormal vitamin A profile in the liver manifested by the appearance of significant amounts of retinyl acetate. This disturbance of vitamin A metabolism in the liver is probably related to an alteration of the hydrolysis and esterification of retinyl palmitate.

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