AN ABSTRACT OF THE THESIS OF

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Title: Hypervitaminosis A: Effects on Reproduction and Interactions with Pyrrolizidine Alkaloids.

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Peter R. Cheeke

In the first experiment the minimum dietary toxic levels of vitamin A were determined in pregnant rabbits over three parities. The treatments consisted of basal diet plus 10,000, 30,000, 60,000, and 90,000 IU/Kg added vitamin A. The basal diet was formulated to be free of vitamin A and beta-carotene; rice hulls were used in place of alfalfa meal as the dietary fiber source. It was found that during storage, considerable destruction of vitamin A occured, probably stimulated by lipoxidases in the rice hulls. As a result, the 10,000 IU/Kg diet became deficient and the rabbits on this diet showed vitamin A deficiency symptoms such as fetal resorptions and fetal hydrocephalus. Rabbits on the 90,000 IU/Kg diet exhibited symptoms associated with toxicity. The diets with intermediate amounts of vitamin A supported relatively normal reproduction, and the does on these diets were healthier in terms of incidence of respiratory and enteric disease. Of

these two diets the 30,000 IU/Kg yielded the highest total grams of kits weaned over three parities.

The liver vitamin A reserves of the female rabbits increased with increasing dietary vitamin A levels; however, the plasma concentrations did not follow the same pattern. Plasma vitamin A concentrations increased significantly ($P<.05$) as the dietary levels increased from 10,000 to 30,000 IU/Kg. When these levels increased from the latter to 60,000 IU/Kg, the average plasma vitamin A levels increased, but the difference was not significant ($P<.05$). It was found that the plasma vitamin A concentrations dropped markedly in animals fed the 90,000 IU/Kg diet and became significantly ($P<.05$) lower than in those fed the 60,000 IU/Kg diet but not significantly ($P<.05$) different from the vitamin A deficient plasma samples. It was concluded that in rabbits vitamin A at high concentrations inhibits its own secretion from the liver into the circulation. The symptoms, although associated with low plasma vitamin A levels, are attributed to hypervitaminosis A. This might explain why the symptoms of hypo- and hyper-vitaminosis A in rabbits are similar. This condition could then be termed "Secondary hypovitaminosis A" since it is not caused by low vitamin A intake and the liver contains high amounts of the vitamin.

In the second experiment the interactions between Sececio pyrrolizidine alkaloids (PA) present in Senecio jacobaea or tansy ragwort (TR) and vitamin A were investigated in rats. It was found that PA present in a 5% TR diet reduced both plasma and liver vitamin A levels significantly compared to the control ($P<.05$). Increasing the amount of TR in the diet from 5 to 10% did not make a significant change ($P<.05$); suggesting that 5% dietary TR was sufficient for this type of study. It was concluded that the reduction in hepatic and plasma vitamin A was due to either higher catabolism or lower intestinal absorption rate.

In the third experiment the possibility of impaired fat and fat-soluble vitamin absorption was considered. The PA cause biliary hyperplasia and bile duct obstruction, hence reducing bile secretions into the small intestine. Because of the necessity of bile acids and fat emulsification in the small intestine for vitamin A and fat absorption, PA induced liver damage could thus impair absorption of these nutrients. Dietary PAs reduced fat absorption in 5% TR fed rats ($P<.05$) compared to the control.

Hypervitaminosis A:
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Interactions with Pyrrolizidine Alkaloids

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Typed by Nora Peters for Mehran Fallah Moghaddam

Dedicated to my father and my sister,
Ahmad and Mehrnoosh Fallah Moghaddam
but specially to my dedicated mother,

Mrs. Tahereh Karimzadeh

for without her continuous support
and neverending encouragement and
concern throughout my life,
none of this would have been possible.

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Introduction

Vitamin A can become toxic if its rate of intake exceeds the rate of excretion and catabolism (Mallia et al., 1975). The symptoms of hypervitaminosis A result from metaplasia of the epithelial cells at different sites of body. Acute hypervitaminosis A, although only observed in rare situations, can be potentially fatal (Mahoney et al., 1980; Moore, 1957). Chronic hypervitaminosis A is much more common. With the advent of vitamin A therapy for acne (Anon., 1982b) and the still controvertial preventative roles of this vitamin in cancer, chronic hypervitaminosis A is becoming increasingly more common in humans as a result of supplementations. The most important adverse effect of chronic hypervitaminosis A is probably impaired and abnormal reproduction, since vitamin A can be abortifacient and/or teratogenic (Cheeke et al., 1984; Fell and Mellanby, 1952; Larente et al., 1977; Roels et al., 1969). Most other symptoms of this disorder are reversible upon discontinuing vitamin A intake.

Vitamin A metabolism can be affected by hepatotoxins, since liver is the storage site for this vitamin. Some hepatotoxins like ethanol (Sato and lieber, 1982) and 2,3,7,8-Tetrachlorodibenzo-p-Dioxin (Thunberg et al., 1979) can increase plasma and decrease hepatic vitamin A levels. This can predispose to hypervitaminosis A since plasma levels increase. On the other hand, there are substances like colchicine which do exactly the opposite because they inhibit the synthesis of retinol binding protein (vitamin A carrier protein) by the liver (Smith et al., 1980).

The objectives of this study were to: 1) Determine the minimum toxic levels of dietary vitamin A in pregnant rabbits, and 2) Investigate the interactions between the hepatotoxic pyrrolizidine alkaloids present in Senecio jacobaea (tansy ragwort) and vitamin A metabolism in rats.

Chapter 1

LITERATURE REVIEW

1.1. LIVER PHYSIOLOGY AND ANATOMY

In order to better understand hepatoxicity, a brief overview of liver anatomy and physiology is provided.

The liver contains four types of cells: endothelial, Kupfer, parenchymal, and Stellate cells (Figure 1.1.1).

a. Endothelial cells. These cells are in contact with the blood in the sinusoidal lumen. They are generally thought to be a different functional state of Kupffer cells and contribute to the immune system.

b. Kupffer cells. These have projections reaching into the sinusoidal lumen which apprehend older red blood cells and foreign material in circulation and phagocytize them. Both types of sinusoidal cells are well supplied with lysosomal enzymes, reflecting their role in degradation of various blood particulates.

c. Hepatocytes or parenchymal cells. Hepatocytes constitute the greatest proportion of total cells and total space within the liver lobules. These cells have a wide profile of enzymes that no other cell in the body has, permitting a wide variety of biochemical reactions. The cell membrane exposed to the circulation bears

microvilli for increasing surface area. The membranes on the other sides of the cells form tight junctions with the other hepatocytes, particularly where mutual association makes up the bile cannaliculi, the canals in which bile is secreted. Small fat vacuoles are found when circumstances encourage lipogenesis, such as with the laying hen, in which the Golgi complex is more active for lipoprotein "packaging" than in non-laying birds (Moran, 1982).

d. Stellate cells, also often referred to as lipocytes, perisinusoidal, fat-storing, interstitial, and Ito cells, are located between the Kupffer-endothelial composite and hepatocytes. Various stresses such as carbon tetrachloride toxicity, alcoholic cirrhosis, protein deficiency, etc., that cause extensive damage to the liver increase the prominance of Stellate cells (Moran, 1982). There is evidence indicating collagen production by these cells when the lobule is structurally damaged; therefore, these cells are fibroblasts (Moran, 1982). The cytoplasm of Stellate cells contains lipid droplets that alter in size and number with dietary vitamin A status (Moran, 1982). These cells are the main vitamin A storage site in the liver; however, hepatocytes have also been noted to contain minor amounts (Anonymous, 1982).

A functional unit of liver tissue is called a liver lobule (Figure 1.1.2). The liver lobule is made up of a portal triad (which consists of a branch of the hepatic artery, a branch of the portal vein, and a bile ductule), a central vein, hepatocytes (parenchymal cells) which separate these two units, sinusoids that separate layers of hepatocytes, and bile canaliculus. Blood flows into the sinusoids from portal triads to the central vein and bile flows through the bile canaliculus in the opposite direction into the bile ductules which empty into bile ducts. The region surrounding the portal triads is called Zone 1. The cells in this area are rich in oxidative enzymes used in carbohydrate metabolism. The cells surrounding the central veins are considered to make up the Zone 3 cells. These cells, on the other hand, are rich in microsomal pathway enzymes (P450 enzymes). Therefore, toxins that are bioactivated by the P450 system would exert their necrotic effects more in zone 3 (centrilobular necrosis) than zone 1. The region between Zones 1 and 3 is Zone 2. These cells have enzyme profiles ranging from more oxidative to more microsomal depending on the zone to which they are closer.

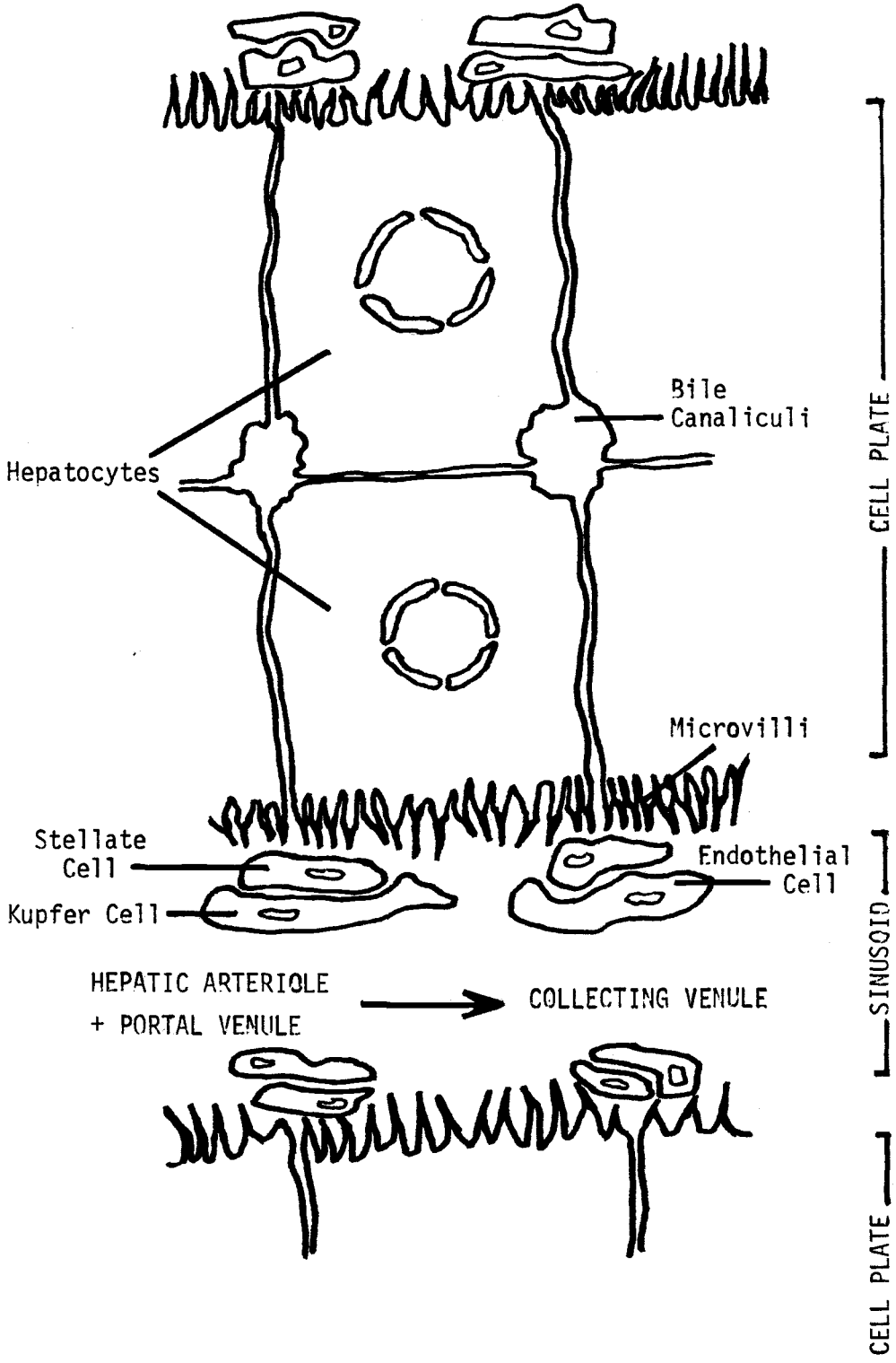


Figure 1.1.1. Diagram of the cells comprising the liver lobule.

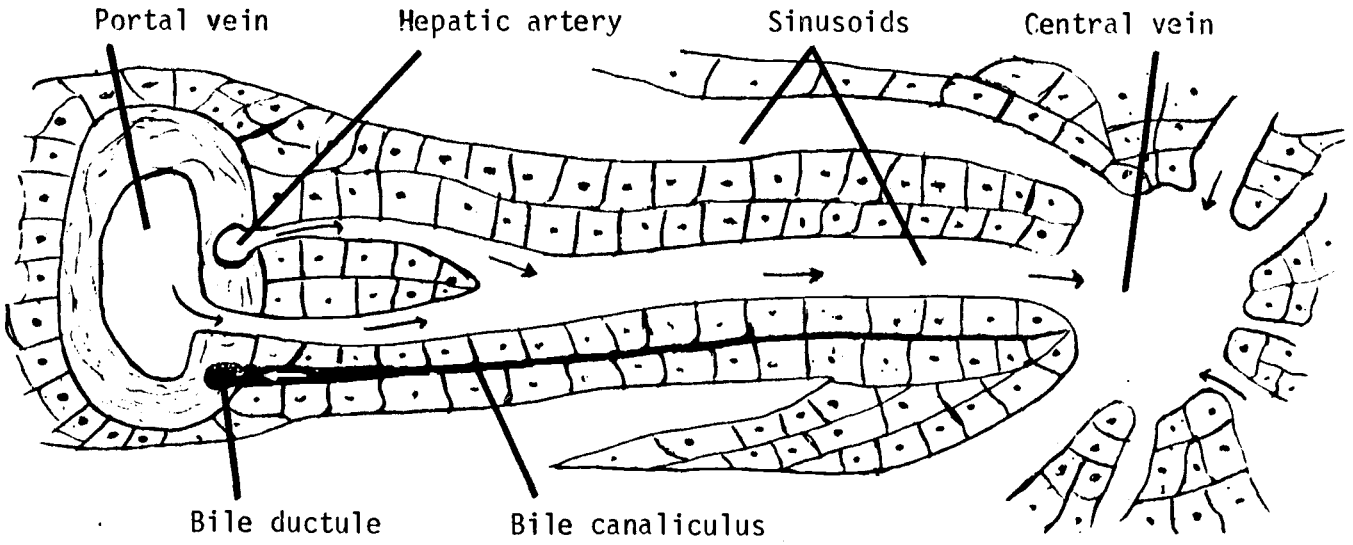


Figure 1.1.2. Diagram of liver lobule.

1.2. VITAMIN A METABOLISM.

Introduction

Vitamin A is one of the few vitamins for which both deficiency and excess presents serious health hazards. These two conditions arise under different circumstances. In humans, deficiency occurs in endemic proportions in many developing countries and is seen occasionally in technologically developed societies in patients with severe malabsorption, transport disorders or liver disease. Toxicity, however, usually arises from the abuse of vitamin supplementation and therapy. In animals, however, these conditions arise as a result of under or over supplementation of vitamin A when in confinement.

Deficiency of vitamin A has been extensively studied in humans and animals. Xerophthalmia, the term generally used to cover all the ocular manifestations of vitamin A deficiency, is the most common cause of blindness in young children throughout the world (Dorea et al., 1984). At least 250,000 children become blind annually from this disease. A large proportion of children with severe corneal destruction, especially the ones untouched by health services, will die while, or soon after, becoming

blind. This mortality, which is usually due to secondary infection (a result of vitamin A deficiency), is estimated at between 30-80 percent and drastically reduces the number of blind children remaining in the population. In animals, conditions such as altered cerebrospinal fluid pressure, skin and reproductive problems and anorexia (loss of appetite) can be observed as a result of vitamin A deficiency. These and other serious manifestations of vitamin A deficiency will be discussed later.

Vitamin A toxicity symptoms vary considerably depending on the age of the subject and the duration of the excessive intake. Young children are especially susceptible. Acute vitamin A toxicity has resulted from single large doses. There have been cases of acute toxicity associated with intakes of several hundred thousand micrograms of vitamin A, from the consumption in a single meal by polar explorers of the liver of a seal or a polar bear, which contains about 18,000 IU/g (Mahoney et al., 1980). Death is known to have occurred in some instances but in most cases headache, vomiting, vertigo, blurred vision and peeling of the skin are the usual consequences and they rapidly subside (Mahoney et al., 1980; Moore, 1957). Eskimos have long been aware of the danger. In subacute or chronic toxicity, doses usually ranging from about 10,000-50,000 micro-grams have been

given daily for several months to years. Hypervitaminosis A can occur in infants fed chicken liver. Although manifestations of hypervitaminosis A subside gradually following cessation of intake, there is some evidence of irreversible damage to the liver (Hruban et al., 1974; Weber et al., 1982). Vitamin A toxicity can cause alterations in cerebrospinal fluid pressure, dermatitis and reproductive problems, which will be discussed in more detail later.

I. Chemistry of Vitamin A

Vitamin A, discovered by McCollum in 1915, is a fat soluble, unsaturated 20 carbon cyclic alcohol containing a B-ionine ring and an unsaturated side chain (Fig. 1.2.1). Vitamin A has different isomeric forms with different biological activities. However, all-trans retinol (vitamin A) is the most active and abundant form. Vitamin A (retinol) derivatives with terminal aldehyde (retinal) or carboxyl groups (retinoic acid) are active biological intermediates. Retinol is converted to retinal in reactions involved in visual cycles which were described by Wald (1968). Retinoic acid is formed by irreversible oxidation of retinol and supports the biological activities

of vitamin A with the exception of vision and reproduction. Vitamin A esters, predominately of palmitate esters, are found as storage forms of vitamin A in the body. Figure 1.2.1, contains structures of some of these compounds.

II. Sources of Vitamin A

Vitamin A can be provided by the vitamin itself, called retinol, by retinyl esters, mainly retinyl palmitate, and by its precursor beta-carotene.

Retinol and its ester-forms are available in animal products like liver, eggs, and milk. beta-carotene, which is the main dietary source of vitamin A in most third world countries and among strict vegetarians (Brubacher and Weiser, 1985), is an accessory pigment associated with chlorophyll in green plants. This reddish pigment absorbs light around 450nm, where chlorophyl does not absorb. Other than green plants, some sources of beta-carotene include animal fat, milk fat, egg yolk and liver (Bondi and Sklan, 1984). Absorption efficiency of beta-carotene decreases as the dietary intake increases. In rats, in the range of about one to 10 times the daily requirement, beta-carotene is completely absorbed and transformed to vitamin A. With higher intake levels, absorption and conversion rates decrease. The unabsorbed carotene is excreted. In man,

30-80% of carotene taken in high doses is passed out in the feces; whereas low doses are completely absorbed (Brubacher and Weiser, 1985). According to Wolf and Phil (1982), about 12% of fed beta-carotene is absorbed by humans, of which 60-70% is converted to retinyl esters and 20-30% enters the circulation as beta-carotene. In some other species like birds, cattle, and horses, some carotenoids also escape conversion to vitamin A in the intestine and are taken up intact into the circulation. However, in animals like rats, goats, sheep, cats (Bondi and Sklan, 1984) and rabbits (Kormann and Schlachter, 1984), all of the beta-carotene absorbed by the intestine is converted to vitamin A (table 1.2.1). When adequate levels of vitamin A stores are reached in man, the conversion of beta-carotene to vitamin A is further reduced and excess beta-carotene accumulates in the body (Mahoney et al., 1980). High levels of beta-carotene in blood caused by high dietary levels or low conversion rates in species which can absorb it as such, leads to hypercarotenemia, which causes a yellowish-orange color of the skin. beta-carotene is not toxic even in high levels (Wolf and Phil, 1982) and hypercarotenemia is, therefore, not harmful. In cattle, differences between breeds are noted with respect to carotene absorption and conversion to vitamin A. In Holsteins, less carotene reaches circulation than in Guernseys and Jerseys. This is manifested in the more

yellow color of the milkfat and butter in the latter breeds. The decrease in the potency of beta-carotene with higher intakes could represent a natural regulation mechanism to prevent toxicity by vitamin A (Brubacher and Weiser, 1985).

In general, dietary beta-carotene by itself is a poor source of vitamin A for humans since a low percentage of it is absorbed and converted to vitamin A in an average person (Wolf and Phil, 1982).

Retinol, retinyl esters and beta-carotene are all very unstable in the presence of oxygen, moisture, heat and UV light. Under these conditions, oxidation of these compounds which leads to loss of biological activity is likely to occur. This is an important factor when considering different sources of dietary vitamin A. Trace metals and unsaturated, easily oxidized fats have deleterious effects on vitamin A and beta-carotene stability in animal feed. The oxidation process can be reduced by adding antioxidants like ethoxyquin or BHT to the vitamin premix or to the diet. Storage of the feed in moist air can double or even treble oxidation of these compounds. Carotenoids from plants are subject to both enzymatic oxidations through lipoxygenase systems and to non-enzymatic processes accelerated by heat and light. Slow

drying of forages in the sun may cause an up to 80% loss of beta-carotene but high temperature, short-term drying deactivates the lipoxygenase enzymes and reduces the loss. Ensiling the forage preserves the carotenoids and leads to about 10% loss. Pasteurization, a process used to kill microbial organisms in milk, destroys vitamin A. Therefore, pasteurized milk is fortified with appropriate amounts of vitamin A esters, as well as other vitamins (Bondi and Sklan, 1984).

III. Vitamin A Metabolism

a. Intestinal absorption and liver storage.

Dietary vitamin A from animal and synthetic sources is usually in the form of retinyl esters, the palmitate being the major natural ester. These esters are hydrolyzed to retinol in the intestinal lumen by pancreatic hydrolases. Retinol and dietary beta-carotene are taken up from the micellar phase into the intestinal mucosa cells. Dietary fat (Brubacher and Weiser, 1985) and bile acids (Olson, 1961; Sharma and Dostalova, 1986) are essential for this process. In the mucosal cells beta-carotene is cleaved by two soluble enzymes to retinol. All of retinol in the mucosal cells is esterified as retinyl esters, mainly retinyl palmitate. Retinyl esters are then incorporated

into chylomicrons, which in mammals reach the circulation via the lymphatic system (Hollander, 1980). In the liver, hydrolysis of retinyl esters occurs during uptake. Vitamin A is then stored following re-esterification by deposition of mainly retinyl palmitate in lipid droplets of liver Ito cells (lipocytes, Stellate cells). Newly absorbed vitamin A is secreted and metabolized prior to "older vitamin A" stored in Ito cells (Goodman, 1980). There seem to be two pools of vitamin A inside the Ito cells. When vitamin A deficient rats are administered vitamin A, it accumulates in the mitochondrial-lysosomal fractions at a faster rate than in nuclear, microsomal and cytosolic fractions of liver cells. However, during depletion, vitamin A is mobilized from all fractions at a constant rate. The nuclear fraction accommodates about 40% and the mitochondrial-lysosomal fraction about 35% of total cellular retinyl palmitate hydrolase activity, which needs bile salts for stimulation, of rat liver cell homogenates (Periquet et al., 1985). This may explain why they mobilize retinyl palmitate at about the same rate.

Vitamin A metabolism is under control of nutritional, hormonal and physiological stimuli. Liver is the main storage site of vitamin A and more than 90% of vitamin A is found there, but it can also be found in the kidneys (1-2% of liver vitamin A levels), the cortex of adrenal glands,

and in the pigment epithelium of the eye, in much lower levels than found in the liver. The concentration of vitamin A varies in different parts of the liver (Dorea et al., 1984). Samples taken from the right lobe are consistantly higher in vitamin A than the other sections of the liver (Olson J.A. et al., 1979). Although vitamin A is stored mainly in the liver, it is also deposited in the kidneys, fat, adrenal glands, lungs and intestine (Mahoney et al., 1980). In neonatal rabbit lungs, retinol is esterified to retinyl palmitate at a lower rate than in liver (Zachman, 1985).

Hepatic damage can alter vitamin A metabolism by affecting the liver. Rats treated with 2,3,7,8-Tetrachlorodibenzo-p-Dioxin (TCDD) had a total liver vitamin A storage level of about 30% that of control rats after eight weeks. The serum retinol was considerably higher for treated animals (Thunberg et al., 1979). In acute ethanol administration to rats and baboons the same results were obtained (Sato and Leiber, 1982). This may be due to either excessive release of vitamin A from liver or reduced absorption of vitamin A esters entering the liver via the portal vein.

b. Plasma transport.

Under normal conditions, vitamin A is transported in the plasma as retinol bound to a specific binding protein (20,000-21,000 MW) called retinol binding protein (RBP). The RBP is synthesized in liver endoplasmic reticulum. It has been found in day-13 rat fetuses and day-6 chick embryos. Thus, the vitamin A transport system is present in fetuses at mid-gestation (Lorente and Miller, 1977). Hepatic dysfunction can cause reduced serum RBP levels (Weber et al, 1982). Colchicine administration retards RBP secretion as well as that of the very low density lipoprotein (VLDL) and high density lipoprotein (HDL) and albumin (Smith et al., 1980).

In rats, retinoic acid supplementation (Underwood et al., 1979) and excessive retinol administration (Mallia et al., 1975) cause significant reductions in RBP and retinol levels in the blood. The reduction in blood retinol following retinoic acid treatment has also been reported in rabbits (Lorente and Miller, 1977). The mechanism regulating hepatic secretion of RBP appears to be generated extrahepatically, perhaps in response to tissue catabolism and uptake rate of retinol (Underwood et al., 1979). This might explain why blood levels of RBP are reduced by retinoic acid since it can satisfy all vitamin A functions

but vision and reproduction and therefore there is less need for retinol mobilization. The mechanism by which excessive doses of retinol reduce blood RBP levels seems to be by reduction in hepatic synthesis (Mallia et al., 1975).

Retinol binding protein has a short half life and so it can be a good indicator of protein malnutrition (Weber et al., 1982). In rats, when protein quantity and quality were restricted, plasma vitamin A levels were maintained in a range above a normal 30 ug/dl only when vitamin A was added. Diets deficient in both vitamin A and protein quantity could still support normal plasma vitamin A levels if they contained high quality protein even when liver vitamin A levels were in the deficient range (Underwood et al., 1979). After hydrolysis of the stored retinyl esters, RBP binds retinol; probably somewhere prior to the Golgi apparatus. Binding to RBP renders retinol stable in aqueous solutions towards both chemical and enzymatic attack. The RBP also seems to protect normal tissues against the surface-active properties of vitamin A. Retinol bound to RBP does not exhibit its surface-active and membranolytic effect on membranes (Mallia et al., 1975). Holo-RBP (RBP bound to retinol) will complex with pre-albumin (PALB) in circulation after it has been secreted out of liver cells. The PALB-RBP-retinol complex reaches the target tissues where the uptake of retinol, but

not RBP or PALB, has been postulated to occur through specific RBP receptors. Retinol crosses the cell membrane. Affinity of the apo-RBP (unbound to retinol) for PALB is then lower than that of holo-RBP so they dissociate. Apo-RBP is mainly filtered out and catabolized by the kidneys. One of the factors important in regulating delivery of vitamin A to tissues is synthesis (Huang and Hembrel, 1979; Weber et al., 1982) and release of RBP (Hollander, 1980).

c. Intracellular transport.

Both retinol and retinyl palmitate are found in most cells (Goodman, 1980). In cytosol both entities are found in high molecular weight lipid-protein aggregates which contain retinyl palmitate hydrolase and intracellular retinol binding protein (cRBP) that is distinct from RBP (Ross and Goodman, 1979). Intracellular retinol binding proteins appear to transport retinol to the nucleus where specific interactions occur that affect gene expression. This is manifested by an increase in RNA synthesis of deficient rat testes cells after vitamin A administration.

d. Intracellular retinol metabolism.

Enzymatic oxidations:

The alcohol group of retinol can be reversibly oxidized to retinal by alcohol dehydrogenase. Retinal in turn can be irreversibly oxidized to retinoic acid. Both retinol and retinoic acid can be glucuronidated in the presence of UDP-glucuronic transferase in liver. These glucuronides which are water soluble constitute a large portion of the retinol excreted through the bile (Bondi and Sklan, 1984).

Membrane glycoprotein synthesis:

In rat small intestine, vitamin A deficiency causes a reduction in the synthesis of a fucose-containing glycopeptide (Fuc-glycopeptide) and a decrease in the number of the goblet cells (DeLuca et al., 1971). Fucose is an unusual monosaccharide occurring as L-fucose (6-deoxy-L-galactose) in a number of mucopolysaccharides and mucoproteins. This glycopeptide which is present in goblet cells contains D-fucose, D-galactose, D-glucosamine, D-galactosamine, and D-sialic acid (DeLuca and Wolf, 1972).

Biosynthesis of a lipid containing mannose from GDP-mannose by the membrane of goblet cells is reduced in vitamin A deficiency. Addition of retinol to the system restores the biosynthesis of mannolipids to normal levels. Addition of phosphorylated radioactive mannose yields one radioactive site to the mannolipid while adding radioactive retinol makes double-labeled mannolipid (DeLuca and Wolf, 1972). The mannolipid can function as a sugar donor for the synthesis of specific glycopeptides and the mannose can be metabolized to fucose (DeLuca and Wolf, 1972). One can then speculate that vitamin A is the carrier of the carbohydrate moieties in the biosynthesis of glycoproteins within membranes (DeLuca and Wolf, 1972). This might explain the membrane protection role of vitamin A. As a result of defects in this process, membranes might become less stabilized and this might explain the reduction in the number of goblet cells which would in turn reduce mucin production in the small intestine.

Retinol affects nucleic acid and protein biosynthesis:

Synthesis of RNA is stimulated when vitamin A is administered to deficient animals, cells and nuclei (Tsai

and Chytil, 1977). Zile et al. (1979) noted an increased DNA/protein ratio in vitamin A deficient animals and postulated a direct role of vitamin A in cell replication (Becking, 1973).

IV. Regulation of Vitamin A Metabolism.

Absorption of vitamin A or beta-carotene and formation of vitamin A esters occurs in the upper two-thirds of the small intestine (Olson, 1961) and requires bile and pancreatic secretions (Sharma et al., 1986). There is a negative feedback on conversion of beta-carotene to vitamin A, because the efficiency of conversion declines as vitamin A reserves of the body increase.

The main excretory pathway of vitamin A is the conversion of retinol to polar water soluble metabolites, principally retinyl-β-glucuronide, in the liver and excretion through the bile and hence via feces (NRC, 1987). Some of the vitamin A excreted this way undergoes enterohepatic circulation and is reabsorbed (Sklan, 1983). However, glucuronide formation may follow irreversible oxidation to retinoic acid (NRC, 1987). Small amounts of glucuronides conjugates and chain-shortened metabolites may be eliminated via urine.

The levels of plasma vitamin A appear to be kept at a particular range (Table 1.2.2) under normal conditions. Increases in plasma levels occur when retinyl esters appear in the circulation with the onset of hypervitaminosis, and decreases occur when liver stores are completely exhausted. Therefore, plasma vitamin A levels are not good indicators of dietary intake levels except in severe hypo- or hypervitaminosis A (Wolf and Phil, 1982).

The RBP and vitamin A need to be bound to each other in order to be secreted from lipocytes into the circulation. Under normal conditions RBP is synthesized in the liver at a rate independent of vitamin A status. Therefore, in vitamin A deficient animals, RBP levels build up in the lipocytes. Administration of retinol to these animals stimulates RBP secretion by the lipocytes which induces more RBP synthesis (Smith et al., 1980), since a reduction in hepatic RBP level is experienced. The synthesis of RBP, however, proceeds independently of the vitamin A status under normal situations. Protein deficiency which could reduce RBP synthesis can affect vitamin A secretion and hepatic levels (Underwood et al., 1979).

Factors that affect vitamin A or beta-carotene absorption (and/or conversion), bile production and secretion, hepatic function, RBP production and secretion can affect vitamin A metabolism.

V. Physiological functions of vitamin A and effects of deficiency.

a. Vision

Retinol functions in the eye in transmission of the light stimuli to the brain. All-trans retinol is converted to 11-cis retinol by the action of retinol isomerase. Retinol reductase then converts (oxidizes) 11-cis-retinol to 11-cis-retinal which binds with opsin to produce rhodopsin in the retina. The pigment rhodopsin is produced in the dark and light energy will bleach this light sensitive pigment, causing dissociation of opsin and 11-cis-retinal and also conversion of 11-cis-retinal to trans-retinal. The isomerization of 11-cis-retinal to the trans form triggers a nerve impulse to the brain via the optic nerve and seeing of color is registered. In the dark rhodopsin is regenerated (1.2.2).

Vitamin A is stored in the retina pigment epithelium. Vitamin A deficiency caused by inadequate intake of vitamin A is usually first manifested by night blindness due to insufficient vitamin A present to regenerate rhodopsin. Prolonged lack of rhodopsin may result in degeneration of the retina epithelium and ultimately in irreversible blindness.

b. Maintenance of the integrity of normal epithelial tissue.

As mentioned previously, vitamin A has an important role in forming membrane glycoproteins (DeLuca and Wolf, 1972). In the absence of vitamin A, cell surface glycoproteins are found to be under-glycosylated and glycolipids are also affected. The most general morphological change occurring in vitamin A deficiency is the replacement of the mucus lining of epithelial tissue by a squamuous metaplastic epithelium which eventually produces large amounts of keratin. These changes in epithelial cells in vitamin A deficiency are widespread and include the respiratory and alimentary tracts and corneal epithelium of the eye. Salivary glands are also affected. Loss of appetite in vitamin A deficiency may be connected to these changes. In the small intestine during hypovitaminosis A, the number of goblet cells which are

responsible for mucus production in order to lubricate the lumen and also buffer the environment decrease. Also, the protein and mucin production capacity of these cells in rats diminishes (DeLuca et al., 1969). The vitamin A deficient rats had a pH of 5 in this area of the intestine. The acidic environment and lack of mucus protection favors the enteritis complex. The mucosal changes and reduced protein synthesis take place at a very early stage of the deficiency, before the weight plateau stage begins (DeLuca et al., 1969). Furthermore, according to Moran (1982), the greater the number of goblet cells and, in turn, the amount of mucin in the crypts, the less the opportunity for small molecules like enterotoxins produced by colibacilli to enter the gaps between the cells to be absorbed and manifest enterotoxemia. Xerophthalmia is due to depressed mucus secretion by the corneal epithelium. Also, changes in the integrity of epithelial cells may affect resistance to bacterial infections. In milking Holstein cows deficiency in vitamin A is associated with a higher incidence of mastitis (Chew et al., 1982). Potential pathogens are regularly in the cow's teat but do not always cause infections. The functional state of epithelium regulates transfer of immunoglobulins and polymorphonuclear leukocytes and production of bacteriocidal agents like keratin. When this regulation mechanism breaks down as a result of impairment of epithelial integrity, infections

develop (Chew et al., 1982). In vitamin A deficient rabbits, ataxia, diarrhea, emaciation and respiratory disturbances frequently accompany the ocular lesions and can often be the cause of death. The secondary infections in these rabbits may also be attributable to breakdown of epithelial cell integrity (Payne et al., 1972).

c. Bone growth

Vitamin A plays a role in the maintenance of mesenchymal structures, with considerable changes occurring in cartilage and bone in deficiency and excess vitamin A. In growing animals when deficient in vitamin A, bones become shorter and thickened (Roels et al, 1969). Hypervitaminosis A results in bone lesions, decreased length and width and decreased osteoblasts. Excess vitamin A acts through a detergent effect which solubilizes lysosomal membranes and results in release of proteolytic enzymes. Vitamin A deficiency can also impair stability of lysosomal membranes by making them more fragile (Roels et al., 1969).

d. Cerebrospinal fluid pressure

In mammals, cerebrospinal fluid is mainly formed at choroid plexuses, flows through the ventricular system to the subarachnoid space and, in part, returns to the blood via arachnoid villi. Changes in permeability of arachnoid villi cells (route of absorption), choroid plexuses (formation site) and faulty bone growth, as a result of vitamin A imbalance, can cause abnormalities in cerebrospinal fluid pressure.

In man and rats both hypo- and hypervitaminosis A cause elevated CSF pressures. However, in calves, puppies, and pigs, low vitamin A causes elevated pressures but high vitamin A causes reduced CSF pressures (Eaton, 1969).

The increased cerebrospinal fluid pressure in human vitamin A intoxication gives rise to headaches, vomiting, diplopia, visual field defects and papilledema in adults and hydrocephalus in newborns (Mahoney et al., 1980) and fetuses. In rabbits, hydrocephalus is caused by both hyper- (Cheeke et al., 1984) and hypovitaminosis A (Payne et al., 1972).

e. Reproduction

In males, vitamin A deficiency leads to testicular degeneration of germinal epithelium and cessation of spermatogenesis (Chaudhary and Nelson, 1985; Payne et al., 1972; Unni and Rao, 1985). Vitamin A deficient rats also have lowered circulating androgen levels. The target cell appears to be the Leydig cells of the testes. This can be reversed by administration of retinoic acid (Bondi and Sklan, 1984). In vitamin A deficiency of rats, in addition to cessation of spermatogenesis of the primary spermatocyte stage, the structural integrity of Sertoli cells is also impaired. These cells are responsible for androgen binding protein (ABP) production in response to FSH binding to FSH receptors, is also impaired. This damage to Sertoli cells would reduce the ABP production and result in higher plasma FSH since FSH-membrane receptor binding is decreased. Administration of vitamin A enhances this binding (Unni and Rao, 1986). Vitamin A deficiency before sexual maturity is more effective in reducing viability of the sperm and volume of ejaculate. Payne et al., (1972), observed no significant changes in libido of male rabbits with change in dietary intakes of vitamin A, perhaps because Leydig cells which produce testosterone were not damaged in rabbits.

In females, vitamin A is required for vaginal epithelium maintenance. Vitamin A deficiency during pregnancy results in resorption of the placenta or spontaneous abortions in rats (Lorente and Miller, 1977). Vitamin A deficiency causes necrosis of the junctional zone of placenta and also a decline in steroid and mucopolysaccharide synthesizing enzymes which is probably the cause of abortion in rats (Sharma et al., 1986). In humans, abruptio placentae has a common occurrence in populations with poor socio-economic situations. Vitamin A, beta-carotene and vitamin E were all shown to be lower in abruptio placentae than those in normal pregnancy (Sharma et al., 1986). Whether multiple vitamin deficiency causes abruptio placentae or is itself the result of some other metabolic disorder is yet to be found. However, low levels of these fat soluble compounds in abruptio placenta could also result from inadequate intake of these nutrients, impaired gastrointestinal (G.I.) absorption which might be caused by metaplasia of G.I. eptithelial cells during vitamin A deficiency or enhanced catabolism of the compound (Sharma et al., 1986). Also, under vitamin A deficient conditions malformed fetuses may result. Excess retinol or retinoic acid can have teratogenic effects (Donaghue and Copp, 1981) even in humans. However, in general, transplacental vitamin A transfer is highly regulated with minimal changes in fetal vitamin A status.

Vitamin A does not appear to have a physiological role in either maintenance of rat mammary epithelium or in its potential for hormone-dependent phenotypic expressions like casein formation in response to glucocorticoids (Sankaran and Topper, 1982). There are vitamin A esters in milk which help bring up the relatively low levels of vitamin A reserves in newborns.

f. Growth

Vitamin A affects both cellular proliferation and differentiation. Therefore, growth is reduced with vitamin A deficiency . The reduced regeneration of injured liver cells after partial hepatectomy in vitamin A deficient rats can be enhanced by vitamin A administration (Ganguly et al., 1980). In swine, vitamin A-supplemented dams had heavier litters at birth and weaning than deficient ones (Brief and Chew, 1984). Excessive vitamin A intakes can also result in depression of growth (Donoghue et al., 1981).

g. Infectious Diseases

Vitamin A deficient animals are more susceptible to bacterial, viral, protozoal and rickettsial infections, possibly due to changes in epithelial tissue.

Vitamin A increases non-specific resistance to infection. Reversal of post-surgical immunodepression by administration of vitamin A in man has recently been demonstrated (Bondi and Sklan, 1984).

VI. Dietary factors affecting vitamin A.

a. Protein

Adequate dietary protein is required in order to synthesize the proteins associated with both the absorption and transport of carotenes and retinol. Under conditions of inadequate protein intake conversion of beta-carotene to retinol in the intestinal mucosa is depressed (Underwood et al., 1979). Also, the hydrolysis of retinyl esters in the lumen of the intestine is decreased. Furthermore, RBP production is sensitive to inadequate intakes of protein (Underwood et al., 1979). Children with xerophthalmia also

suffering from protein-energy malnutrition do not respond to vitamin A supplemention unless also supplemented with protein (Mclaren, 1984).

b. Vitamin E (alpha-tocopherol)

High dietary vitamin A intake depresses tissue and plasma vitamin E in chicks, rats and calves (Frig and Broz, 1984). High dietary vitamin E, on the other hand, enhances vitamin A levels in rats but prevents the signs of hypervitaminosis A by reducing liver depletion (Sklan and Donoghue, 1982). When both vitamins are fed orally, tissue oxidation of vitamin E occurs; however, if vitamin A is administered orally and vitamin E is injected, this does not happen. Therefore, the basis for antagonistic effects of high levels of vitamin A on vitamin E seems to be at the level of the gut prior to intestinal absorption. Excess dietary vitamin A seems to cause a greater fraction of vitamin E to be oxidized prior to the digesta reaching the duodenum. Vitamin E glucuronide secretions are also enhanced (Frigg and Broz, 1984). Therefore, excess vitamin A seems to increase vitamin E requirements and the dosage of the two vitamins should not be considered independently of each other.

c. Vitamin K

Retinoic acid is a remarkably potent antagonist to vitamin K in rats. It does not significantly alter excretion of vitamin K but rather impairs absorption. This form of vitamin A inhibits only part of the absorption capacity for vitamin K and further ingestion of it is without effect on prothrombin concentration (Matschiner et al., 1967).

d. Zinc

Zinc deficient animals have lower blood retinol and RBP and higher liver vitamin A than zinc adequate animals. This may be because of zinc involvement in production of RBP and also hyrolysis of retinyl-palmitate to retinol in the liver. Another area of zinc-vitamin A interaction may be the oxidation-reduction of retinol in peripheral tissues. The conversion of retinol to retinal involves an alcohol dehydrogenase which is a zinc metalloenzyme and in zinc deficiency both retinol oxidase and reductase are depressed (Bondi and Sklan, 1984).

e. Copper

Beta-carotene is cleaved at carbon-15 to yield two molecules of retinal in the intestinal mucosa cells by a copper containing dioxygenase enzyme. This activity may be reduced in copper deficiency. Copper and retinol behave similarly in being stored preferentially in the liver and are carried out into the plasma associated with similar but different alpha-globulins. Other than this the interrelationships of these two nutrients are often inverse. Factors that increase the concentration of one often decrease the concentration of the other (Moore et al., 1972). For example, as cited by Moore et al. (1972), the blood of healthy men contained on average about 20% more vitamin A than that of women but for Cu, the average is 10% higher for women. Also, in both sexes fever is usually associated with a greatly decreased blood retinol concentration while the Cu concentration is increased. This inverse relationship did not exist in normal sheep in the field and a direct correlation between copper and retinol was established. However, the liver of a single lamb that died of Cu deficiency contained only traces of Cu but had a retinol content at the top of the normal range (Moore et al., 1972).

VII. Vitamin A Toxicity.

Factors that determine toxicity of vitamin A include species, age, weight, health and duration and dose of intake. The rate of vitamin A excretion from the body can be readily exceeded when intakes of vitamin A are much more than the requirements for a variable length of time. Table 1.2.3. lists some vitamin A requirements for different species. According to Mclean (1984), in humans the RDA for vitamin A is set at 3300 IU for adult males and about 2664 IU for adult females. However, Weber et al. (1982) suggest 5000 IU as the RDA for adult humans. For infants and children up to 12 years of age, 1500-4500 IU is the recommended daily allowance. When liver storage becomes saturated, increasing amounts of esterified retinol appear in the plasma, transported by lipoproteins instead of RBP. The main lipoprotein fraction responsible for this is VLDL. However, vitamin A has been detected associated in lower amounts to LDL and even with HDL in much lower concentrations (Schindler and Klopp, 1986). This could result in non-specific delivery of either retinyl esters or retinol by lipoproteins to peripheral sites of cytotoxic action (Halevy and Sklan, 1986; Schindler and Klopp, 1986). This would result in membranolytic action of retinol and cell damage. The RBP seems to have a protective action, since retinol bound to RBP does not appear to manifest its

surface-active effects on biological membranes (Mallia et al., 1975). Some symptoms of hypervitaminosis A appear at about the same time as ester levels increase in the plasma. Table 1.2.2. supplies some normal blood vitamin A levels in different species. In general, symptoms of vitamin A toxicity include hepatic disorders like fatty liver, bone fragility, changes in cerebrospinal fluid pressure, secondary vitamin K and E deficiency, loss of hair, anorexia, reproductive problems and hydrocephalus.

a. Liver disorders

Hypervitaminosis A does not significantly reduce the rate of hepatic triglyceride secretion; rather, it produces fatty liver by stimulating the synthesis of triglycerides in the liver and their release into the plasma (Singh and Singh, 1978). The hypertriglyceridemia can then be caused by reduction in periphery uptake of triglycerides. It has been demonstrated that excess intake of vitamin A increases levels of corticoids in the plasma and their synthesis in the adrenal cortex. Glucocorticoids are known to cause impairment of peripheral utilization of plasma triglycerides by inhibiting lipoprotein lipase activity in the adipose tissue (Singh and Singh, 1978). Other possible

liver problems are hepatomegaly (Anonymous, 1982) and cirrhosis due to deposition of collagen produced by Ito cells or fibroblasts (Weber et al., 1982).

b. Bone disorders

Cells and subcellular organelles are penetrated and subsequently lysed by retinol when it is present in large doses. Administration of large doses of vitamin A to rabbits causes destruction and depletion of their cartilage matrix, increased blood chondroitin levels, and increased sulfur excretion (Roels et al., 1969). This proteolytic activity can also be demonstrated in vitro and is a result of lysosome enzyme activation. Retinol and retinoic acid are active in release of cathepsins, proteolytic enzymes that are normally contained in cytoplasmic particles called lysosomes (Roels et al., 1969). In young rats, hypervitaminosis A renders the long bones fragile, so that they sometimes suffer spontaneous fracture. In the limb bones of young rats and guinea pigs the maturation and degeneration of the cartilage cells and the replacement of cartilage by bone cells is greatly accelerated. As a result the fracture occurs because of the extensive loss of previously formed cortical bone before the newly deposited

bone has acquired sufficient firmness to meet mechanical requirements (Fell and Mellanby, 1952).

c. Cerebrospinal fluid (CSF) pressure alterations

Alterations in CSF pressure are different depending on species. As mentioned before, hypervitaminosis A increases CSF pressure in species like humans and rabbits and reduces it in other species like cattle and pigs. In humans, elevated CSF pressure gives rise to headaches, vomiting, diplopia, visual field defects, papilledema, and bulging fontanelles without focal signs of cerebral damage (Mahoney et al., 1980) and this may also be the cause of hydrocephalus in fetuses. These conditions would generally subside as vitamin A reserves of the body are depleted.

d. Anorexia

Vitamin A affects the epithelial tissues of the alimentary tract. Changes in salivary glands and epithelial cells might account for loss in appetite in both vitamin A deficiency and toxicity (Anonymous, 1982a; Bondi and Sklan, 1984).

e. Reproductive problems and hydrocephalus

The types of vitamin A found in fetuses are the same as those in their dams. The site of vitamin storage in fetuses is also the liver. According to Lorente and Miller (1977), vitamin A affects the fetuses directly rather than through damage to the placenta. In rats, high levels of vitamin A given to the dam increase the fetal liver to maternal serum vitamin A ratios, allowing it to reach levels capable of causing malformation but not death. However, rabbits protect their fetuses by not allowing this ratio to rise as quickly (Lorente and Miller, 1977). At high enough maternal serum levels this barrier breaks down and embryos are subjected to very high levels of vitamin A which can cause embryonic mortality (Lorente and Miller, 1977). Therefore, rabbits are more resistant to teratogenic effects of vitamin A than rats. Hydrocephalus is one of the teratogenic effects of excess vitamin A in rabbits (Cheeke et al., 1984). This, as mentioned before, may be caused by elevated CSF pressures in the cranial area of the fetus. Another embryonic malformation caused by excess vitamin A may be shortened limbs which is related to the adverse effects of excessive vitamin A on bones (Roels et al., 1969; Fell and Mellanby, 1952).

In order to better illustrate the practicality of the hypervitaminosis problem in humans, some case reports will be discussed. A 20 year old female was admitted to hospital for evaluation of a possible brain tumor. Two days before admission she experienced emesis and diplopia and severe headaches. Two months before admission, she became anorectic with increasingly severe headaches and diffuse scaling erythematous dermatitis. Two years before admission she began taking 50,000 IU of vitamin A per day for acne. The severe headache was caused by increased CSF pressure. Elevation of CSF pressure was either due to excessive formation of cerebrospinal fluid because of increased permeability of the choroid plexus or decreased absorption in the subarachnoid space, both attributable to the toxic effects of free vitamin A on cell membranes (Anonymous, 1982b).

A 62 year old male was admitted to hospital with edema, protein malnutrition and abnormal liver function and hepatomegaly. He had had an intake of approximately 50,000 IU/day of vitamin A for seven years. In this case there was an absence of peripheral manifestations of vitamin A toxicity, apparently because he had protein malnutrition which impaired RBP and/or also lipoprotein synthesis and

vitamin A secretion into the circulation. This resulted in accumulation of large amounts of vitamin A in the liver and cholestasis (Weber et al., 1982b).

Another interesting case dealing with RBP is of a 42 year old vegetarian man who consumed about 100,000 IU/day of vitamin A for ten years and showed no symptoms of peripheral symptoms of hypervitaminosis A until he contracted hepatitis B which unmasked the toxicity in about three weeks. The virus injury seemed to have promoted retinyl ester mobilization. Since Ito cells which store vitamin A are not known to be destroyed in hepatitis B, this ester mobilization was not attributable to necrotizing effects of the virus (Hatoff et al, 1982). However, during the course of this disease as well as other types of liver damage (Weber et al., 1982), the RBP level is reduced. The drastic reduction in RBP in hepatitis B in turn could have reduced retinol mobilization and its plasma levels. As a result, retinyl palmitate could have been stored in the liver at increasingly higher rates and the liver approached its retinyl ester storing capacity threshold at an increasing rate, causing overflow of the vitamin A unbound to RBP and hence peripheral manifestations of hypervitaminosis A. Furthermore, hepatocytes are known to store minor amounts of vitamin A (Anonymous, 1982b). Since this patient was a vegetarian, he might not have had enough

dietary protein and this reduced lipoprotein levels as well as RBP in the liver and therefore postponed the mobilization of retinyl esters. This could have been the cause of an even more abnormally high hepatic vitamin A level. It is possible that under such conditions hepatocytes participated more in vitamin A storage and ended up containing much more vitamin A than usually anticipated. In this case, the necrotizing effects of viruses on hepatocytes and not necessarily lipocytes (Ito cells) might have been enough to cause release of extremely high levels of retinyl esters in the blood. One last possibility is that hepatitis B may cause reduced retinyl ester absorption by the Ito cells in the liver as blood was passing through the liver from the intestine to the periphery. This would cause elevated blood and possibly adipose tissue retinyl ester levels. One of the symptoms of hypervitaminosis is anorexia. Anorexia increases production of glucocorticoids in the adrenal cortex and they would in turn mobilize fat and triglycerides. The loss of adipose tissue would release the vitamin A as well as other toxins stored in this tissue into the blood and create toxicity of vitamin A. Children and infants seem to be more sensitive to hypervitaminosis A (Anonymous, 1982). Mahoney et al. (1980) reported a case of dizygotic infants who had a total vitamin A intake of 36,000 IU/day and developed hypervitaminosis A. The main dietary ingredient

implicated was chicken liver, fed to them twice daily by the mother. The symptoms included red, rough skin, vomiting, and bulging of anterior fontanelles due to enlarged brain ventricles and increased CSF pressure.

Fortunately, once the situation is recognized as either vitamin A deficiency or toxicity, it can be corrected simply by either supplementation of or depletion from the diet, except in cases like xerophthalmia, where the pathogenesis is at the irreversible stage.

The cases mentioned here, along with numerous others found in the literature, may only represent a small fraction of the U.S. and European populations with hypervitaminosis A. With the medical indications for treating acne vulgaris with vitamin A and the still controversial preventative role of retinoids in carcinogenesis, we may be on the verge of an epidemic of hypervitaminosis A (Anonymous, 1982b).

Table 1.2.1. Efficiency of conversion of B-carotene to vitamin A in different species.

Species	Conversion Efficiency %
Rat	100
Chicken	100
Rabbit	100
Dog	67
Man	33
Horse	33
Sheep	30
Pig	30
Cattle	24

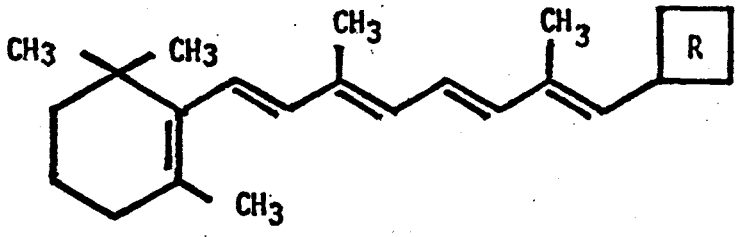
Table 1.2.2. Plasma and liver vitamin A levels for different species.

Species	IU/ml	IU/g liver	Pertaining to	Source
human	1.67	-	normal levels	Anonymous, 1982a
human	.67-2.67	.33-10	normal levels	Anonymous, 1982b
human	.67-2.67	<10	normal levels	Hatoff et al., 1982
human	.67-1.67	-	normal levels	Mclaren, 1984
rabbit	3.53	565.35	experimental control value	Lorente and Miller, 1977
rat	1.20	800.30	experimental control value	"
rat	1-1.6	-	normal values	Underwood et al., 1979
rat	1.3-2.0	-	fed 25000 IU/kg for 3 weeks	Mallia et al., 1975

Table 1.2.3. Dietary vitamin A requirements.

Species	Vitamin A		β-Carotene	Source
	ug/kg feed	IU/kg feed	mg/kg	
Swine				Bondi and Sklan, 1984
Growing	500	1667		
Breeding	1330	4433		
Lactation	1100	3667		
Cattle				Bondi and Sklan, 1984
Growing	730	2433	4.0	
Lactation	1070	3567	8.0	
Sheep				Bondi and Sklan, 1984
Growing	500	1667	2.5	
Lactation	730	2433	3.0	
Rabbit				Lebas, 1980
Growing	3000	10,000		
Lactation	3600	12,000		
Rat	1200*	4000*		NRC, 1978
Human	1500*	5000*		Weber et al., 1982
Male	990*	3300*		Mclaren, 1984
Female	799*	2664*		Mclaren, 1984
<12 year old	450-1350*	1500-4500*		Mahoney et al., 1980

* Daily vitamin A requirement.



	R
Retinol	$-CH_2OH$
Retinal	$-C(=O)H$
Retinoic acid	$-COOH$
Retinyl palmitate	$-CH_2OC(=O)-(CH_2)_{14}CH_3$

Figure 1.2.1. Molecular structures of retinol and some of its derivitives.

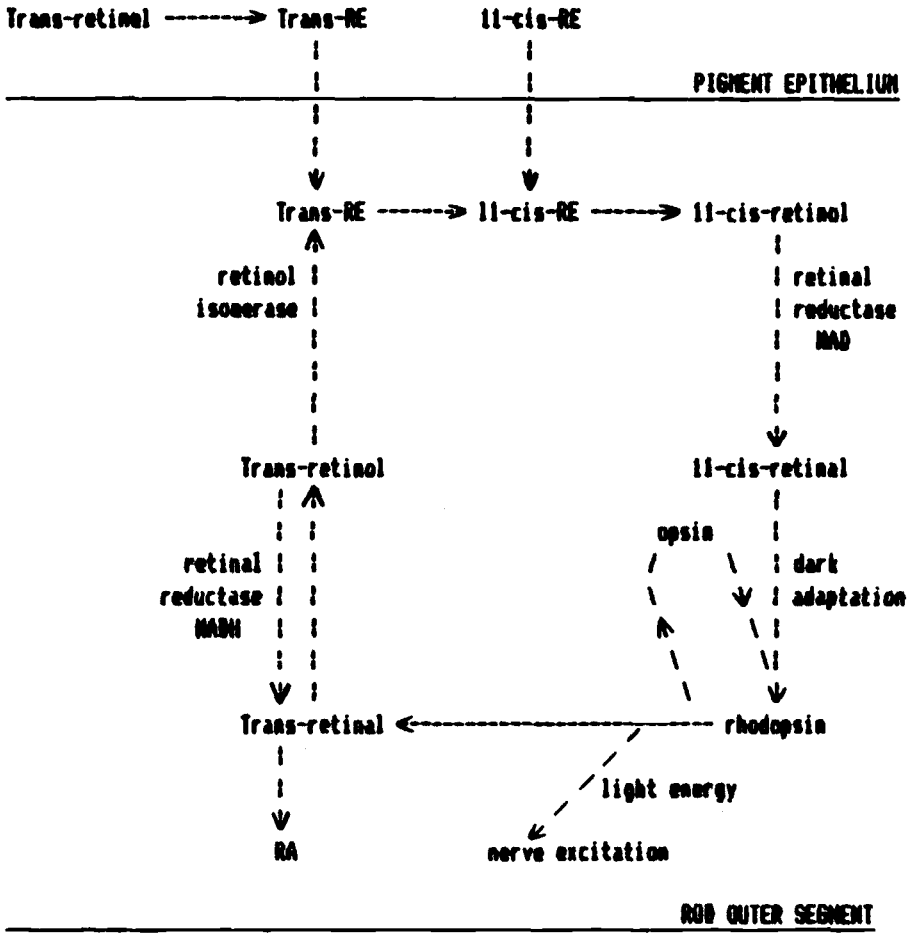


Figure 1.2.2. Role of vitamin A in visual cycle of a frog. Adapted from Bondi and Sklan (1984).

1.3. Pyrrolizidine Alkaloids

Alkaloids are basic substances that contain nitrogen in a heterocyclic ring. Pyrrolizidine alkaloids (PA) contain a pyrrolizidine nucleus and side chains at positions 1 and 7 (Figure 1.3.1). The PAs comprise some 200 substances, many of which are hepatotoxic (Ridker et al., 1985). All hepatotoxic PAs share the common characteristics of a 1,2 double bond in the ring and esterification of the CH_2OH groups in the side chain(s) (Cheeke and Shull, 1985). Most PA-containing plants that have caused livestock poisoning are in the genera Senecio, Crotalaria, Heliotropium and Echium (Cheeke and Shull, 1985).

Symptomatology of Senecio PA poisoning in animals had been referred to by different names in different parts of the world before the underlying etiology was finally discovered by experiments in the early 1900's. In the 1700's a problem called stomach staggers in Wales was thought to be due to consumption of Senecio jacobaea (tansy ragwort). In Nova Scotia a similar problem was called Pictou disease, while in New Zealand it was called Winton disease (Cheeke and Shull, 1985). Liver cirrhosis, jaundice and, in the case of horses, staggering, pressing

the head against objects, and walking in a straight line regardless of obstruction were noticed. Death is always caused by irreversible liver damage (Cheeke et al., 1985).

The PAs are also known to cause poisoning in humans. Senecio contamination of bread and tea in Africa and India have been the cause of "Chiari's syndrome" (Mclean, 1970). In Jamaica, "bush tea" is made from leaves picked from the bush and infused with hot water. These teas often contain PAs and are used as medicine and often drunk by children. As cited by Mclean (1970), Hill et al. (1953) observed a veno-occlusive disease characterized by collagenous occlusion of the small branches of the hepatic venous tree common among Jamaican children. There have also been instances of epidemic veno-occlusive disease in Afghanistan and India which resulted from contamination of millet with Crotalaria and wheat by Heliotropium seeds (Anonymous, 1978). There are numerous other human PA poisoning cases but one of the newest ones was reported in the U.S. by Ridker et al. (1985). According to this report, venocclusive disease, a form of Budd-Chiari syndrome, was diagnosed in a 49 year old woman who regularly consumed large amounts of herbs, vitamins, and "natural" food supplements containing PA. Upon analysis it was found that the main source of PAs was the comfrey (Symphytum

officinale) she used as a natural food supplement. Her estimated PA intake was 14.1 ug/kg body wt./day for about four months or a total of 1700 ug/kg body weight.

It is also worth noting that in certain instances, these alkaloids have been detected in milk and honey, as well as herbal teas and preparations. With respect to contamination of milk with PAs, goats could be of most concern since they are owned by families who drink the milk directly and repeatedly, whereas cow's milk would be diluted when pooled with other batches of milk (Dickinson, 1974). Another important factor to consider is that cows are very susceptible to PAs whereas goats and sheep are not (Table 1.3.1). Therefore, a cow might not be able to contribute to PA contamination of milk for very long. Within families, children drink more milk than others and this could further complicate the problem since younger individuals are more susceptible to toxins than adults. Contamination of honey with PAs from tansy ragwort poses another potential threat to human health. Since the tansy ragwort flowers contain the highest concentrations of PAs in the plant, it is likely that the bees acquire considerable amounts of PAs during nectar gathering. Dickinson (1976) reported values of 0.1 mg/kg honey. Deinzer and Thomson (1977) detected PAs and tansy ragwort pollen in four honey samples collected from western Oregon

in areas where the weed flourishes. Values for alkaloid content ranged from Ø.3 mg/kg to 3.9 mg/kg honey. Sometimes at concentrations above Ø.1 mg/kg the tansy ragwort odor is detectable and the honey becomes unpalatable (Dickinson, 1976). This demonstrates that one does not necessarily have to live in a third world country or be a "natural food" consumer to be exposed to PAs.

The parent pyrrolizidine alkaloids are non-toxic, but mixed function oxidase (MFO) system enzymes in the liver dehydrogenate the 1,2-dehydropyrrolizidine ring, forming pyrrole (dihydropyrrolizidine) metabolites (Figure 1.3.1) which are responsible for the toxicity (Mattocks, 1968). The hepatic effects of PAs are thus due to the reaction of pyrroles and/or other metabolites with tissue components, primarily DNA. As a result, a mixture of DNA-DNA interstrand cross-links and DNA-protein cross-links can be observed (Petry et al., 1984). Recently, Segal et al. (1985) isolated a highly reactive aldehyde, trans-4-hydroxy-2-hexenal (t-4HH), as a metabolite of the PA senecionine. Induction of the MFO system by drugs, such as phenobarbital, increased and inhibition of MFO by drugs like chloramphenicol reduced conversion of PAs to reactive metabolites (Allen et al., 1972). The classic pathological conditions associated with PA poisoning are centrilobular necrosis of hepatocytes (parenchymal cells), megalocytosis,

fibrosis, biliary hyperplasia and venocclusion (Mclean, 1970). Other symptoms include pulmonary, heart, gastrointestinal tract and kidney lesions and ascites (Cheeke and Shull, 1985). Susceptibility is species dependent, as shown in Table 1.3.1 (Shull et al., 1976). According to Shull et al. (1976), the susceptible species have higher rates of liver pyrrole formation than resistant ones and the resistant animals can become susceptible by phenobarbital administration which induces the MFO system. An exception to this, however, is the rabbit. Even though rabbits have a high rate of pyrrole formation, they are resistant to dietary PAs (Shull et al., 1976). In sheep some rumen detoxification of PA takes place. Lanigan (1976) showed that a microorganism present in sheep rumen could detoxify heliotrine, a PA present in heliotropium species, to nontoxic metabolites by reduction of 1,2 double bonds in the nucleus and cleavage of the esters which gave rise to a 1-methylene derivative. The resistance of sheep to Senecio PAs, however, does not appear to be due to rumen metabolism of these PAs. Shull et al. (1976) and Swick et al. (1983) reported that incubation of Senecio jacobaea (SJ) with sheep rumen fluid did not reduce its toxicity, and that no 1-methylene derivative was produced. The difference in Senecio PAs and heliotrine is that heliotrine is a monoester, susceptible to esterases and hydrogenations. The toxicity and stability of PAs depend on a number of

factors, including the number of ester chains, cyclization of side chains, branching of the side chains, and presence of epoxide moieties. The more of these factors a PA posseses, the more toxic it becomes. Senecio PAs are closed cyclic diesters which are more toxic and more resistant to reductions due to steric hindrance. Heliotrope PAs are, on the other hand, monoesters and therefore less toxic (Cheeke and Shull, 1985). However, Bull et al. (1956) demonstrated that long term ingestion of the PA-containing Heliotropium europium predisposed sheep to toxic hepatic accumulation of copper. Miranda et al. (1981b) and Swick et al. (1982) demonstrated accumulation of copper in the liver of rats fed tansy ragwort with supplemental copper. In sheep, PAs cause enlargement of the parenchymal cells (megalocytosis) accompanied by an increased avidity of the cells for copper which leads to the extremely high levels of copper in the liver (Underwood, 1977). Another reason for high levels of copper in the liver is reduced biliary excretion of copper due to biliary hyperplasia caused by the PAs. The PAs are known to cause disruption of cytoplasmic organelles such as mitochondria as well as rupture of plasma membrane (Johnson, 1981). Copper tends to accumulate in mitochondria, lysosomes, and microsomes of rat liver cells (Miranda et al., 1981b). When copper is liberated in sufficient concentration from organic combination within the cell, it becomes toxic to the cell (Underwood, 1977).

The mechanism of copper toxicity as suggested by Metz and Sagone (1972) is oxidative injury to the membranes. Therefore, the hemolytic crisis due to copper toxicity may be enhanced by PA as follows. The PAs would cause reduced biliary excretion of copper, causing elevated liver copper levels. The megalocytosis caused by the PAs would enhance the capacity of the hepatocytes to sequester and store copper because of the increase in size. The PAs, on the other hand, have the capability of rupturing cells by their effects on membranes (Johnson, 1981). This would release high levels of copper previously stored in mitochondria and lysosomes into the cytoplasm. At this point there would be elevated levels of both copper and proteolytic enzymes (from lysosomes), both capable of destroying membranes, enhancing the membrane rupturing effects of PA metabolites. Once the cell has lysed, there would be high levels of copper present in the blood which could oxidize RBC membranes, causing release of hemoglobin (Swick et al., 1982). The result is hemoglobinemia and hemoglobinuria, the most prominent signs of the hemolytic crisis.

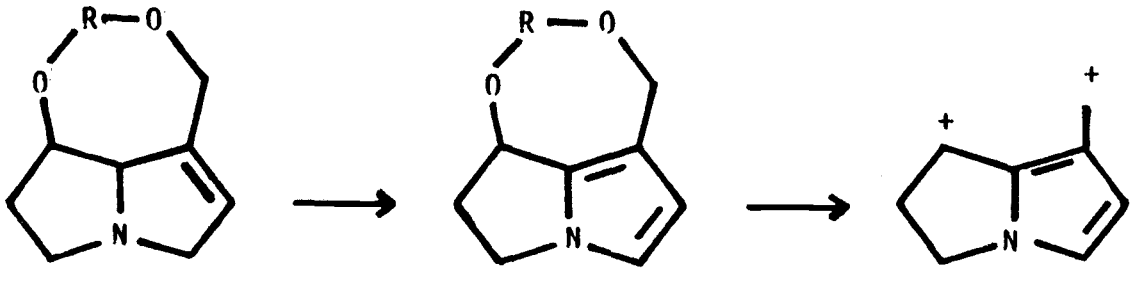
Some dietary supplements like the antioxidants butylated hydroxyanisole or BHA (Miranda et al., 1981c) and ethoxyquin (Miranda et al., 1981a), and vitamins B6 and B12

(Mclean, 1970) have shown some protective effects against PA toxicosis. However, a practical dietary supplement to protect farm animals against PA toxicosis is yet to be discovered (Garrett et al., 1984; Cheeke et al., 1985).

Table 1.3.1. Order of susceptibility of animal species to pyyrolizidine alkaloid toxicity.

Species	Susceptibility to PA toxicosis	Lethal dose as % of body wt.
Cow	High	3.6
Horse	High	7.3
Rat	High	21.0
Chicken	High	39.0
Mouse	Intermediate	?
Rabbit	Low	>113.0
Guinea Pig	Low	119.0
Goat	Low	205.0
Sheep	Low	302.0
Hamster	Low	338.0
Japanese Quail	Low	2450.0
Gerbil	Low	3640.0

Adapted from Cheeke and Shull, 1985 and Shull et al., 1976.



Alkaloid

Pyrrole derivitive

Pyrrole (alkylating agent)

Figure 1.3.1. Conversion of pyrrolizidine alkaloids to derivitive alkaloids to pyrrole, the toxic metabolite.

Chapter 2

METHODOLOGY:

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

ANALYSIS OF VITAMIN A

In order to analyze blood, liver, feed and fecal samples for vitamin A and possibly its esters, high performance liquid chromatography (HPLC) methods were used. An extensive search through the literature and assistance from the Oregon State Department of Agriculture led to finding of such methods. Changes were made when appropriate. The need for these changes was brought about as a result of the differences in vitamin A levels and the nature of our experimental samples and those of others (Miller et al., 1984 and Muniz, 1986). The feed analysis method suggested to us by Muniz (1986) was adapted to analysis of liver and fecal samples.

Cautions: Vitamin A, its esters, and beta-carotene are all unstable compounds. To minimize their loss the samples and standards should be kept frozen and under nitrogen when not in immediate use. It is wise to use amber glassware to block ultraviolet light, and keep the temperature as low as possible when working with these compounds.

2.1. STANDARD PREPARATION

Vitamin A, its esters and beta-carotene are unstable compounds, therefore, the working standards were prepared daily except in the case of retinyl acetate which is more stable (Miller, 1984) and was prepared weekly.

The compounds were purchased from Sigma Chemical Company. The compound of interest was dissolved in 37/63, acetonitrile/methanol (HPLC grade). A combination of these two solvents seemed to make the compounds of interest more stable (Muniz, 1986). The concentrations of the compounds in the standard solutions were determined spectrophotometrically using the extinction coefficient and observed biopotency (O.B.) values provided in table 2.1.1 and the formula in the calculation section. Retinol and beta-carotene linearity plots are provided in figures 2.1.1 and 2.1.2, respectively.

Table 2.1.1. Important parameters to consider for vitamin A.

Compound	MW	Extinction coefficient $E^{1\%}_{1\ cm}$	Biopotency X10⁴	ug equivalent to 1 IU	Multiplication factor to convert to IUs of retinol	Optimal UV absorption in 37/63,Acn/meoH (nm)
Retinol	286.44	1835.0	3.330	.300	1.0000	325.0
Retinyl acetate	328.00	1550.0	2.904	.344	1.0014	323.0
Retinyl palmitate	496.34	975.0	1.817	.550	1.0576	325.0
β-carotene	536.85	2518.0	1.670	.600	2.1342	451.0

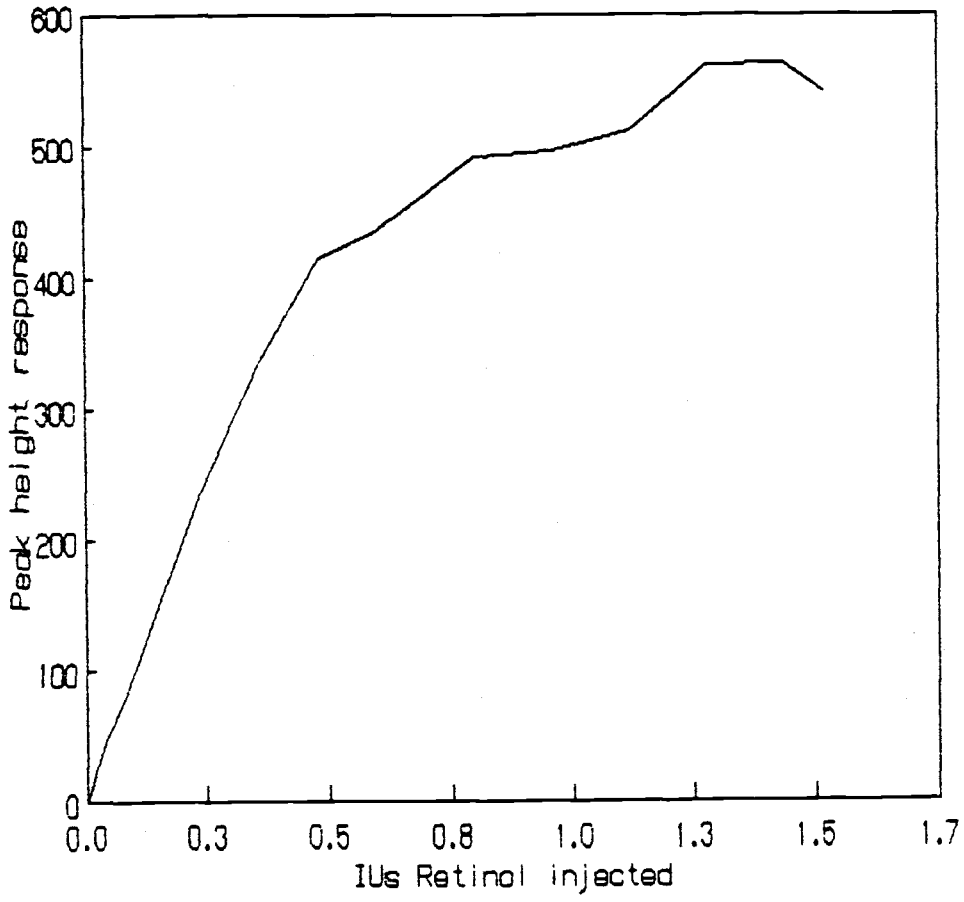


Figure 2.1.1. Retinol standard linearity plot.

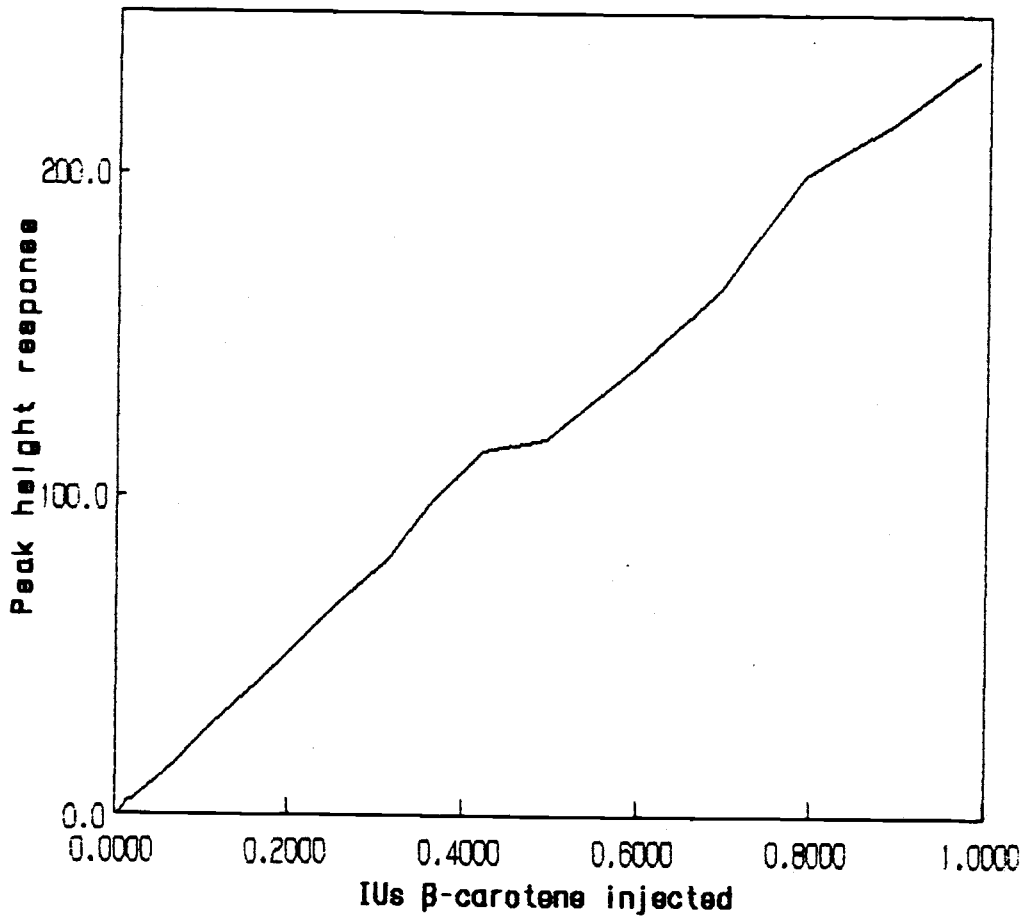


Figure 2.1.2. β-carotene standard linearity plot.

2.2. INSTRUMENTATION

Reverse phase HPLC analysis was performed, using a two pump solvent delivery system which included two model 510 pumps, model 680 automated gradient controller, model 740 data module, model 710B WISP, model 441 UV absorbance detector, and a C-18 guard column all purchased from Waters Associates Company and also a C-18 Vydac column, and Baker analyzed HPLC grade solvents. The detector used was a fixed wave-length detector; 340 nm and 436 nm UV filters were used to detect retinol (and its esters) and beta-carotene, respectively. The solvent flow rate was set at 1 ml/min for retinol and 3 ml/min for beta-carotene. For simultaneous determination of retinol and retinol palmitate a gradient program was used. In this program the flow rate reached 1 ml/min in 30 seconds and was maintained there for 3 minutes at which time retinol had emerged. The flow rate then reached 3 ml/min in 60 seconds and was maintained at that level for the next 4 minutes to allow the palmitate ester to emerge. The solvent composition was always 37/63, acetonitrile/methanol.

2.3. EXTRACTION METHODS

A. Plasma and serum

1. Thaw and mix.
2. Put 200 ul of plasma in test tube.
3. Add 200 ul HPLC water.
4. Vortex for 5 sec.
5. Add 400 ul EtoH.
6. Vortex for 5 sec.
7. Add 2500 ul n-hexane.
8. Seal the tubes.
9. Vortex for 30 sec.
10. Shake for 5 min.
11. Centrifuge at 2000 rpm for 5 min.
12. Take 2000 ul n-hexane for HPLC analysis.
13. Dry under N2.
14. Redissolve in 500 ul 37/63 Acn/MeoH.
15. Inject onto a C-18 Vydac column.

Note: The volume of plasma used can be changed according to how much plasma is available, however, the ratios should be kept the same. Also, the volume of Acn/MeoH used in step 14 is subject to changes depending on the concentrations of the compounds in plasma. External standards should be used to calculate % recovery.

B. Liver, feed and feces carotene and total retinol (Vitamin A)

1. Weigh out about 5 grams of ground sample.
2. Put into a 100 ml amber volumetric flask.
3. Add 40 ml 95% EtoH.
4. Add 10 ml 50% (W/V) KOH.
5. Saponify for 30 minutes on a boiling steam bath (100 ^{o}C).
6. Cool down to room temperature in a cool water bath.
7. Transfer the saponified sample into a 125 ml separatory funnel.
8. Rinse the 100 ml amber volumetric flask with 50 ml of distilled water and add to the 125 ml separatory funnel.
9. Rinse the 100 ml amber volumetric flask with 70 ml n-hexane and add to the separatory funnel.
10. Shake slowly for 2 minutes and wait till the two layers separate.
11. Aspirate out as much of the n-hexane layer (upper layer) into a 250 ml amber Erlenmyer flask as possible without including any of the lower layer.
12. Add 70 ml n-hexane to separatory funnel, and repeat steps 10 and 11.
13. Add 40 ml of n-hexane to the separatory funnel and shake once more for 1 minute and then wait for separation.
14. Discard as much of the aqueous (lower) layer as possible without discarding any of the n-hexane layer. It may be necessary to force out the feed sample using a wire.
15. Add all the asparated n-hexane fraction that is in the 250 ml amber volumetric flask to the separatory funnel.
16. Rinse the n-hexane in the separatory funnel 3x with 50 ml of distilled water until the water layer which is the lower layer is clean and clear. Dump the lower layer in between rinses.

17. Pour about 5 grams anhydrous Na_2SO_4 into the separatory funnel and shake to get rid of any residual water in the separatory funnel.

18. Pour all of the n-hexane in separatory funnel into a 250 ml amber volumetric flask.

19. Dry under N_2 current on a steam bath.

20. Dilute to 25 mls with (37/63, V/V) Acn/MeOH, immediately and inject onto a C-18 Vydac column. Detect under 326 nm for retinol and about 436 nm for beta-carotene.

Note: The amount of sample in step 1 and all dilution and injection volumes in step 20 varied depending on the concentrations of the compounds in the sample. For liver analysis 1-2 grams of sample were used.

2.4. CALCULATION FORMULAS

A. Standard solution concentration

$$IU/ml = \frac{(\text{Spectrophotometric abs.})(O.B.)(.01)}{\text{Extinction coefficient}}$$

(Roels and Trout, 1972)

B. Liver, feed, and feces

$$IU/g = \frac{A\ B\ C\ F\ H}{D\ E\ G\ W}$$

C. Serum or plasma

$$IU/ml = \frac{A\ B\ C\ F\ H}{D\ E\ G\ V}$$

D. Key

Acn = Acetonitrile

MeoH = Methanol

O.B. = Observed biopotency

A = standard solution concentration (IU/ml)

B = Peak height of the compound from injected sample

C = Volume of injected standard solution

D = Peak height of the compounds in injected standard solution

E = Volume of injected sample (ul)

F = Total volume of n-hexane used (ml)

G = Volume of n-hexane dried after extraction (ml)

H = Volume of Acn/MeoH used (ml)

W = Sample weight (g)

V = Sample volume (ml)

Chapter 3

EXPERIMENTAL

3.1. DETERMINATION OF THE MINIMUM TOXIC DIETARY VITAMIN A LEVEL FOR PREGNANT RABBITS.

SUMMARY

Vitamin A, an essential nutrient, is a fat soluble vitamin which can be toxic. In this experiment, four dietary levels of vitamin A were used. These consisted of 10,000, 30,000, 60,000 and 90,000 IU/kg of diet. The diet supplemented with vitamin A to contain 90,000 IU/Kg of diet produced definite signs of toxicity. Storage of these feeds led to major oxidations of vitamin A, probably because of the lipoxidase activity of the rice hulls used in the basal diet. As a result the 10,000 IU/Kg diet became a vitamin A deficient diet and produced characteristic signs of vitamin A deficiency. The group of does on the 30,000 IU/kg diet performed best and produced the highest total weaning weight in three parities. The average plasma vitamin A value of this group which could be considered as normal was 2.8 $\pm$ 0.4 IU/ml which is more than two times greater than that of human and rat normal values. Hepatic vitamin A values increased with increasing dietary vitamin A levels. The plasma levels did not follow the same pattern. As dietary vitamin A increased up to 60,000 IU/Kg, the plasma

concentrations increased but with the dietary levels of 90,000 IU/Kg, there was a decrease in the plasma levels. The plasma levels of rabbits on the 90,000 IU/Kg diet was significantly ($P<.05$) lower than the 60,000 IU/Kg diet and insignificantly higher than the plasma values for the vitamin A deficient group. This showed that the reproductive problems attributable to high dietary levels of vitamin A in rabbits may actually be associated with low rather than high plasma vitamin A concentrations, even though the liver concentrations were extremely high. Rabbits feeding on extremely high dietary vitamin A levels may suffer from "Secondary hypovitaminosis A". It is important to note that according to these results plasma vitamin A analysis per se would not reveal the true status of vitamin A metabolism in rabbits and should always be complemented by the liver analysis results, specially when vitamin A toxicity is suspected.

Key words: Rabbits, Vitamin A toxicity, Reproduction

INTRODUCTION

In rabbits, hypovitaminosis A can cause signs of ataxia, diarrhea, emaciation and respiratory disturbances frequently accompanied by ocular lesions. This condition can progress to death, often as a result of secondary infections (Payne et al., 1972). Adverse effects on reproduction include reduced conception rates, fetal resorption, abortion, hydrocephalus and low birth weight. In hypervitaminosis A the same reproductive symptoms are observed (Cheeke et al., 1984). Adverse effects on reproduction occur at much lower dietary levels of vitamin A than are needed to cause other obvious and detectable signs of toxicity (Cheeke et al., 1984). The study by Cheeke et al. (1984) demonstrated that a dietary level of 191,000 IU vitamin A/Kg diet resulted in severe reproductive damage, including fetal resorption, abortion, hydrocephalus and low viability of neonates.

The objective of this study was to evaluate lower levels of excess vitamin A, to more clearly define the minimum dietary toxic level.

MATERIALS AND METHODS

Animals. Twenty-eight New Zealand White nulliparous does were used, with seven does assigned to each treatment. The protocol was to obtain data for three parities from each doe. However, some does were not able to complete these parities. Does were put on the experimental diets for a two week adaptation period before the first mating. They were bred on a random basis to one of the two bucks which were fed standard OSU diet. After breeding, does were palpated on days 11, 13, and 28 post-coitus. If diagnosed as non-pregnant after the first two palpations the individual was bred again immediately. Does with two positive palpations which did not give birth were considered to have resorbed the fetuses. Does that did not conceive after three consecutive breedings were culled. Rebreeding was attempted one week after kindling. Kits were weaned at five weeks of age. Does were euthanized after completion of the experimental period; livers and blood were collected and frozen to be analyzed for retinol and retinyl palmitate.

Diets. Four diets, OSU 27, 28, 29, and 30, differing only in retinyl palmitate supplementation levels, were used. These supplemental levels were 10,000, 30,000, 60,000, and

90,000 International Units (IU) of Retinyl palmitate per kilogram of diet, respectively. The composition of the basal diet is shown in table 3.1.1. Rabbits were fed at libitum.

Vitamin A analysis. Vitamin A analysis was performed as described in section 2.

Statistical analysis. The ANOVA was performed on SAS statistical package.

RESULTS AND DISCUSSION

The reproductive data is shown are tables 3.1.2 and 3.1.3. No significant differences were observed in the parameters measured with different vitamin A levels in the diet. This was because of the high degree of variation among the does. Nevertheless, some conclusions can be made. For example, under practical situations one might only be concerned about how much total weight of rabbits can be weaned from does feeding on a particular diet rather than how many kits are born per doe, how many kits would die before weaning, or even how many would be weaned. This is because the total weight of animals weaned is a reflection of all the other parameters. Another parameter a rabbit raiser should be concerned about is the health of the does.

The does fed the diet containing 30,000 IU/kg vitamin A produced 46.11 kg of weaned rabbits (Table 3.1.2), which is about 4.5 times more weight than from the does fed the diet supplemented with 10,000 IU/kg. The 60,000 IU/kg diet yielded 42.99 kg of weaned rabbits, which is similar to the 30,000 IU diet while the 90,000 IU diet produced only 2.6 kg more than the 10,000 IU diet (note figure 3.1.1). These

figures suggest that the two extreme diets represent deficient and toxic diets and the optimal dietary level of vitamin A required for rabbit reproduction lies close to 30,000 IU/kg of diet.

The does on the diet supplemented with 30,000 IU/kg seemed to have the lowest incidence of health abnormalities (Table 3.1.4). On the deficient diet with 10,000 IU/kg only one doe, survived to be used in generating data for the third parity, whereas five rabbits survived in each of the other treatments. In the two highest vitamin A groups, reproductive problems such as abortions and resorbed fetuses which are characteristics of hypervitaminosis A (Cheeke et al., 1984) were observed, whereas the 30,000 IU/kg diet did not cause any of these problems. The deficient diet caused abortion in the second and also hydrocephalic and xeropthalmic kits in the third parities. These symptoms are manifestations of hypovitaminosis A in rabbits (Cheeke et al., 1984; Payne et al., 1972). Diarrhea was a cause of death among rabbits on diets containing 60,000 IU/kg or less, even in the 30,000 IU/kg diet. The diarrhea has previously been noted as a sign of hypovitaminosis A in rabbits (Payne et al., 1972). The high level of rice hulls in the diets (note table 3.1.1) may have exacerbated enteric problems. Rice hull contains high levels of silica which may cause enteritis and

erosions in the gut and lead to diarrhea (Robinson, 1987). This stress may have raised the vitamin A requirements of the rabbits for maintenance of the gut epithelial tissue. If this is true, then the only diet capable of providing enough vitamin A to protect the GI tract against the severe physical insults may have been the reproductively toxic diet. Pneumonia was observed in both toxic and deficient diets but not the intermediate ones, probably because rabbit lung epithelium, as any other epithelial cells, are sensitive to both extreme levels of vitamin A. Both excess and deficiency may cause loss of the integrity of these cells and reduction in the number of mucous secreting cells. This reduction in mucous secretion could then lead to increased susceptibility to infections (Moran, 1982).

All of this suggested that the ideal diet for rabbit reproduction might be the 30,000 IU/kg diet. However, this contradicts with the levels previously suggested. Lebas (1980) suggested a requirement level of 12,000 IU/kg of diet for reproduction. One of the possible explanations for this difference could be that perhaps the extra stress presented by the rice hulls elevated the vitamin A requirements. Another possibility could be that the experiment was conducted over a one year period and extensive losses of vitamin A in the feed may have occurred. Furthermore, the rice hulls contain lipoxidases

which can oxidize lipids and cause rancidity (Cheeke, 1987). This would accelerate the oxidation of vitamin A and beta-carotene even further. As a result, the animals were actually exposed to less vitamin A than anticipated and the actual levels for the 30,000 IU/kg diet could very well be close to 12,000 IU/kg. Furthermore, the actual levels the animals were consuming changed throughout the year, as vitamin A destruction occured. The deficient and toxic diets were analyzed at the end of the study. The values obtained were about ten times lower than anticipated.

The plasma and serum of five rabbits were compared to see if they contained comparable vitamin A levels. Vitamin A is bound to retinol binding protein (RBP) in blood and so preparation of serum which requires coagulation of the fibrinogen may trap some of the RBP and therefore result in serum levels of vitamin A lower than plasma values. However, our findings shown in table 3.1.5 suggested there was no significant difference between the plasma and serum vitamin A levels. One explanation could be that as blood proteins which include RBP coagulated and denatured, vitamin A was released into the lipid fraction of the blood. In fact, during analysis of blood plasma (includes RBP) for vitamin A, it is routine to use ethanol before the extraction steps because ethanol acts as a RBP denaturant.

The denaturation of this protein supposedly releases all the vitamin A into the plasma lipid fraction. It might be advantageous to use plasma instead of serum, if the samples are to be stored, because RBP is known to protect vitamin A against enzymatic and oxidative attack (Bondi and Sklan, 1984).

The results obtained from analyzing the plasma and liver samples are shown in table 3.1.6. The plasma vitamin A levels of the rabbits on the diets with 30,000 and 60,000 IU/kg were significantly different than those on the "deficient" and "toxic" diets and from each other. As the dietary vitamin A levels were increased from 10,000 to 60,000 IU/kg diet the plasma vitamin A of the rabbits exhibited an increasing trend. However, it was surprising that feeding the "toxic" diet caused a lower plasma vitamin A, insignificantly different from those of rabbits on the "deficient" diet. (see Figure 3.1.2). A possible explanation can be offered by looking at liver levels in table 3.1.6. The liver samples that were analyzed were not taken from the same sites on the livers among different individuals. Because the concentration of vitamin A varies in different parts of the liver (Dorea et al., 1984), the sampling technique used might in part explain the high variability observed in liver vitamin A levels within each treatment. Our data showed significant differences between

all treatments. The liver vitamin A levels increased as the dietary vitamin A levels increased (Figure 3.1.3). In order for vitamin A to be mobilized from liver storage and enter circulation, it needs to be bound to RBP which is produced in the liver. Factors which reduce RBP production cause reductions in mobilization and subsequent accumulation of vitamin A in the liver (Underwood, 1979). Mallia et al. (1975) noted that administration of excessive vitamin A to rats caused significant reductions in RBP and retinol levels in the blood. It is possible that this happened to the rabbits on the "toxic" (90,000 IU/kg) diet. The RBP production capacity of the liver might have been impaired or depressed by the high liver vitamin A levels (Figure 3.1.4). Therefore, the symptoms of hypervitaminosis A in rabbits might infact be manifestations of a condition which could be termed "secondary hypovitaminosis A". This condition which has not been previously reported would then refer to low plasma but high liver vitamin A levels with contrast to hypovitaminosis A which requires both values to be low or hypervitaminosis A in which both values are high. This experiment demonstrated the importance of complementing plasma vitamin A analysis data with that of liver for precise diagnosis of vitamin A status of rabbits, particularly if hypervitaminosis A is suspected.

For studies of vitamin A requirements, it may be advisable to administer vitamin A by injection rather than via the diet, to accurately control the level administered. This is particularly true of long-term reproductive studies, because loss of vitamin A activity of the experimental diets during storage is very difficult to prevent entirely.

Table 3.1.1. Composition of the basal diet.

Ingredient	%
Alfalfa meal	10.0
Rice hulls	25.0
Oats	13.5
Wheat mill run	25.0
Soybean meal	20.0
Molasses	3.0
Fat	2.0
Dicalcium phosphate	1.0
Trace mineralized salt	0.5

Table 3.1.2. Reproductive data (Total production over three parities).

Diet vitamin A content (IU/kg)	Parity	Number of does	Total kits born	Kits born alive	Kits alive after first week	kits weaned	Total birth weight of live litter (g)	Total weaning weight (g)	Total weaning weight produced in three parities (g)
10,000	1	6	39	23	12	10	1108	7080	
	2	2	17	8	7	6	403	3164	10244
	3	none	-	-	-	-	-	-	
30,000	1	7	50	33	25	24	1655	17936	
	2	6	43	25	18	13	1538	10313	46112
	3	5	36	31	26	23	1672	17863	
60,000	1	7	49	31	25	25	1652	19126	
	2	5	29	15	14	13	1046	12120	42985
	3	5	36	17	16	16	1001	11739	
90,000	1	7	45	22	13	13	1042	9394	
	2	6	36	19	12	1	983	951	12893
	3	2	10	3	3	3	200	2548	

Table 3.1.3. Reproductive data (Average production per doe).

Diet vitamin A content (IU/kg)	Parity	Number of does	Average kits born	Average kits born alive	Average kits alive after first week	Average kits weaned	Average total birth weight of live litter (g)	Average total weaning weight (g)
10,000	1	6	6.5 ± 2.6	3.8 ± 1.7	2.0 ± 1.3	1.7 ± 1.5	184.7 ± 82.1	1180.0 ± 1079.1
	2	2	8.5 ± .7	4.0 ± 5.7	3.5 ± 4.9	3.0 ± 4.2	201.5 ± 285.0	1582.0 ± 2237.3
	3	none	-	-	-	-	-	-
30,000	1	7	7.1 ± 3.8	4.7 ± 3.9	3.6 ± 3.0	3.4 ± 3.1	236.4 ± 172.2	2562.3 ± 2159.7
	2	6	7.2 ± 2.6	4.2 ± 2.6	3.0 ± 3.3	2.2 ± 2.4	256.3 ± 218.6	1718.8 ± 1941.2
	3	5	7.2 ± 5.2	6.2 ± 5.2	5.4 ± 4.5	4.6 ± 4.3	334.4 ± 245.3	3572.6 ± 3369.3
60,000	1	7	7.0 ± 3.1	4.4 ± 4.6	3.6 ± 3.5	3.6 ± 3.5	236.0 ± 220.9	2732.3 ± 2347.6
	2	5	5.8 ± 3.1	3.0 ± 2.8	2.8 ± 2.9	2.6 ± 3.1	209.2 ± 191.6	2424.0 ± 3072.9
	3	5	7.2 ± 4.8	3.4 ± 4.8	3.2 ± 4.4	3.2 ± 4.4	200.2 ± 275.3	2347.8 ± 3219.3
90,000	1	7	6.4 ± 3.0	3.1 ± 3.9	1.9 ± 3.3	1.9 ± 3.3	148.9 ± 178.4	1342.0 ± 2296.5
	2	6	6.0 ± 4.4	3.2 ± 3.4	2.0 ± 2.8	.2 ± .4	163.8 ± 164.7	158.5 ± 388.2
	3	2	5.0 ± 1.4	1.5 ± 2.1	1.5 ± 2.1	1.5 ± 2.1	100.0 ± 141.4	1274.0 ± 1801.7

Means ± SD
No significant difference between any treatment.

Table 3.1.4. Health and reproductive status of does on different vitamin A diets.

Diet vitamin A content IU/kg	Doe(s) that finished the third parity	Reasons for exclusion before third parity	Cases of abortion, hydrocephalus, and resorbed fetuses
10,000	1	Lack of libido (2)[a] Lack of conception (1) Deaths due to pregnancy toxemia, bilateral pneumonia, and diarrhea (3)	Abortion on second parity (1) Hydrocephalus on third litter (1)
30,000	5	Death due to diarrhea (2)	none
60,000	5	Lack of conception (1) Death due to diarrhea (1)	Abortions on second and third parities (2)
90,000	5	Lack of conception (1) Death due to pneumonia (1)	Abortion on third parity (1) Resorbed fetuses, one on second and three on third parities (4)

[a] number of rabbits.

Table 3.1.5. Comparison of rabbit plasma and serum vitamin A concentrations.

Animal	Plasma	Serum
1	0.40	0.44
2	0.20	0.20
3	2.00	2.20
4	2.50	2.40
5	0.30	0.30

No significant difference ($P<.05$).

Table 3.1.6. Average plasma and liver vitamin A levels for rabbits.

Diet vitamin A content (IU/Kg)	Average plasma vitamin A (IU/ml)	Average liver vitamin A (Total IU)
10,000	0.5 ± 0.2[a]	209.3 ± 112.5[a]
30,000	2.8 ± 0.4[b]	3,962.5 ± 3098.5[b]
60,000	4.2 ± 1.0[c]	42,890.9 ± 16,436.2[c]
90,000	0.9 ± 0.3[a]	153,945.0 ± 60,850.5[d]

Mean ± SD
Means bearing common superscripts in the same column are not significantly different ($P<.05$).

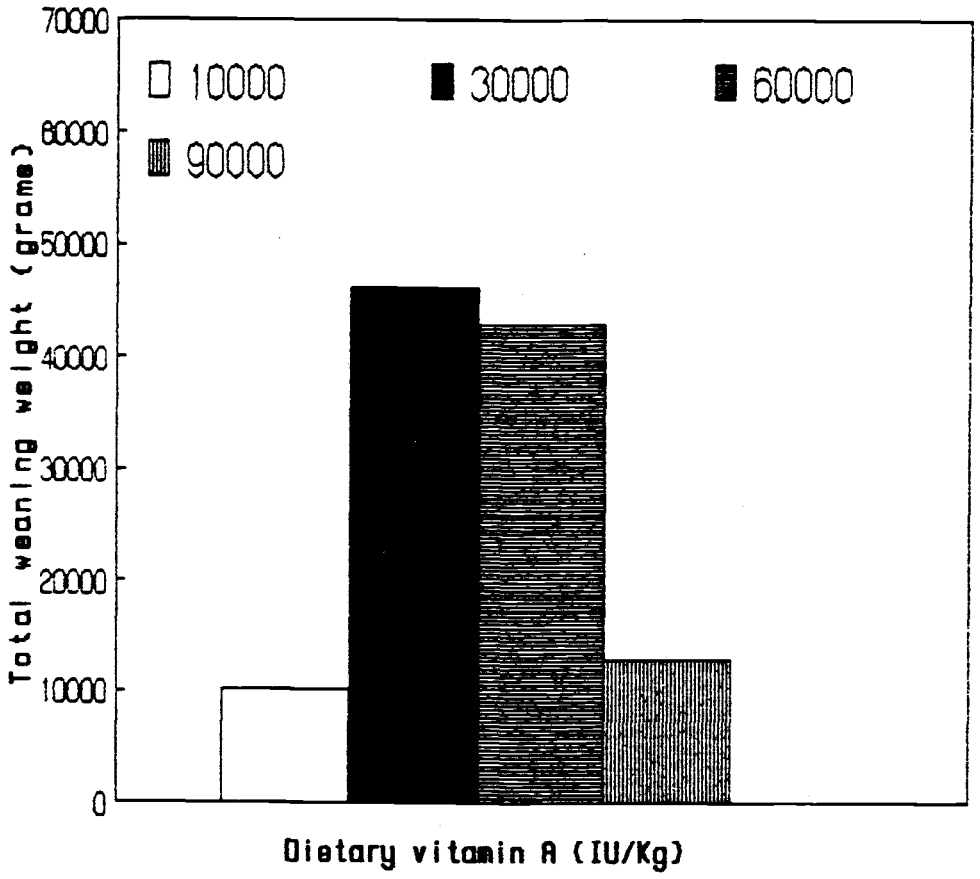


Figure 3.1.1. Total weaning weights of kits born to does on different vitamin A treatments.

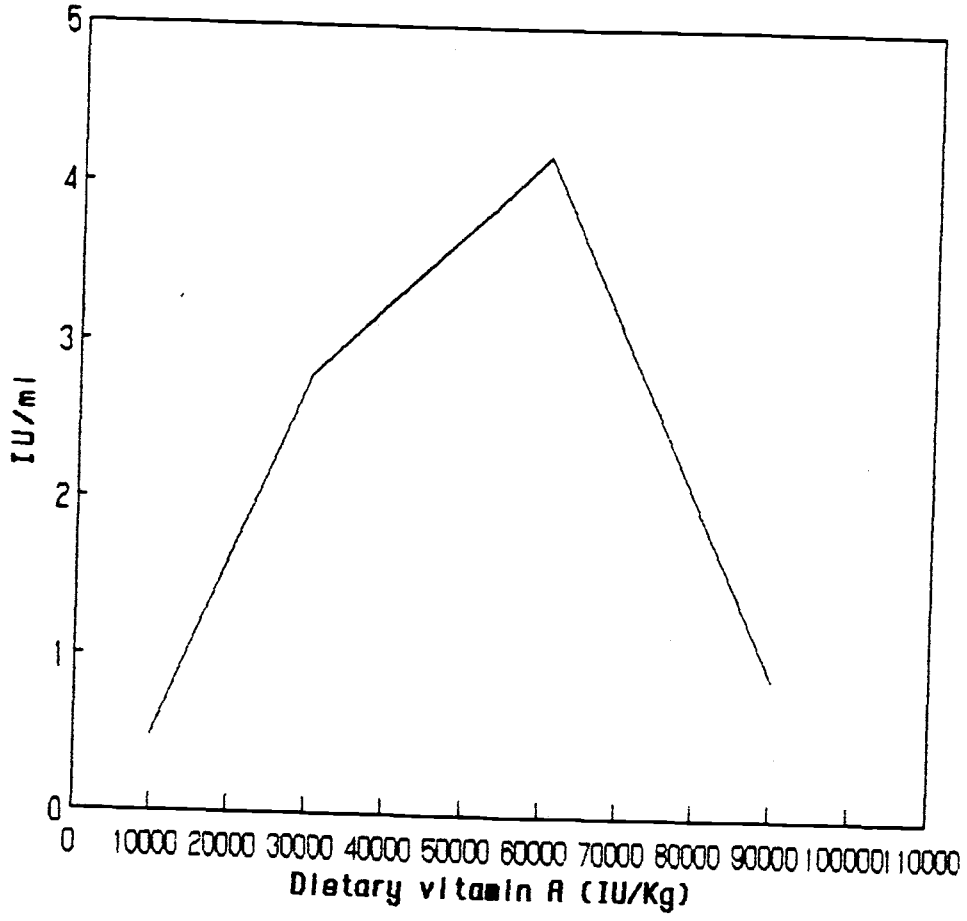


Figure 3.1.2. Average plasma vitamin A concentrations of rabbits on different vitamin A treatments.

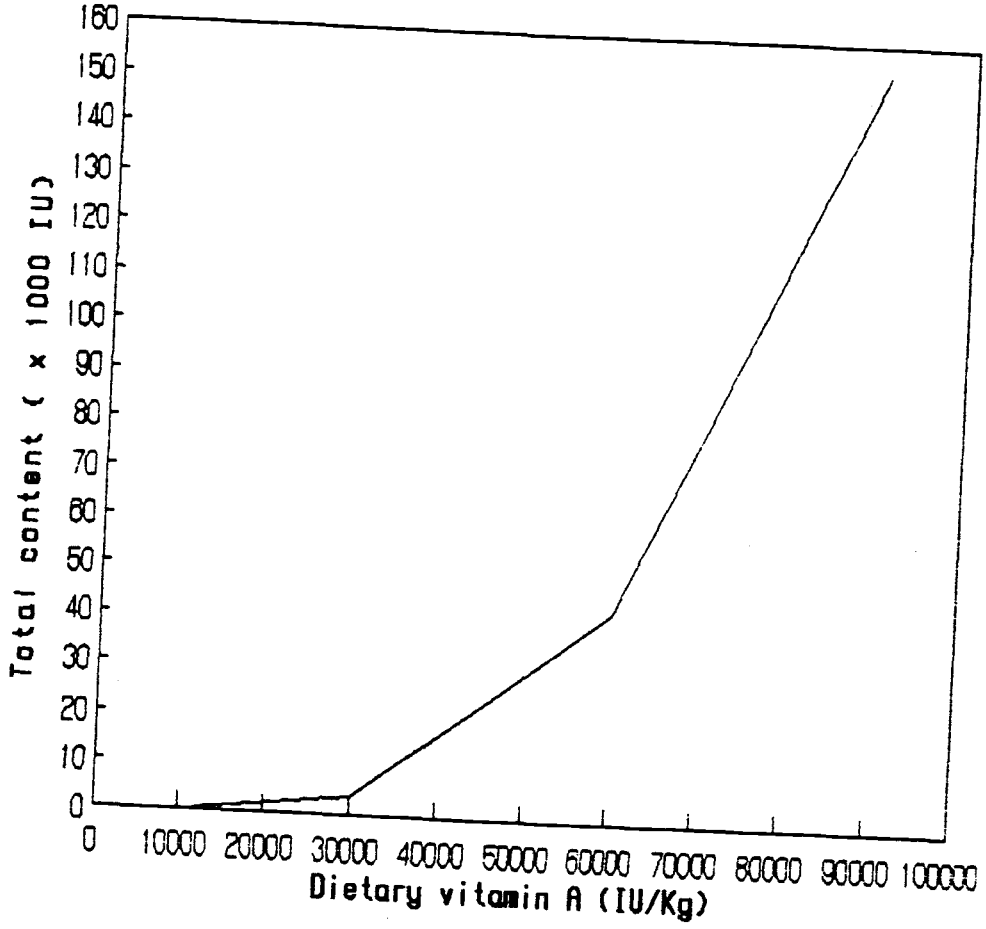


Figure 3.1.3. Total liver vitamin A levels of rabbits on different vitamin A treatment.

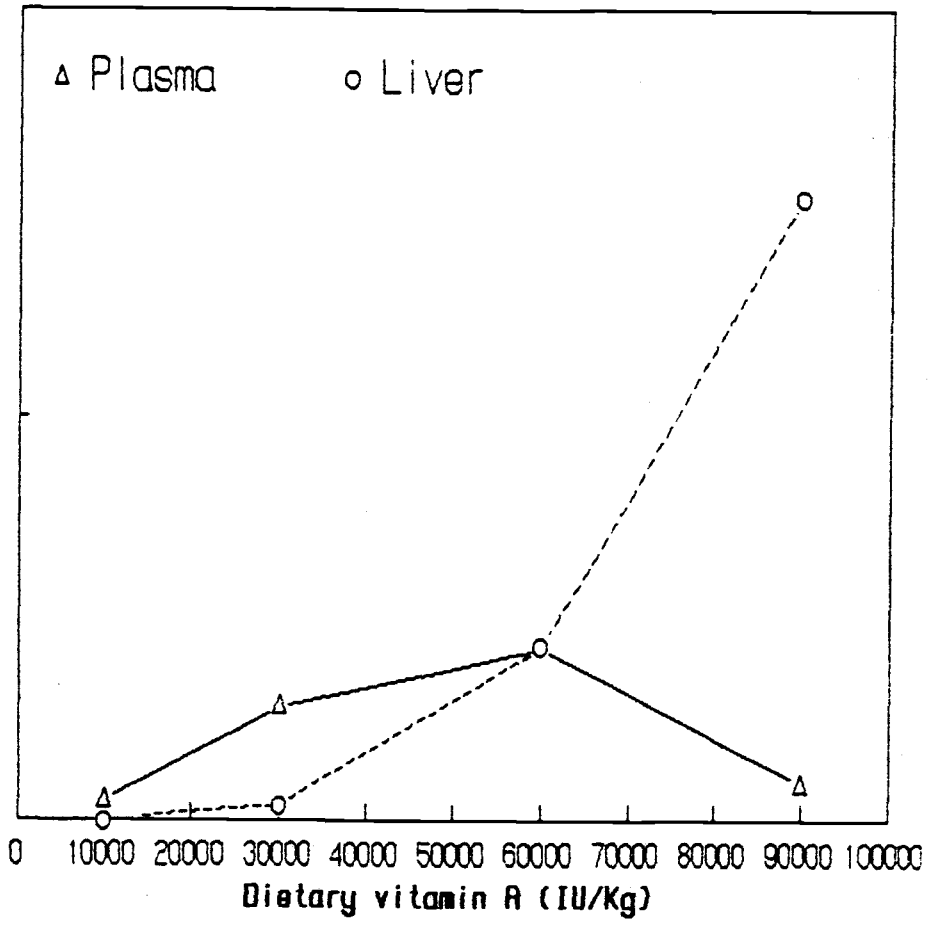


Figure 3.1.4. Comparing the response of liver and plasma to increases in dietary vitamin A in rabbits.

3.2. INTERACTIONS BETWEEN PYRROLIZIDINE ALKALOID AND VITAMIN A TOXICITY AND METABOLISM.

SUMMARY

Possible interactions between vitamin A and the pyrrolizidine alkaloids (PA) present in Senecio jacobea (tansy ragwort) were investigated in rats. There was a significant (P<.Ø5) reduction in liver and plasma vitamin A in PA-fed rats as compared to the control group. The rats fed tansy ragwort (TR) had significantly lower feed intakes (P <.Ø5) than controls. However, this did not explain the depressed liver and plasma vitamin A levels in the body. The total vitamin A intake of each group was calculated and compared to their liver and plasma vitamin A levels. The intake/liver ratio of vitamin A was significantly higher for TR-fed groups compared to the control (P <.Ø5). However, the difference in this ratio was not significantly different (P<.Ø5) among the 5 and 1Ø% TR-fed groups. This shows that in order for TR-fed rats to have the same concentration of vitamin A in their livers as the controls, they have to consume more vitamin A. Since the plasma

vitamin A levels of the TR-fed rats were also lower than those of the control rats, the possible explanations for low liver vitamin A reserves of the TR group include either higher catabolism or lower intestinal absorption of vitamin A.

Key words: Hepatoxicity, Tansy ragwort, Senecio jacobea, Pyrrolizidine alkaloids, Vitamin A, Rats

INTRODUCTION

Vitamin A, a fat soluble, unsaturated 2Ø carbon cyclic alcohol, is one of the few vitamins for which both deficiency and excess present serious health hazards. These two conditions arise under different circumstances. Vitamin A deficiency or hypovitaminosis A occurs as a result of malnutrition and malabsorption, transport disorders or liver disease. Vitamin A toxicity or hypervitaminosis A, on the other hand, usually arises from the abuse of vitamin A supplementation and therapy and in some cases because of liver damage.

Sources of vitamin A include dark green plants which contain beta-carotene, the vitamin A precursor, and animal sources like liver, milk, egg yolk and fat (Bondi and Sklan, 1984). Depending on the species, liver can contain high or low levels of vitamin A. Cases of acute hypervitaminosis A have been reported as a result of consumption of polar bear, dog and chicken liver by humans (Mahoney et al., 198Ø). beta-carotene is not always a good source of vitamin A (Brubacher and Weiser, 1985). The other source of vitamin A can be the synthetic forms commonly sold over the counter in drug stores, and used as supplements in the feed maufacturing industry.

Vitamin A is absorbed in the upper two thirds of the small intestine (Olson, 1961). Dietary fat (Brubacher and Weiser, 1985) and bile acids (Olson, 1961; Sharma and Dostalova, 1986) are essential for its absorption. Vitamin A then travels to the liver via the portal artery where it is deposited in the form of retinyl palmitate. Liver contains four different types of cells: endothelial, Kupffer, parenchymal, and Stellate cells. The Stellate cells, also known as fibroblasts, lypocytes, perisinosoidal, fat storing, interstitial and Ito cells, are the cells normally responsible for fat and vitamin A storage. However, parenchymal cells (hepatocytes) have also been observed to accommodate fat vacuoles when the circumstances encouraged lipogenisis (Moran, 1982). When needed, vitamin A (retinol) leaves the Ito cells bound to retinol binding protein (RBP). The RBP renders retinol non-toxic (Mallia et al., 1975) to cell membranes.

Generally, vitamin A in normal levels functions to protect the epithelial cell membranes. More specific functions, however, include vision (Bondi and Sklan, 1984), bone growth (Roels and Trout, 1969), normal cerebrospinal fluid pressure maintenance (Eaton, 1969), normal reproduction maintenance (Lorente and Miller, 1977; Unni

and Rao, 1985), growth (Brief and Chew, 1984), and general health and prevention of infections (Chew et al., 1982). Any deviations from normal levels of vitamin A in the body would cause changes and deviations in vitamin A functions.

Hypovitaminosis A arises when liver reserves are exhausted and there is not enough vitamin A in the circulation to meet metabolic needs. As a result, symptoms such as Xerophthalmia (Dorea et al., 1984), excessive secondary infections, increased or decreased cerebrospinal fluid pressure depending on the species, reproductive problems, impaired growth and diarrhea as a result of impaired epithelial cell membrane maintenance in the gut (Payne et al., 1972), can occur. Hypervitaminosis A, on the other hand, takes place when liver vitamin A storage capacity is saturated and retinyl palmitate is secreted into the blood. Since this form of vitamin A is not bound to RBP it can exert a membranolytic action and cause peripheral cell damage wherever there are esterases to convert it to retinol (Halvey and Sklan, 1986; Schindler and Klopp, 1986). The peripheral manifestations of hypervitaminosis A include bone problems, alterations in cerebrospinal fluid pressure, anorexia, and reproductive problems.

Acute ethanol administration (Sato and Lieber, 1982) and treatment with 2,3,7,8-Tetrachlorodibenzo-p-Dioxin or TCDD (Thunberg et al., 1979) reduce liver and elevate plasma vitamin A levels.

The pyrrolizidine alkaloids (PA) present in Senecio jacobea (tansy ragwort) are well-known hepatotoxins. While PA have a diversity of biological effects, the principal problems are irreversible liver cirrhosis with pronounced fibrosis and biliary hyperplasia which would lead to death as a result of liver injury (Cheeke and Shull, 1985). The PA are not toxic unless they are biotransformed to their toxic metabolites, the pyrroles and the recently isolated highly reactive aldehyde, trans-4-hydroxy-2-hexenal (Segal et al., 1985). This is why the liver lesions are observed in the centrilobular hepatocytes containing high concentrations of the mixed function oxidase enzymes. As a result of necrosis of these hepatocytes, secondary problems may arise which complicate the situation. Even though sheep are relatively resistant to PA toxicosis, ingestion of the PA-containing Heliotropium europium predisposes them to copper toxicity (Bull et al., 1956). This situation was simulated by feeding Senecio PA to rats (Miranda et al.,

1981b; Swick et al., 1982). The induced copper toxicity is partially due to the rupture of copper containing hepatocytes (Underwood, 1977).

Even though the main concern with PA poisoning in the U.S. Pacific Northwest has been mainly with livestock, the potential for human exposure to these toxins should not be overlooked. Important cases of human poisonings by these toxins have already been extensively documented overseas and in third world countries where herbicides are not utilized. Recently, increasing attention has been paid to this problem in the U.S. Ridker et al. (1985) documented for the first time a case of serious human poisoning due to consumption of comfrey (Symphytum officinale) used by many health conscious, "natural food" consumers. The PA have also been recovered from milk (Dickinson, 1974) and honey (Deinzer and Thomson, 1977) produced in areas infested with tansy ragwort.

The objective of this experiment was to investigate the interactions between vitamin A and PA present in tansy ragwort and the possibilities of PA predisposing rats to vitamin A toxicity, perhaps via the rupture of vitamin A containing cells, or as is the case with ethanol and TCDD. The information obtained in this experiment is important both in dealing with PA-resistant and non-resistant

species. Resistant animals which can consume high levels of PA, could perhaps develop chronic vitamin A toxicity. In the case of non-resistant animals and humans, a combination of high vitamin A supplementation and PA exposure may cause the same problem.

MATERIALS AND METHODS

Vitamin A premix preparation. All-trans retinol palmitate was obtained from Sigma Chemical Company. The concentration of this compound in the material obtained was 250,000 IU/g. Vitamin A premix was prepared by mixing 9.2436 g of the vitamin A material with 1,031.0 g ground corn. The vitamin A concentration of the premix was then calculated to be 2222 IU/g.

Animals and diets. A total of 54 male Long Evans rats, weighing 83-117 g, were used. The experiment lasted 32 days. During this time, daily feed intake and weekly body weight data were collected. At the endof the study, animals were euthanized using CO_2. Blood and liver samples were collected and stored frozen until analyzed for vitamin A using the methods outlined in section 2. The animals were assigned to nine different diets. The basal diet (95.5% of total) was composed of the following ingredients on a percentage basis: Ground corn, 53; soybean meal, 30; alfalfa meal, 5; vegetable oil, 3; molasses, 3; dicalcium phosphate, 1; and trace mineralized salt, .5. The remaining 4.5% of the diet was supplemented with vitamin A premix. The medium vitamin A diets received 34 and the high vitamin A diets recieved 135 g of the vitamin A premix. This resulted in 25,183 IU vitamin A/kg of the

medium and 99,990 IU vitamin A/kg of high vitamin A diets. If still needed after addition of premix, ground corn was used to bring up the diets to 100%. In addition to this, tansy ragwort collected in the vicinity of Corvallis, OR. was added in proper amounts to each diet in order to make up 0, 5 and 10% of the diets. Table 3.2.1 contains precise information regarding the vitamin A and tansy ragwort composition of each diet.

Vitamin A analysis. Methods previosly outlined in chapter 2 were used.

Statistical analysis. The ANOVA was performed on SAS statistical package which was capable of analyzing for uneven number of replications in different treatments.

RESULTS AND DISCUSSION

The plasma vitamin A values are presented in table 3.2.2. The plasma vitamin A levels of all rats on Ø% TR diets coincided with the normal values (Mallia et al., 1975; Underwood et al., 1979) and were not significantly ($P < .Ø5$) different from each other regardless of vitamin A supplementations. This proved the absence of hypo- and hypervitaminosis A in all levels of vitamin A supplementations in Ø% TR-fed group. Contrary to our hypothesis, PA toxicosis reduced plasma vitamin A. Thus, in contrast to substances like TCDD (Thurberg et al., 1979) and ethanol (Sato and Leiber, 1982) which would reduce liver but elevate plasma vitamin A, PA appear to reduce both values. This would suggest that the PA act via a different mechanism. The plasma data from rats on the 1Ø% TR diets is limited because of deaths due to PA toxicity prior to the end of the experiment. However, from the limited data obtained with the 1Ø% TR treatments, there did not seem to be a significant difference between the plasma vitamin A levels of rats on the 5 and 1Ø% TR diets. The 5% TR diet seems to be sufficient for creating the type(s) of damage which could reduce plasma vitamin A to about half that of the control (note Figure 3.2.1). Vitamin A

supplementation did not cause any significant changes in plasma vitamin A levels of rats on either of the TR supplemented diets.

The possibility that PAs may cause the liver to sequester more vitamin A than control, as seen in the case of copper (Underwood, 1977), was ruled out after liver analysis. The liver concentration data are shown in table 3.2.3. In the Ø% TR-fed groups there were significant differences ($P < .Ø5$) between all vitamin A supplementation levels. In the 5 and 1Ø% TR-fed groups, however, the Ø and intermediate vitamin A supplementation level groups did not differ from each other significantly. The high vitamin A supplemented groups had significantly higher ($P<.Ø5$) liver vitamin A values than the others. Table 3.2.3 also allows for studying effects of PA on liver vitamin A under different vitamin A supplementation levels. First, the liver vitamin A levels for the two TR-fed groups were not significantly different from each other. Secondly, by increasing the dietary vitamin A levels the difference between the control and TR-treated groups became more significant (note Figure 3.2.2). The liver vitamin A concentration varies depending on the sampling site, with the right lobe having the highest concentrations (Dorea et al., 1984). In the present study, liver samples were not consistently taken from the same site in all individuals,

which contributed to variability in the data. However, since a large part of the total liver was used, this sampling difference is of limited importance.

The TR-fed rats receiving the high vitamin A diets had plasma vitamin A levels of about half those of control rats with no vitamin A supplementation (table 3.2.2). Furthermore, on average their livers contained more than thirteen times more vitamin A than those of the latter group. In order to investigate the possibilities of impairment of vitamin A mobilization in the PA intoxicated rats, a liver:plasma ratio was calculated (table 3.2.4). A group with a relatively higher liver:plasma ratio than others could have had an impairment of liver vitamin A mobilization capabilities. This is because within the same vitamin A treatment a higher liver:plasma ratio for one TR treatment versus another would indicate higher liver and/or lower plasma concentrations, hence impairment of vitamin A mobilization for that group. As can be observed in table 3.2.4, there was no significant difference between the control and the 5% TR-fed rats in any of the vitamin A treatments for the liver:plasma ratios (Figure 3.2.3). This rules out the liver mobilization impairment theory.

Another possibility to account for the lower liver vitamin A levels in PA-fed rats could be that the lower feed intake (table 3.2.5) caused by PA toxicosis lowered vitamin A intake which in turn reduced the total body vitamin A content. In order to examine this possibility the total vitamin A intake of each rat was calculated. For the rats which were not supplemented with vitamin A and whose only source of vitamin A was the B- carotene available in the diet, a vitamin A equivalent intake value was calculated. This was done by measuring the amount of beta-carotene available in the diet (section 2) and considering a 100% conversion rate (table 1.2.1). Table 3.2.6 shows the total vitamin A intake of each group. There was a significant difference ($P < .05$) between all vitamin A and TR treatments in total vitamin A intakes. The only exception to this was that when there was no vitamin A supplementation there was no significant difference in vitamin A equivalance intake which was based on beta-carotene levels. The reason for this was that the TR which was added to the TR-diets probably contributed to the beta-carotene concentrations of these diets and this may have compensated for the significantly reduced dietary intake of these rats (table 3.2.5). Thus, there was a decreased vitamin A intake as a result of decreased feed intake with addition of TR to the diet. However, this did not seem to cause the depressed liver and vitamin A levels.

If the total vitamin A intake of the rats in the high vitamin A, high TR group is compared to that of the intermediate vitamin A, no TR group (table 3.2.6), it is apparent that the latter group consumed about half as much vitamin A as did the first group, but their liver vitamin A contents are similar. To further substantiate that the reduced vitamin A levels were not solely due to reduced intakes a vitamin A intake to liver vitamin A ratio was calculated (table 3.2.7). Overall, treatment with TR increased this ratio significantly ($P < .05$) which would mean that the rats fed the TR-diets had to consume significantly more vitamin A in order to maintain the same liver vitamin A levels as the controls. In other words, the livers of these rats contained relatively less of the vitamin A they consumed than did the livers of the controls. This would suggest either a lower absorption rate or higher catabolism rate of vitamin A in the TR-treated rats.

Vitamin A is an unsaturated alcohol with five double bonds (see figure 1.2.1). Pyrroles which are the toxic metabolites of pyrrolizidine alkaloids possess positive sites (see figure 1.3.1) which make them alkylating agents seeking electronegative sites, such as the phosphate sites of DNA. Theoretically, it is possible that the pyrroles bind the electronegative double bonds on vitamin A and/or

its esters by addition reactions and catabolize them. The vitamin A complexed in such manner would have not been chromatographically detected because of a different retention time.

Percent liver weight was calculated for each group of animals (table 3.2.8). In the vitamin A supplemented groups, TR consumption did not result in significant differences in % liver weight. However, when not supplemented with vitamin A, TR reduced % liver weight significantly ($P < .05$). This might suggest some protection offered by vitamin A against hepatic effects of PA (Figure 3.2.4).

Average daily gain (ADG), shown in table 3.2.9, decreased for all groups as dietary TR increased, which is typical of TR poisoning (Cheeke and Shull, 1985).

Table 3.2.1. Additions to basal diets (95.5%).

Diet #	Vitamin A Premix Added (% of diet)	Ground corn Added (% of diet)	Total amount of vitamin A (IU/kg)	Tansy Ragwort added (% of diet)
1	-	4.50	-	-
2	1.13	3.37	25183	-
3	4.50	-	99990	-
4	-	4.5	-	5
5	1.13	3.37	25183	5
6	4.50	-	99990	5
7	-	4.50	-	10
8	1.13	3.37	25183	10
9	4.50	-	99990	10

Table 3.2.2. Plasma vitamin A values of rats fed various levels of vitamin A and tansy ragwort.

%dietary tansy ragwort	Vitamin A in diet (IU/kg)		
	0	25,000	100,000
0	1.57 ± 0.3^{ad}	1.65 ± 0.2^{af}	1.68 ± 0.3^{an}
5	0.74 ± 0.2^{bk}	0.87 ± 0.1^{bi}	0.81 ± 0.1^{bi}
10	0.82 ± 0.03^{ck}	0.71 ± .02^{ci}	0.71 ± 0.02^{ci}

Means (IU/ml) ± SD
Means in the same columns or rows, bearing common superscripts are not significantly different (P<.05).
SEM = .08

Table 3.2.3. Total liver vitamin A content of rats fed various levels of vitamin A and tansy ragwort.

%dietary tansy ragwort	Vitamin A in diet (IU/kg)		
	0	25,000	100,000
0	1053.3 ± 325.2^{a,h}	11838.0 ± 3520.7^{b,i}	49076.4 ± 13417.0^{c}
5	627.5 ± 506.5^{a,h}	4467.3 ± 1259.2^{d,i,j}	19226.9 ± 7407.8^{e,k}
10	866.8 ± 182.5^{f,h}	3016.7 ± 352.0^{f,j}	14458.8 ± 10220.6^{e,k}

Means (IU) ± SD
Means in the same columns or rows, bearing common superscripts are not significantly different (P<.05).
SEM = 2804.2

Table 3.2.4. Vitamin A liver:plasma ratios.

%dietary tansy ragwort	Vitamin A in diet (IU/kg)		
	0	25,000	100,000
0	714.4 ± 272.2^{ag}	7425.7 ± 2516.0^{ah}	29906.9 ± 9525.3^{bi}
5	1175.0 ± 1266.4^{cg}	5119.0 ± 1404.8^{ch}	26181.0 ± 7431.5^{di}
10	1326.6 ± 13.45^{kg}	4288.9 ± 219.6^{kh}	33027.4 ± 15837.6^{fi}

Means ± SD
Means in the same columns or rows, bearing common superscripts are not significantly different (P<.05).
SEM = 2383.5

Table 3.2.5. Average daily feed intake of rats fed various levels of vitamin A and tansy ragwort.

%dietary tansy ragwort	Vitamin A in diet (IU/kg)		
	0	25,000	100,000
0	21.3 ± 1.1^{ap}	21.5 ± 1.2^{ah}	20.6 ± 0.9^{ak}
5	15.7 ± 1.2^{bf}	15.4 ± 1.6^{bci}	13.7 ± 1.0^{ci}
10	10.5 ± 1.0^{dg}	10.1 ± 1.1^{dj}	11.5 ± 1.4^{dm}

Means (g) ± SD
Means in the same columns or rows, bearing common superscripts are not significantly different (P<.05).
SEM = .52

Table 3.2.6. Total vitamin A intakes of the experimental rats.

%dietary tansy ragwort	Vitamin A in diet (IU/kg)		
	0	25,000	100,000
0	1288.2 ± 64.0^{a,i}	19457.2 ± 1149.4^{b,j}	70791.2 ± 3140.2^{c,m}
5	1417.3 ± 117.9^{c,i}	14462.9 ± 1468.8^{d,k}	47751.0 ± 3339.2^{e,n}
10	1286.9 ± 121.4^{f,i}	9876.4 ± 4435.5^{g,l}	40135.5 ± 4097.2^{h,o}

Means (IU) ± SD
Means in the same columns or rows, bearing common superscripts are not significantly different (P<.05).
SEM = 972.2

Table 3.2.7. Vitamin A intake:liver ratio in rats.

%dietary tansy ragwort	Vitamin A in diet (IU/kg)		
	0	25,000	100,000
0	1.22 ± 0.7^{ap}	1.64 ± 1.3^{ag}	1.44 ± 0.5^{ai}
5	2.26 ± 2.8^{bf}	3.24 ± 2.1^{dk}	2.50 ± 0.8^{cj}
10	1.49 ± 0.8^{dp}	3.27 ± 0.4^{hk}	2.78 ± 1.1^{hj}

Means ± SD
Means in the same columns or rows, bearing common superscripts are not significantly different ($P<.05$).
SEM = 0.63

Table 3.2.8. Liver weight as percentage of body weight in rats fed various levels of vitamin A and tansy ragwort.

%dietary tansy ragwort	Vitamin A in diet (IU/kg)		
	0	25,000	100,000
0	4.6 ± 0.2^{ad}	4.3 ± 0.5^{af}	4.5 ± 0.7^{ak}
5	3.6 ± 0.4^{bh}	3.9 ± 0.6^{bf}	4.3 ± 0.5^{bk}
10	3.8 ± 1.1^{ch}	$3.7 \pm .34^{cf}$	4.0 ± 0.4^{ck}

Means (%) ± SD
Means in the same columns or rows, bearing common superscripts are not significantly different ($P<.05$).
SEM = 0.25

Table 3.2.9. Average daily gains of rats fed various levels of vitamin A and tansy ragwort.

%dietary tansy ragwort	Vitamin A in diet (IU/kg)		
	0	25,000	100,000
0	6.22 ± 0.4^{ap}	5.22 ± 0.5^{bh}	5.40 ± 0.5^{bk}
5	3.02 ± 0.7^{ef}	2.72 ± 0.9^{ei}	2.28 ± 0.7^{ei}
10	0.98 ± 0.8^{dg}	0.75 ± 0.3^{dj}	0.48 ± 0.3^{dm}

Means (g) ± SD
Means in the same columns or rows, bearing common superscripts are not significantly different (P<.05).
SEM = 0.27

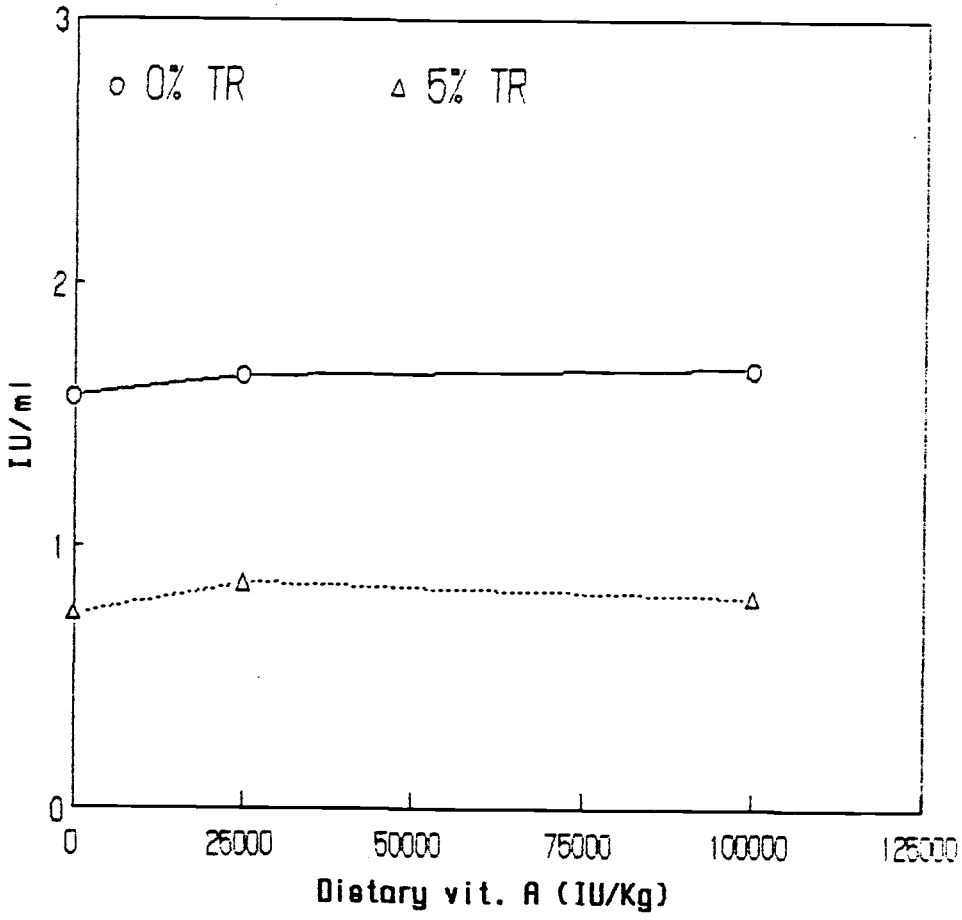


Figure 3.2.1. A comparison between the plasma vitamin A concentration of control and tansy ragwort-fed rats.

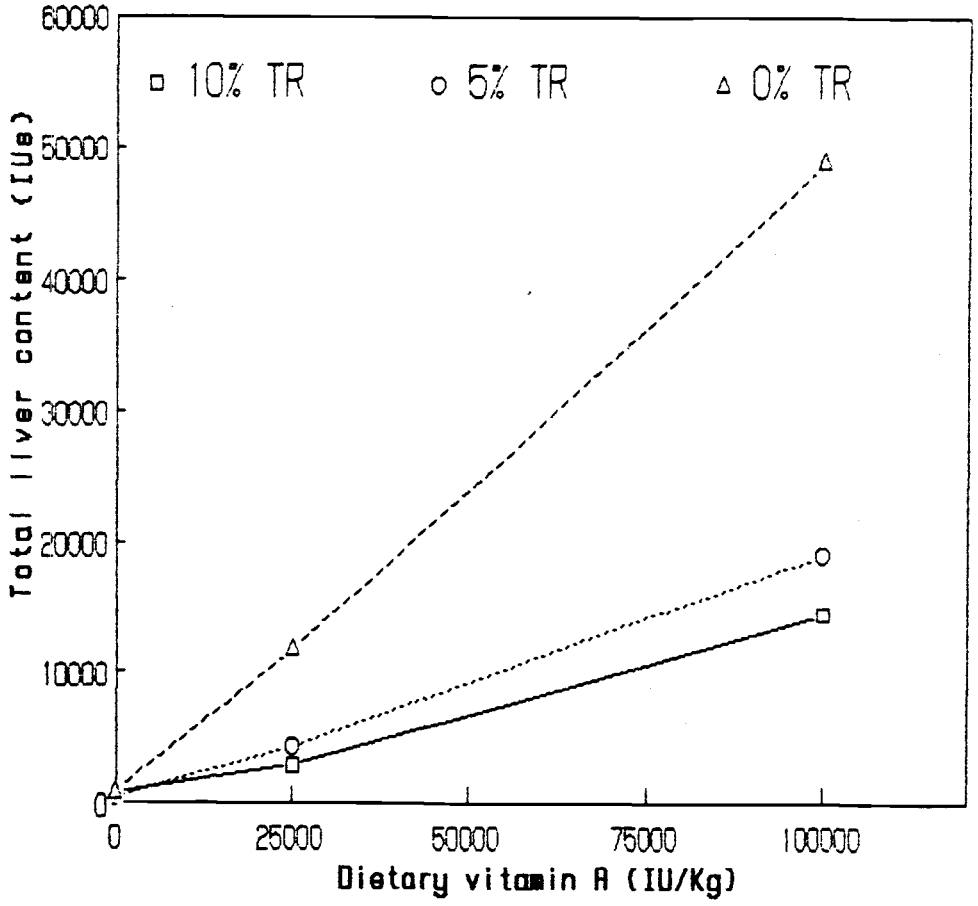


Figure 3.2.2. Total liver vitamin A contents of rats on different vitamin A diets.

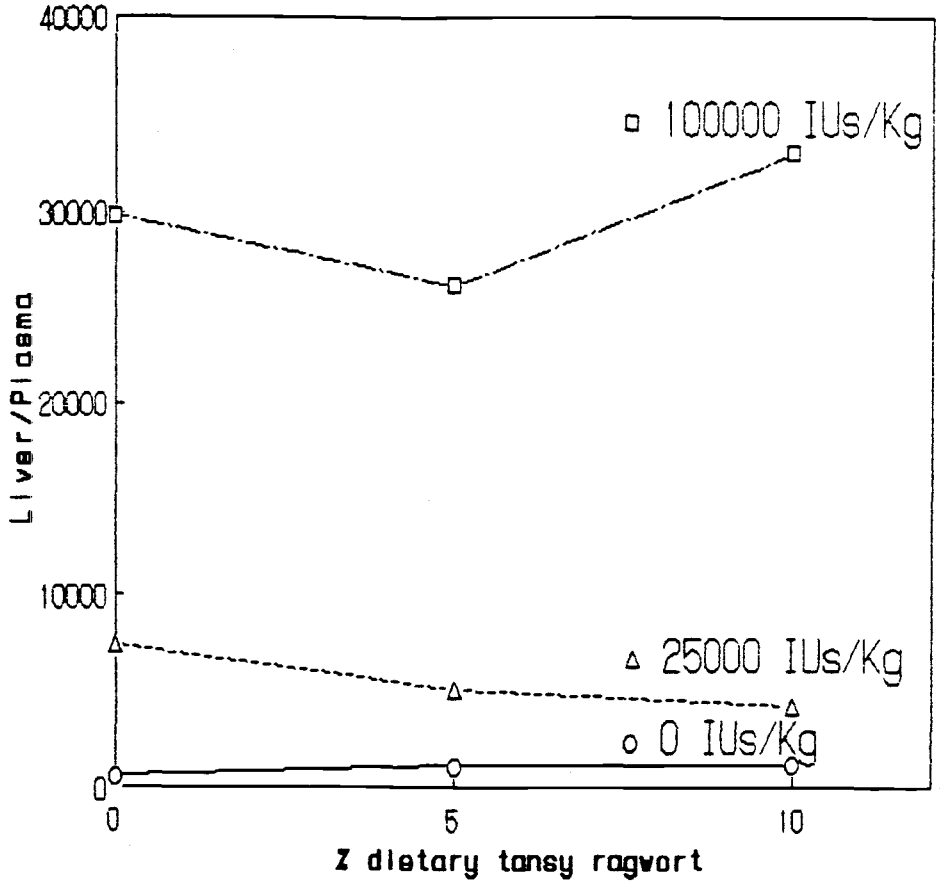


Figure 3.2.3. The liver/plasma ratio of vitamin A in rats on different vitamin A and tansy ragwort treatments.

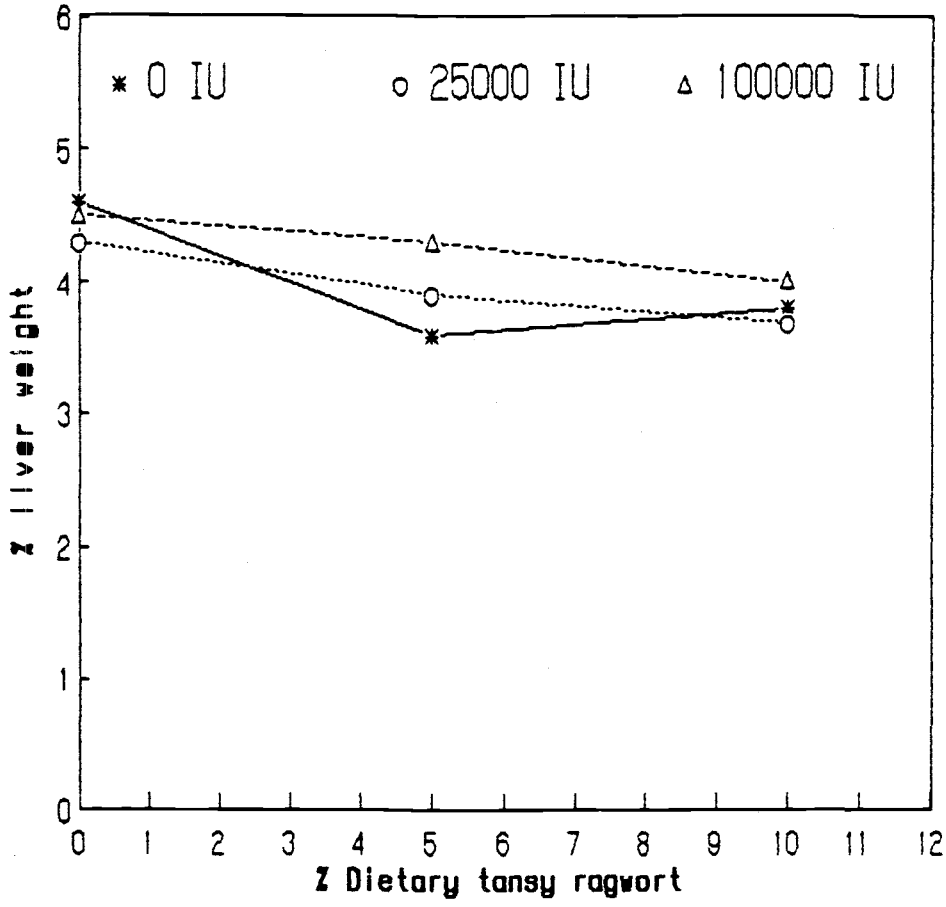


Figure 3.2.4. The effect of tansy ragwort on %liver weight of rats.

3.3. EFFECT OF PYRROLIZIDINE ALKALOIDS ON FAT AND VITAMIN A METABOLISM IN RATS.

SUMMARY

The effect of dietary pyrrolizidine alkaloids (PA) on the absorption of fat and vitamin A in rats was measured, to determine if the reduction in plasma and liver vitamin A levels observed in rats fed Senecio jacobaea (tansy ragwort) is due to impaired vitamin A absorption. The total liver fat levels were reduced ($P<.05$) in rats fed PA. Also, the liver:intake ratio for fat was significantly ($P < .05$) reduced for tansy ragwort treated rats. The lower liver:intake fat ratio for tansy ragwort fed rats seems to suggest that a lower fraction of consumed dietary fat was deposited in the liver. Since the PA intoxicated rats had a reduced feed intake and were emaciated, the low liver fat content may simply reflect their inadequate energy intake and lack of excess energy to store as fat. The control and treated groups did not differ significantly ($P < .05$) with regard to fat and vitamin A absorption. The fecal collection period of 48 hours may not have been adequate to account for all of the vitamin A excreted. The results do

not suggest that the depressed plasma and liver vitamin A levels in rats fed tansy ragwort are a result of impared vitamin A absorption.

Key words: Pyrrolizidine alkaloids, Tansy ragwort, Fat absorption, Vitamin A absorption.

INTRODUCTION

Pyrrolizidine alkaloids (PA) present in Senecio jacobea (tansy ragwort), reduce levels of plasma and liver vitamin A in rats (Section 3.2). The PA cause biliary hyperplasia and decreased bile secretion. This could adversely affect fat emulsification and absorption. Dietary fat (Brubacher and Weiser, 1985) and bile acids (Olson, 1961; Sharma and Dostalova, 1986) are essential for absorption of vitamin A in the small intestine. Thus, the reduced vitamin A levels observed in the rats consuming tansy ragwort may be due to depressed biliary secretions which result in reduced absorption of fat and fat soluble vitamins such as vitamin A.

The objective of this study was to investigate the possibility of PA reducing the vitamin A level in plasma and liver via lowering its absorption as a result of depressed absorption of lipids.

MATERIALS AND METHODS

Animals and diets. Twenty two Sprague Dawley rats weighing 112-136 g were used, 5 for initial data collection (weanlings), 8 for the control group, and 9 for the tansy ragwort (TR) treatment. The weanlings were sacrificed immediately in order to get base line values for plasma and livers. The rest of the animals were sacrificed after twenty-eight days. On the tenth day, six animals per treatment were anesthetized using ketamine and about 1 ml of blood was withdrawn from each animal by heart puncture technique. These blood samples were analyzed for vitamin A; the values are referred to as the "mid-experiment" plasma data. At the end of the third week, two rats from each group were dosed in order to observe the pattern of vitamin A excretion. At the end of the fourth week all of the rats which were left were dosed with vitamin A and placed in collection cages. Feces were collected for 48 hours, and analyzed for vitamin A. At the end of 48 hours all rats were sacrificed in a CO_2 chamber.

Dosing. Retinyl palmitate was purchased from Sigma Chemical Company. This material was in granular form and contained 250,000 IU/g retinyl palmitate. Hexane was used in dissolving enough of this material into a 3600 IU per ml solution. Exactly eight ml of this hexane solution were

added to 30 ml of vegetable oil. The hexane was evaporated under nitrogen and retinyl palmitate remained in the oil. Retinyl palmitate concentration of oil was then 960.0 IU/ml which was equivalent to 1015.3 IU/ml of retinol (multiplication factor in table 2.1.1 was used for conversion). Each rat was dosed with 2 ml of this oil solution orally.

Blood and liver preparation. After sacrificing the animals, blood and liver were collected for vitamin analysis. In order to prepare plasma, heparin was used at the time of blood collection to prevent coagulation. These samples were immediately spun to obtain plasma. The liver samples wre divided into three sections; the right lobes for vitamin A analysis, the left lobe for lipid analysis, and the mid-lobe for histological examination (if needed).

Vitamin A analysis. The instructions presented in Section 2 were followed.

Lipid analysis. The ether extract method presented in Association of Analytical Chemists (AOAC, 1970) was followed.

Qualitative hemoglobin detection. As suggested by Lemberg and Legge (1949), presence of hemoglobin was detected under 555 nm UV light, after simply diluting plasma with distilled water.

Statistical analysis. The ANOVA was performed on SAS statistical package which was capable of analyzing for uneven number of replication in different treatments.

RESULTS AND DISCUSSION

The plasma vitamin A levels of 6 rats after 10 days on each dietary treatment were 0.89 ± 0.22 and 0.58 ± 0.16 IU/ml for control and TR-fed rats, respectively. Thus an effect of PA on reducing plasma vitamin A levels was observable by 10 days; this trend continued throughout the experimental period (Figure 3.3.1.).

To determine if the TR feeding period was adequate to modify vitamin A metabolism, two rats from each group were randomly selected to be administered vitamin A after 3 weeks, before dosing all of the rats. These animals inadvertently received about 10 times the dosage of the main group (21,839 IU/rat vs. 2,031 IU/rat). About 82% of the vitamin A dose that was excreted, was excreted in the first 24 hours in the control animals, whereas only 57% of the excreted dose was in the first 24 hours in the TR-fed group (Figure 3.3.2). In retrospect, the fecal collection period should have been longer than 48 hours. For the control animals, 5.88% of the total dose was excreted, while for the TR-fed group, 2.52% was excreted. The liver contents were 39.3% and 13.7% of the total dose for the control and TR-fed rats, respectively (Table 3.3.1). Thus 54.8% and 83.8% of the administered dose was unaccounted for in the two groups. Presumably the remainder of the dose was still in the

digestive tract. The much lower percent of the dose that could be accounted for in the TR-fed rats may indicate a slower rate of absorption and fecal excretion in rats receiving PA. This pattern suggests that PA may reduce gut motility and prolong the time of digesta passage.

For the main group of animals, the percent apparent absorption of vitamin A was very high in both groups (Table 3.3.3). As discussed for the animals receiving the vitamin A after 3 weeks, this may in fact simply be a lack of fecal excretion within the 48 hour collection period, rather than representing absorption. In retrospect, the collection period should have been longer, and the digestive tract contents should have been analyzed for vitamin A at the conclusion of the collection period. As with the rats receiving the high dose of vitamin A after 21 days, the pattern of fecal vitamin A excretion differed between the control and TR-fed groups. About 8.5% of dosed vitamin A was excreted in 48 hours by the control animals, whereas in the TR-fed rats 12.8% of dosed vitamin A was excreted in that period of time. Considering the total amount of vitamin A present in the livers and plasma samples of each group, a greater proportion of the dose can be accounted for in the control animals. This again suggests an effect of PA on reducing gut motility if the unaccounted vitamin A was to

be found in the gut contents, or an alteration in hepatic metabolism of vitamin A such as addition reactions between pyrroles and vitamin A which reduced the hepatic vitamin A levels.

The liver:intake ratio for fat was calculated. This parameter is a function of total intake during the course of the experiment and not just during the collection period. The liver is not the only site of fat accumulation and also it is a dynamic fat storage site. However, since the TR-fed rats were emaciated and had less fat both in liver and peripheral sites than the control, using this parameter as a means to compare the two groups for retention of fat in the body is useful. The ratio was significantly ($P<.05$) higher for control rats than the TR-fed group (Table 3.3.2). However, this might simply be a reflection of their inadequate energy intake and lack of excess energy to store as fat.

Vitamin E is also a fat soluble vitamin. This vitamin protects unsaturated membrane lipids against oxidation (Stryer, 1981). In vitamin E deficiency, hemolysis may occur and result in anemia (Church, 1984). All of the plasma samples obtained from the TR-fed rats were hemolyzed whereas those from the control groups were all normal. Qualitative hemoglobin detection seemed to suggest a much

higher degree of red blood cell hemolysis in the TR-fed rats than the control. This might further substantiate an impaired fat and fat soluble vitamin absorption. This could have further implications in the pathogenesis of PA toxicity. Segall et al. (1985) have demonstrated that hepatic metabolism of PA can yield reactive aldehydes which are pro-oxidants. Thus the PA-induced liver necrosis may in part be peroxidation damage. Vitamin E is the principal intracellular antioxidant. Impairment of vitamin E absorption or transport with exposure to PA could thus intensify the hepatotoxic effects. This could account for the beneficial effects of synthetic antioxidants in alternating PA toxicity (Miranda et al., 1981a,c).

Plasma and liver vitamin A and liver fat contents were compared between the weanling, control, and TR-treated rats (Table 3.3.2 and 3.3.3). The TR-treated rats had significantly ($P<.05$) lower plasma and liver vitamin A than the controls. The control plasma values, as well as other plasma values, were considerably lower than the control values obtained in the previous experiment or values reported in table 1.2.2. The same was true for the total liver vitamin A levels. Since the only source of vitamin A in the diet was beta-carotene, this might indicate a beta-carotene deficiency in this diet. This could have increased the vitamin A absorption efficiency after dosing the rats.

Figure 3.3.1 shows that even though the animals were dosed with about 2000 IU of vitamin A at the end of the experiment, the mid-experiment blood plasma values were higher than both the zero-day and the terminal plasma vitamin A levels.

Table 3.3.2 also gives total liver fat contents for the three groups. The weanling and control groups had significantly ($P<.05$) higher total liver fat contents than the TR-fed group. The very low fat content of the livers of the PA-fed rats may indicate a PA-induced alteration of fat metabolism. An alternate explanation is that because of low feed intake, presumably because of the pronounced unpalatability of TR, the TR-fed animals were deficient in energy intake, so there was no surplus energy to deposit as fat. Rats fed TR are characteristically emaciated in appearance.

Table 3.3.1. Vitamin A metabolism of rats administered a high dosage of vitamin A after 3 weeks exposure to tansy ragwort.

Group	Dose (IU)	Plasma Vitamin A (IU/ml)	Total liver Vitamin A (IU)	Fecal vitamin A excretion (IU)		
				0-24 hr	24-48 hr	0-48 hr
Control	21839.1[a]	.93 ± .02[a]	8576.5 ± 776.6[a]	1056.8 ± 314.7[a]	228.8 ± 36.8[a]	1285.6 ± 278.0[a]
Tansy Ragwort	21839.1[a]	.34 ± .09[b]	2983.6 ± 500.9[b]	316.0 ± 272.5[b]	235.1 ± 181.6[a]	551.1 ± 91.0[b]

Means ± SD
Means in the same column bearing common superscripts are not significantly different ($P<.05$).

Table 3.3.2. Fat metabolism of rats fed tansy ragwort.

Group	Total feed intake (g)	Total fat intake (g)	Fat Liver:Intake ratio	Total feed intake during collection period (g)	Total fat intake during collection period (g)	% Fat absorption for collection period	Total liver fat (g)
Weanlings							.14 ± .03[a]
Control	607.3 ± 11.8[a]	29.2 ± .6[a]	.58 ± .31[a]	29.0 ± 1.9[a]	3.4 ± .10[a]	90.7 ± 1.5[a]	.17 ± .02[a]
Tansy	451.1 ± 10.9[b]	21.7 ± .5[b]	.28 ± .10[b]	18.3 ± 1.8[b]	2.9 ± .10[b]	92.6 ± 1.4[a]	.06 ± .02[b]

Means ± SD
Means bearing common superscripts in the same column are not significantly different.

Table 3.3.3. Vitamin A metabolism of rats fed tansy ragwort for 4 weeks.

Group	Vitamin A dose (IU)	Total vitamin A excretion (IU/48 hrs)	% Vitamin A absorption in collection period	Plasma vitamin A (IU/ml)	Total Liver vitamin A (IU)
Weanlings				.64 ± .06[a]	1487.5 ± 88.6[ab]
Control	2030.6[a]	171.8 ± 106.6[a]	91.5 ± 2.1[a]	.68 ± .05[a]	1727.8 ± 80.9[a]
Tansy	2030.6[a]	259.6 ± 76.6[a]	87.2 ± 1.9[a]	.27 ± .05[b]	1324.0 ± 74.9[b]

Means ± SD
Means bearing the same superscripts in the same column are not significantly different ($P<.05$).

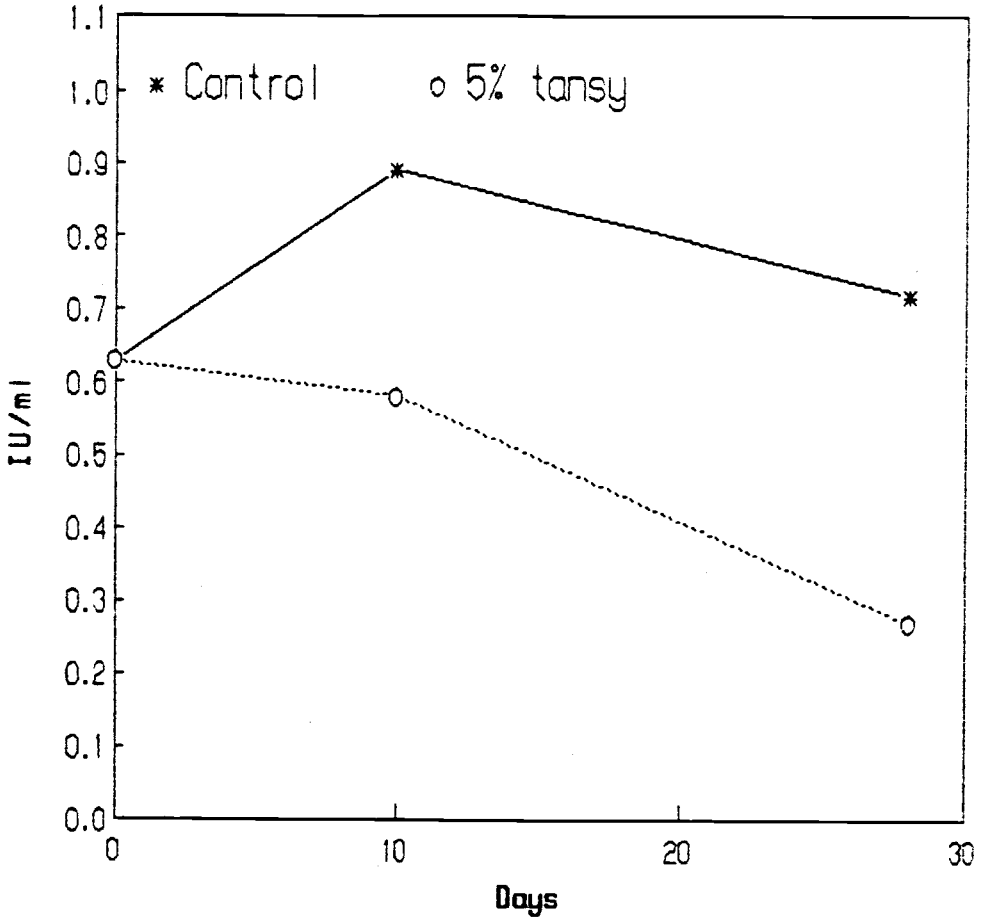


Figure 3.3.1. Plasma vitamin A levels in rats fed a control or tansy ragwort-containing diet.

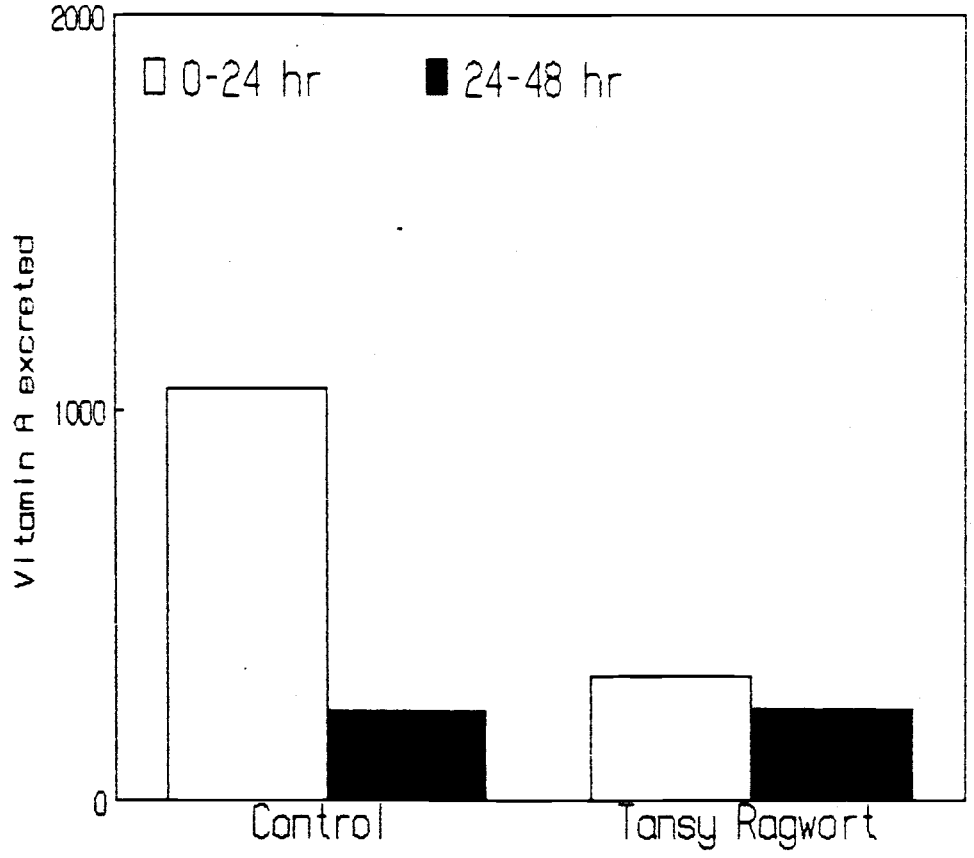


Figure 3.3.2. Vitamin A excretion pattern of control and tansy ragwort-fed rats.

CONCLUSION AND SUGGESTIONS FOR FURTHER WORK:

Vitamin A can have adverse effects on reproduction of rabbits when fed in extremely high levels. The same effects can be observed when there is a deficiency in vitamin A intake. The symptoms of hypo- and hypervitaminosis A which are similar are ataxia, diarrhea, emaciation and respiratory disturbances frequently accompanied by ocular problems. With regard to rabbit reproduction, these symptoms include reduced conception rates, fetal resorption, abortion, hydrocephalus, low birth weights, and low weaning weights. Because of vitamin A losses from the feed during storage the toxic and deficient levels of vitamin A for pregnant rabbits could not be accurately determined in this study, except that the toxic level is less than 90,000 IU vitamin A/Kg diet. This is markedly lower than vitamin A levels reported to be toxic in other species. Of interest was the finding that with the highest level of vitamin A fed, the plasma vitamin A level was similar to that seen in deficient animals. This situation, which has not been reported before, may be termed "secondary hypovitaminosis A" and may explain why the symptoms of hypo- and hypervitaminosis A in rabbits are the same.

The pyrrolizidine alkaloids (PA) were found to reduce both liver and plasma vitamin A in rats. Rats fed Senecio

jacobea (tansy ragwort) had a significantly lower feed intake. However, the reduced plasma and liver levels of vitamin A did not result because of lower vitamin A intake. Under the conditions used, lower absorption of vitamin A as a result of lower bile secretion into the small intestine did not seem to be the reason either. Further study is necessary to elucidate the mode of action of PA in modifying vitamin A metabolism.

To further pursue this work the following studies might have immediate priorities:

1. Investigate the possibility of addition reactions between pyrroles and vitamin A.
2. Study the effects of PA on:
    a. B-carotene conversion.
    b. Synthesis and secretion of RBP.
    c. Cellular distribution of vitamin A.
    d. Distribution of vitamin A among different organs (ie. liver vs. lungs, body fat and, testes).
    e. Plasma vitamin A of rats dosed with vitamin A orally vs. intravenously.
    f. Plasma vitamin A when liver stores of vitamin A are already high.
3. Study the role of hepatocytes in vitamin A storage during hypervitaminosis.
4. Confirm reduction of plasma vitamin A in rabbits in hypervitaminosis A.

5. Establish a correlation between liver and plasma vitamin A levels in rabbits. Follow that with establishing a liver vitamin A clearance rate in rabbits in order to develop an optimum vitamin A injection interval for keeping plasma vitamin A levels within the normal range.

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