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Title: Dietary Factors Influencing the Toxicity of Tansy Ragwort (Senecio jacobaea) in Animals

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Abstract approved: Peter R. Cheeke

Senecio jacobaea (tansy ragwort) is a poisonous plant responsible for livestock deaths in the Pacific Northwest. Horses and cattle are susceptible, while sheep are resistant to the toxic action of the pyrrolizidine alkaloids which the plant contains. Hepatic enzymes are responsible for the biotransformation of pyrrolizidine alkaloids into tissue damaging pyrrole metabolites. The objective of this investigation was to determine the relationship between diet and the toxicity of tansy ragwort.

In Part A of the study, toxicity of tansy ragwort was not found to be significantly altered ($P<.05$) after prolonged periods of storage in excess of 2 years, or after heating during the pelleting process.

Tansy ragwort flowers, leaves, stems and roots were found to differ in toxicity to rats. Flowers and leaves were most toxic to rats, followed by roots. Stems were devoid of toxicity during the 150 day feeding trial. Differences in the relative distribution of two major alkaloids, seneciphylline and jacobine were found in extracts of the plant parts after gas chromatographic analysis. Flowers were highest

in seneciphylline while leaves were highest in jacobine. Roots and stems had lower total alkaloid levels. Seneciphylline was most abundant in roots and was the only alkaloid detectable in stems.

In a trial where rats were fed diets containing ten percent tansy ragwort for varying lengths of time, the chronic nature of pyrrolizidine toxicity was demonstrated. A 1 week feeding period of the toxic diet was enough to kill sixty percent of the rats, some dying months after exposure. The chronic lethal dose for rats was determined to be between 10 and 53 g tansy ragwort per kg body weight ($P<.05$).

The experiments in Part B were designed to determine if tansy ragwort is detoxified in the sheep rumen. Tansy ragwort was fermented in rumen fluid-buffer mixtures, freeze dried and then fed to rats at the ten percent level. As assessed by survival time of the rats, significant detoxification ($P<.05$) did not occur, even after pretreatment of the rumen fluid donor with tansy ragwort for 1 or 5 weeks. Likewise, autoclaving the rumen fluid or the addition of iodoform to inhibit rumen methanogenic bacteria had no effect on survival of the rats. While pretreatment and addition of iodoform decreased the acetic:propionic ratio, favoring alkaloid reduction, no detoxification occurred. When incubated and unincubated rumen fluid-tansy ragwort extracts were compared using gas chromatography, neither reduction of alkaloid level or formation of metabolites could be detected as a result of rumen fermentation. These results indicate that tansy ragwort is not detoxified as a result of sheep rumen fermentation.

In Part C, the interrelationship between nutritional status and the toxicity of tansy ragwort was examined. Addition of one percent dietary cysteine had a significant ($P<.05$) protective effect against tansy ragwort toxicity while phenothiazine, a microsomal enzyme inducer was

detrimental. The protective effect of cysteine was not enhanced by phenothiazine, suggesting that cysteine exerts its effect before pyrrole production. A high level of dietary fat (14 percent), significantly ($P<.05$) decreased survival time in male and female rats consuming diets containing five percent tansy ragwort. Rats fed either deficient amounts of low quality protein (12 percent gelatin) or large amounts of high quality protein (25 percent casein) were significantly ($P<.05$) protected against tansy ragwort toxicity as assessed by survival time. This suggests that the activity of pyrrole forming enzymes are not as dependent on protein nutrition as are the detoxifying enzymes. In another experiment, significant ($P<.05$) accumulation of spleen and liver copper occurred in rats fed five percent tansy ragwort with added dietary copper (50 or 250 ppm). Zinc metabolism did not appear to be affected, while iron levels in spleen and liver tissue were changed significantly ($P<.05$) between the treatments. The trend for iron in these tissues was to increase as tansy ragwort was increased. Liver weights were depressed while spleen and kidney weights were elevated by consumption of tansy ragwort. Soybean meal significantly ($P<.01$) decreased assimilation of copper when compared to casein in rat diets containing 250 ppm added dietary copper.

Dietary Factors Influencing the Toxicity of Tansy Ragwort (*Senecio jacobaea*) in Animals

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INTRODUCTION

Plants containing pyrrolizidine alkaloids have been responsible for the insidious poisoning of livestock throughout the world. In the Pacific Northwest region of the United States, *Senecio jacobaea* or tansy ragwort has become a prolific pasture pest. This exotic, noxious weed contains six known pyrrolizidine alkaloids which are metabolized by liver enzymes in most animals to highly reactive toxic pyrroles. Cumulative damage to the liver results, usually ending in death, sometimes months after consumption of the weed. Unlike cattle and horses, sheep and goats are exceptions, apparently due to rumen detoxification, low activity of liver enzymes or both. Despite the fact these animals are resistant enough to be promoted as biological control agents, they are not totally immune.

Rumen microbial fermentation and hepatic enzyme activity are altered by nutrient and non-nutrient changes in the diet. It was the objective of this research to investigate diet-toxin interrelationships with regard to *Senecio jacobaea* and to determine whether dietary factors might be favorably adjusted to decrease toxicity of this plant.

LITERATURE REVIEW

Background

Senecio jacobaea, commonly called tansy ragwort, ragwort, or stinking Willie, has been responsible for innumerable livestock deaths in various parts of the world. Apparently indigenous to the high rainfall areas of the British Isles, this yellow flowered weed has been unwittingly introduced to each of the continents, where widespread infestation has occurred at various times in the wet temperate areas.

In New Zealand, Senecio jacobaea appeared in the mid 1800's where it produced "Winton" disease. Gilruth (1904) was the first to demonstrate that the hepatic cirrhosis occurring in horses and cattle could be reproduced by feeding these animals on fresh or dried ragwort. While it was noted that sheep could graze infested pastures in New Zealand without difficulty, Gilruth (1904) determined that sheep were not completely resistant in that a hemolytic crisis would occur if the animals were grazed for over a year on such contaminated pastures.

In Nova Scotia, tansy ragwort was introduced near the town of Pictou, from the ballast of a Scottish ship around 1860 (Cushny, 1911). Disease symptoms and deaths in cattle and horses in the surrounding district became apparent a few years later, especially on farms where the plant was prevalent. Investigations by the Canadian government in 1882 failed to correlate "Pictou" disease, as it was called, with the occurrence of the weed. Animals affected with liver cirrhosis were considered to have an infectious disease and thus slaughtered along with the rest of the herd. Microorganisms were isolated from livers of affected animals, but did not produce disease

symptoms in healthy animals upon innoculation. Ford (1901) showed this microorganism (a colon bacillus) to be of secondary nature to the disease as it was also isolated from eighty percent of the healthy animals tested. Pethick (1906) carried out experiments where cattle fed hay from contaminated pastures near Pictou were compared to control cattle fed Senecio-free hay from Quebec. Even though all the cattle were kept in the same barn and on occasion the controls injected with ascitic fluid from the Pictou group, the controls remained healthy while all the Pictou-fed cattle developed cirrhosis Cattle fed hay from Quebec to which chopped ragwort was added also developed the disease. These early experiments by Gilruth and Pethick indicated that without doubt, Senecio jacobaea was the cause of the disease. Similar diseases to stock in South Africa (Molteno disease), Norway (Sirasyke disease), Holland and Australia have been attributed at various times to the consumption of some species of Senecio (Hosking and Brandt, 1936).

In the United States, tansy ragwort occurs in the high rainfall areas of the Pacific states. In Oregon, more livestock deaths are due to tansy ragwort than from all other poisonous plants combined (Snyder, 1972). The occurrence of tansy ragwort in the Pacific Northwest was first noted in Nanaimo, British Columbia, in 1913, and later appeared in a Portland, Oregon ballast dump in 1922 (Isaacson, 1973). It was of little concern until the early 1950's when rapid infestation of crop and pasture land became apparent. In 1972 it was estimated that 3,500,000 acres in western Oregon were contaminated (Snyder, 1972).

Recent serious concern has been expressed over the possibility of poisoning in the human population by tansy ragwort and other plants which contain pyrrolizidine alkaloids. In certain instances, the alkaloids have been detected in milk, honey and herbal teas, and may also be present in meat. Schoental (1959) found the milk transfer of pyrrolizidine alkaloids to occur in rats. The experiments showed that these alkaloids (retrorsine and lasiocarpine) given to lactating rats could poison the young without having noticeable effects on the mother or on her lactation. Dickinson (1974) fed milk from cows ingesting tansy ragwort to mice and reported no damage occurring to the mice. Johnson (1976) fed chronic lethal doses of tansy ragwort to cows and found no histopathologic changes in calves which were allowed to suckle for up to 126 days. Clinical chemical tests, however, did suggest the presence of biochemical lesions. Rats gavaged daily with the milk for 30 days were not affected. In later work by Dickinson et al (1976) ragwort was given to cows at a dose of 10 g/kg/day via rumen cannula and the effects on the nursing calves monitored. No changes of any type were observed in calves, even though the pyrrolizidine alkaloids were detected in the milk at concentrations ranging from 9.4 to 16.7 mg/100 ml of milk. Jacoline was the only alkaloid which was isolated from the milk.

In 1978, Dickinson and King studied pyrrolizidine alkaloid milk transfer in goats. Does were dosed with one percent of body weight per day tansy ragwort via rumen cannula. No significant changes occurred in the does or the kids which were fed the milk although chemical assay revealed 100 ml milk to contain a mean concentration of 233 mg of pyrrolizidine alkaloid.

Goeger et al. (1979, unpublished data) fed milk from goats consuming tansy ragwort in the ration to rats and two Jersey bull calves. Evidence of liver pathology in the calves was found. Using chemical assay, 100 ml milk was estimated to contain 4.25 mg pyrrolizidine alkaloid.

From a health standpoint, milk transfer of pyrrolizidine alkaloids in goats is probably more important than in cows. Cattle, a susceptible species, do not find tansy ragwort to be palatable, whereas goats, a resistant species, readily consume the plant in infested pastures. Furthermore, most cows are milked commercially where any contaminated milk would be diluted greatly after mixing with other milk before reaching the consumer. Goats, on the other hand, are usually owned by families who drink the milk directly with no chance for dilution. The problem is further complicated by the fact that children drink more milk than adults, and would be considered to be more vulnerable to poisoning as are young animals.

Contamination of honey by pyrrolizidine alkaloids constitutes another threat of human poisoning by tansy ragwort. Since the blossom contains the highest concentration of the alkaloid, it is reasonable to suspect that honeybees could pick up significant quantities of alkaloids during nectar gathering. Dickinson (1976) reported values of 0.1 mg/kg of honey in samples collected and stated that in some instances the honey with higher values of alkaloid did carry the odor of tansy ragwort and was not considered palatable. Dienzer and Thomson (1977) detected pyrrolizidine alkaloids and tansy ragwort pollen in four honey samples collected from western Oregon in

areas where the weed flourishes. Values for alkaloid content ranged from 0.3 mg/kg to 3.9 mg/kg. The highest value was taken from honey collected near Salem, Oregon. Total concentration of alkaloids was measured spectrophotometrically by Mattocks' procedure. When subjected to gas chromatography, it was determined that the honey contained all the alkaloids present in tansy ragwort. Mass spectral analysis of the honey revealed the presence of an additional unidentified alkaloid.

Human poisoning by direct consumption of tansy ragwort and other Senecio species has been reported. Senecio contamination of bread and tea in Africa and India have been responsible for "Chiari's" syndrome (McClean, 1970). In Jamaica, "bush tea" is made from leaves picked from the bush and infused with hot water. Used as a medicine often drunk by children, these teas often contain pyrrolizidine alkaloids. Hill et al. (1953) described an illness common among Jamaican children which he termed veno-occlusive disease characterized by collagenous occlusion of the small branches of the hepatic venous tree. Recently in India and Afghanistan, epidemics of veno-occlusive disease have occurred, caused by contamination of millet with (Crotalaria) seeds and wheat by Heliotropium (Anon., 1978).

In the U.S., several cases of human intoxication have been reported in Arizona (Huxtable et al. 1977; Fox et al., 1978; Stillman et al., 1977). Senecio longilobus, a weed containing alkaloids similar to those found in tansy ragwort were found to be in herbal remedies used by Mexican-Americans. The toxic weed is apparently often mistaken for the harmless herb Gnaphalium (gordlobo), an ingredient in the tonic

remedy. In at least one case, the condition of the child closely mimicked Reye's Syndrome. Several fatalities have been reported to date.

Tansy ragwort could be mistaken for common tansy, Tanacetum vulgare, sometimes used as a spice or in teas; however no reported cases of intoxication due to this confusion have been reported.

Another plant, comfrey (Symphytum officinale), has been determined to contain pyrrolizidine alkaloids (Furuya and Araki, 1968). This plant has been used as a tonic in Europe and Japan and is now gaining acceptance and use in the United States. The toxicity of this plant has not yet been fully investigated.

Natural History

Tansy ragwort (Senecio jacobaea Linn.), a member of the Compositae family, is a biennial plant which under some conditions, such as mechanical damage, lives over as a perennial. Most seeds germinate in the fall, form a rosette the following year and blossom the next year. First year rosette plants are prostrate with 8 to 25 cm irregular lobed leaves attached to the mainstalk in a whorl. Inflorescence occurs during the summer of the second year. The plants grow to a height of ½ to 2 M depending on conditions and become covered with many yellow 1 to 2 cm compound flowers. Yellow petals emerge as rays from a central darker button. Individual plants produce as many as 150 thousand seeds which are carried by wind, water and animals over great distances (Hepworth and Guelette, 1977). Tansy ragwort thrives in disturbed areas such as logged over forest land or in pastures

where the soil is disrupted by burrowing rodents. It prefers a mild climate with high rainfall, but can withstand hot dry summers and winter temperatures as low as -40 C. surviving under most soil conditions (Hepworth and Guelette,1977).

Control

Several strategies for control of tansy ragwort have been initiated. The Cinnabar moth, whose larvae feed on the plant parts, particularly the flower buds, has been imported from California as a biological control agent. While unable to totally eradicate the weed, this insect has been effective in limiting the encroachment of tansy ragwort by reducing the number of viable seeds produced. The flea beetle (Longitarsus jacobaea) has recently been introduced as another control agent. The larvae of this insect burrows into the stalk and roots of the weed where it causes damage (Hawkes, 1979). The activity of the flea beetle compliments that of the Cinnabar moth since feeding by each insect occurs at different months of the year (Hawkes, 1979). Sheep and goats have also been advocated as biological control agents for the Senecio species. Pethick (1906) observed that sheep and goats could utilize tansy ragwort and thus control growth of the weed without apparent injury to the goats and with only minor losses in sheep. In feeding trials, sheep and goats consumed relatively large doses of Senecio plants before signs, lesions and fatal poisoning occurred (Dollahite, 1972). In southern Oregon, bands of sheep have been successfully utilized, with proper management, for controlling tansy ragwort in cattle ranges (Mosher, 1979). Accumulation of toxic levels

of liver copper have been reported to occur in Australian sheep grazing the pyrrolizidine alkaloid containing plant, Heliotropium (Bull et al., 1956); however, it has yet to be determined if this effect occurs in sheep grazing tansy ragwort.

Application of herbicides is the most efficient and economical method of controlling tansy ragwort in accessible areas. 2,4-D amine is recommended for control of rosettes, while dicamba is most effective after the flower stalk has elongated (Forbes,1978).

Botanical Distribution

Other plants containing pyrrolizidine alkaloids are widely distributed botanically and geographically. Most important are the families Compositae (contains genus Senecio), Boraginaceae (contains genus Heliotropium, Amsinckia, Echium) and Leguminosae (contains Crotalaria).

There are few areas of the world where grazing animals are not exposed to one or more species within these three main pyrrolizidine containing groups. Senecio is considered to have more species (about 1,450) than any other genus of plants (Bull et al., 1968). The function of the alkaloids in these plants is not known, though having a bitter taste they may serve as protection from grazing animals (McLean, 1970).

Toxicity

Tansy ragwort is not an acutely toxic plant. The effects on animals that consume it are insidious in nature and in the early stages loss of productivity may go unnoticed. In cattle and horses there is

no elevation of body temperature, icterus is common, and evidence of central nervous system disturbance associated with the accumulation of metabolic toxins resulting from liver dysfunction occurs (Muth, 1968). Horses tend to walk in a straight line irrespective of obstructions, after which they commonly press their head against the object (Bull et al, 1968). Pica is often noted, much like in phosphorus deprived animals (Duby, 1975). Diarrhea, tenesmus, and prolapse of the rectum are common in cattle poisoned by tansy ragwort and other pyrrolizidine containing plants (Cushny, 1911; Fowler, 1968; Muth, 1968 ; Duby, 1975). Before death animals become emaciated and ascitic with a dry hair coat and labored breathing. In swine, dyspnea and elevated body temperature are the principal signs of poisoning. There is no icterus (Muth, 1968).

Postmortem examination of most pyrrolizidine affected animals usually reveals gross liver damage, with varying amounts of fluid present in the peritoneal cavity (ascites). Findings in calves reveal considerable amounts of subcutaneous edema as well as edema in the mesentery, omentum, rumen and submuscosa of the abomasum (Thorpe and Ford, 1968). Livers are slightly smaller and firmer than normal with white mottling (Cushny, 1911; Muth, 1968; Fowler, 1968). In pigs, livers tend to be firmer than normal with occasional red-brown mottling (Hooper and Scanlan, 1977). In chickens, livers are paler than normal and shrunken with sharp edges. Ascites is present (Gopinath and Ford, 1977). Gross lung lesions including pulmonary edema, petechial hemorrhage, and congestion occur in certain individuals affected (Allen et al, 1963; McLean, 1970; Hooper and Scanlan, 1977).

Gross kidney damage is not usually observed except in swine where this organ usually is firmer and paler than normal and mottled by small red petechiae (Hooper and Scanlon, 1977).

Microscopic histopathological lesions of livers from chronic pyrrolizidine poisoned animals include megalocytosis, fibrosis, centrilobular necrosis, bilary hyperplasia (see pictures appendix), and veno-occlusion.

Thorpe and Ford (1968) studied the effect of dietary tansy ragwort on calves liver at various stages of intoxication. Early signs were karyomegaly or abnormal enlargement of nuclei in hepatic cells, followed later by megalocytosis and portal fibrosis. In the terminal stages, megalocytosis and steatosis were severe and no lumen was observed in the central vein (veno-occlusion). Veno-occlusion has also been reported to occur in the liver of Maccaca speciosa monkeys dosed with the pyrrolizidine alkaloid, monocrotaline (Allen et al., 1967). These workers described this effect as the discharge of many necrotic cell fragments into adjacent hepatic sinusoids along with fibrin deposits partially or completely occluding sinusoids and adjacent veins.

Hepatomegalocytosis has also been reported in rats, chickens, mice, sheep, horses and pigs receiving pyrrolizidine alkaloids (Bull et al, 1968; Hooper, 1977). In this condition, there is a striking absence of cell division with the mitotic index very low or near zero (Bull et al, 1968). Ultrastructural observation by electron microscopy confirmed that these enlarged hepatocytes are not dying cells.

> "They have abundant rough endoplasmic reticulum studded with ribosomes; hence protein synthesis can actively go on. The distribution of intracellular organelles is irregular

> and accumulations may be present in one part of the cell with deficiency in other parts, as if a factor were missing that is responsible for the organization of the cell." (Afzelius and Schoental, 1967).

Because of the abnormalities which occur in the livers of pyrrolizidine poisoned animals, levels of blood enzymes which originate in the liver are altered. Elevations in glutamate oxaloacetate transaminase (G.O.T.), glutamate dehydrogenase (G.D.H.), onithine carbamyl transferase (O.C.T.), sorbital dehydrogenase (S.D.) and gamma glutamyl transaminase (G.G.T.) have been reported during the course of intoxication (Ford et al., 1968; Fowler, 1968; Craig, 1979).
High blood ammonia levels have been found in horses and rats (Bull et al, 1968) due to impaired liver metabolism. This may account for the neurological symptoms in horses. Allen and Carstens (1970) reported increased blood urea nitrogen, proteinuria, and decreased serum albumin in rats fed diets containing pyrrolizidines. Ascites and edema seen in pyrrolizidine poisoned animals is a result of decreased serum albumin which is important in maintaining the osmotic balance of fluid between the blood and tissues. Decreased blood albumin results in the diffusion of fluid into the tissues and body cavities. Elevated serum bilirubin has been reported in rats during tansy ragwort intoxication (Cheeke and Garman, 1974). This results in the characteristic yellow pigmentation of the skin, fat and mucous membranes.

Lung lesions are produced by most of the pyrrolizidine alkaloids which cause liver lesions. Because a higher dose of alkaloid may be required to produce lung damage, animals may die of liver failure before lung damage is noticed (Culvenor et al., 1976). In general, when lung tissue is affected, it becomes edematous and fibrotic with

extensive emphysema occurring in certain instances. Megalocytosis of lung cells have been reported in mice fed tansy ragwort (Hooper, 1975).

Kidney damage due to pyrrolizidine alkaloids is most common in swine. The most common renal abnormality is megalocytosis in the proximal tubules (Hooper and Scanlan, 1977).

Changes in the gastrointestinal tract of various species poisoned by different pyrrolizidine alkaloids have also been studied. Severe intestinal atrophy was caused by lasiocarpine in sheep, rats and mice (Hooper, 1975). Inhibition of mitosis resulting in megalocytosis appeared to be the cause of the irregularities. Similar findings have been reported in Vervet monkeys (Van der Watt et al, 1972). Symptoms of tenesmus and diarrhea seen in livestock may be due to this intestinal atrophy.

The spleen, an organ important in immunity to disease, is also affected. A two- to three-fold enlargement of this organ was reported in chickens during pyrrolizidine poisoning (Hooper, 1978). Spleen enlargement in rats fed tansy ragwort has also been observed (Buckmaster et al., 1976). In swine, congestion of the malpighian corpuscles and connective tissue proliferation was observed in the spleen (Emmel et al., 1935). Damage to the reticuloendothelial system which is responsible for engulfing waste debris and infective agents from the blood could easily lower the productivity of animals ingesting tansy ragwort.

The extent of damage to vital organs during pyrrolizidine intoxication is variable and dependant on a myriad of factors.

Physiological differences, including absorption, metabolism and excretion, account for much of the species differences in susceptibility.

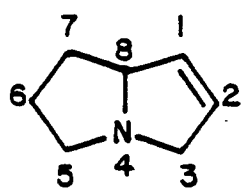
Subtle genetic differences between individuals within a species also causes variation. Site of administration, concentration of dose, and time interval between doses are important. In animals acutely poisoned by intraperitoneal injection of alkaloid, death is usually due to severe hemorrhagic liver necrosis. In hyperacute cases where massive doses are administered intravenously, convulsions occur and result in death with no liver damage (McLean, 1970). Chronic administration produces hepatomegalocytosis. Differences in molecular structure between individual pyrrolizidine alkaloids also affect toxicity. More than 80 different alkaloid derivatives with pyrrolizidine structure have been isolated from various plants. Tansy ragwort contains six individual alkaloids. Since each alkaloid must be metabolized in a slightly different manner, it is not surprising that animal species react differently to tansy ragwort and other pyrrolizidine-containing plants.

Chemistry

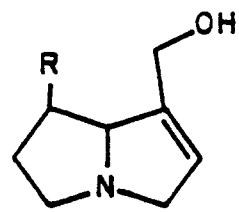
The pyrrolizidine alkaloids consist of two five-membered rings sharing a common nitrogen (pyrrolizidine nucleus) (I) and normally have a hydroxymethyl substituent bound to position one (II) and H or OH bound to position seven. In regard to toxicity, it is well established the molecule must:

1. Contain a double bond between carbon one and carbon two in the ring.
2. Carry esterified hydroxyl groups off of the pyrrolizidine nucleus.
3. And at least one of the ester side chains contain a branched carbon chain (McLean, 1970).

All the alkaloids in tansy ragwort are closed esters (e.g. jacobine - III) whereas in certain other plants such as Heliotropium, alkaloids may be open esters (e.g. Heliotrine - IV). The alkaloids are chemically very stable, with N-oxidation being the reaction most readily available (V) (McLean, 1970). At neutral pH, esters of monocarbosylic acids (IV) are moderately soluble in water as opposed to esters of dicarboxylic acids (III) which are only sparingly soluble in water (Bull et al, 1968). N-oxidation greatly increases solubility in aqueous solvents. The N-oxide and tertiary base forms of pyrrolizidine alkaloids are readily interconvertible in plants and animals, have differing properties relevant to the behavior of alkaloids in biological systems (Bull et al, 1968).

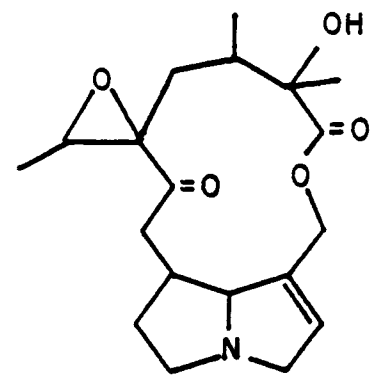


(I)

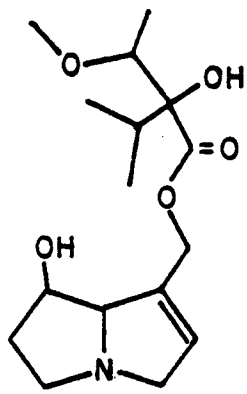


R = H or OH

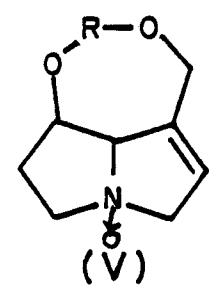
(II)



(III)



(IV)



(V)

Liver

The metabolism of ingested alkaloids in animals was first studied by growing plants in radioactively labeled media. Using nitrogen-15 labeled alkaloids, it was found that up to eighty percent of the pyrrolizidine ring comes out unchanged in the urine. With carbon-14 labeled alkaloids, ten percent of the label appeared as expired CO_2 within 4 hours (McLean, 1970). Liver, lung, kidney, spleen and blood tissue showed highest concentration of labeling after I.V. injections of synthetic tritium labeled alkaloids (Mattocks, 1977). Concentration of radioactivity in the lungs was highest and declined at the lowest rate. Total amount of radioactive label however was greatest in the liver.

Since pyrrolizidine alkaloids are chemically stable, Culvenor *et al.* (1962) suggested alkylation of cell constituents through alkyl oxygen fission of the allylic ester (the ester closest to the 1-2 double bond). While this reaction can be made to occur *in vitro* by adding strong alkali and benzyl mercaptan to the alkaloid, it is unlikely to occur *in vivo*. Mattocks (1968) demonstrated that pyrrolizidine alkaloids are converted to reactive pyrroles by liver enzymes and that the degree of hepatotoxicity is correlated to the amount of pyrrole found in the liver (Figure 1). The pyrrole damages tissue by alkylation. Being electrophylic it becomes bound to nucleophilic macromolecules in the cell such as enzymes and nucleic acids. Since pyrroles are capable of alkylation at both of the ester linkages, they can be compared to nitrogen mustards which are believed to inhibit mytosis by linking themselves across the two strands of DNA (McLean, 1970). The point of

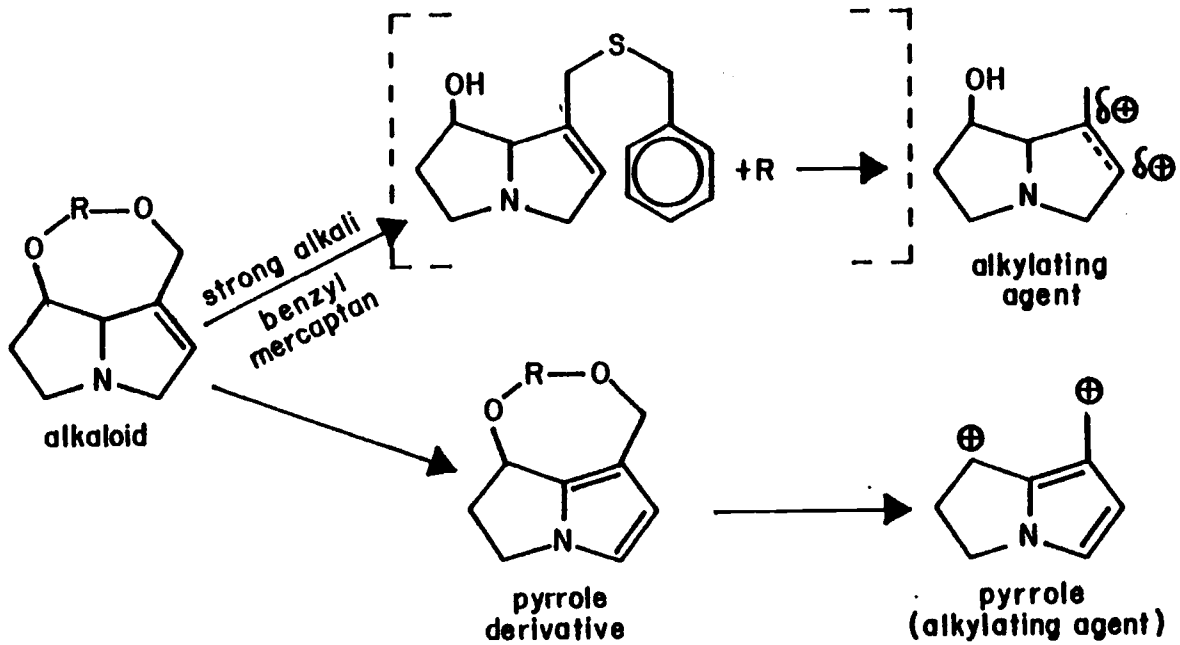


FIGURE 1. FORMATION OF ALKYLATING AGENTS

attack on DNA molecules by alkylating agents is guanine, which becomes ionized and results in mispairing with thymine instead of cytosine. Besides base pair substitution, depurination may result in chromosome breakage. Chromosome breakage by pyrrolizidines has been demonstrated to occur in Allium and Vicia faba root tips (Avanzi, 1962). In animals, chromosome breakage may explain the incidence of necrosis. Base pair substitution causes mutations and has been demonstrated to occur in Drosophila by pyrrolizidine alkaloids (Bull et al, 1968).

In some animals, formation of mutant enzymes and protein in liver cells may account for alterations in metabolism. Pyrrolizidine alkaloids have also been shown to be carcinogenic (Schoental, 1968, 1970; Harris and Chen, 1970; Svoboda and Reddy, 1972). Compounds which contain a flat aromatic ring may be capable of being intercalated into the DNA base pair stack causing a frameshift. The potency of intercalating agents can be increased by orders of magnitude when side chains are available to react covalently with DNA (Ames et al, 1973). Liver enzymes are responsible for the appearance of side chains. Schoental (1970) has suggested that epoxidation of the 1-2 double bond in pyrrolizidine alkaloids by liver microsomal enzymes may be correlated with hepatocarcinogenicity. Since the liver is responsible for enzymatic changes which activate the alkaloid to pyrroles and possibly other derivatives, it is not suprising that the effects are usually first observed in the liver. While the microsomal enzyme system is responsible for activation of many toxins including pyrrolizidines, it's primary function is to detoxify foreign material and metabolic byproducts through different enzyme reactions. Metabolites tend to be more ionized

at physiological pH than parent compounds and hence are likely to be in the form of water soluble salts being more easily excreted (LaDu et al., 1971).

While metabolizing enzymes can occur in the mitochondria, soluble fraction, or microsomal fraction, the microsomal fraction or smooth endoplasmic reticulum has the highest activity. Microsomes contain an electron transport system containing the specialized cytochrome P-450. The reduced form of P-450 reacts with molecular oxygen in such a way that one of the oxygen atoms is reduced to water and the other is introduced into the organic substrate increasing solubility. This is termed phase I reactions (Lehninger, 1976). In phase II reactions, the metabolites are then conjugated with cytoplasmic components such as glucuronides, sulfates, amines, mercapurates or amino acids rendering them detoxified and readily excretable in bile or urine.

Considering species differences with regard to the susceptibility of animals to pyrrolizidine alkaloids, it has been demonstrated that animals with a high microsomal capability for pyrrole formation in vitro are the most susceptible. Shull et al. (1976) found the rates of pyrrole production ranged from high to low in the following order: hamster > rabbit > mouse > male rat > steer > bull > female rat > lamb > chicken > Japanese quail. With the exception of the rabbit, pyrrole production was directly related to toxicity of dietary tansy ragwort. Pierson et al. (1977) suggested that absorption of alkaloids in the digestive tract of rabbits is low, since they are resistant to dietary tansy ragwort, but susceptible to injected alkaloid.

Rumen

Pyrrolizidine alkaloids have also been shown to be metabolized in the rumen. Dick et al. (1963) found heliotrine to be metabolized to 7-α-hydroxyl-1-methyline-8-α pyrrolizidine and the hydrolysis product heliotridine when rumen contents of sheep were examined several hours after receiving a dose of heliotrope. When tested in vitro, it was found that by light centrifugation of the rumen fluid before incubation, no breakdown of heliotrine occurred. Addition of vitamin B_{12} increased the rate of metabolism. These workers synthesized the 1-methylene metabolite and found it devoid of hepatoxic activity. Lanigan (1970) isolated a further reduction product, 1-methyl-8-α pyrrolizidine, from ovine rumen fluid to which heliotrine and lasiocarpine were added. While this metabolite has not been tested for hepatotoxicity, it is unlikely to be toxic since it does not contain the 1:2 double bond (Figure 2).

In further work by Lanigan (1972), the competitive relationship between heliotrine metabolism and methanogenisis in ovine rumen fluid was established. The microorganism Peptococcus heliotrinereductans was found to have the capability of metabolizing heliotrine to the 1-methylene and 1-methyl derivatives, by reduction of the 1:2 double bond and cleavage of the ester (Lanigan, 1976). It was indicated that metabolic hydrogen donors such as reduced coenzymes were essential for metabolism. Inhibition of methane bacteria which use hydrogen to reduce CO_2 to CH_4 was shown to increase formation of nontoxic metabolites. Pretreatment of the rumen fluid donor with heliotrope also had this effect when rate of alkaloid metabolism was measured in vitro. The work of Shull et al (1976) does not support the results obtained by Lanigan.

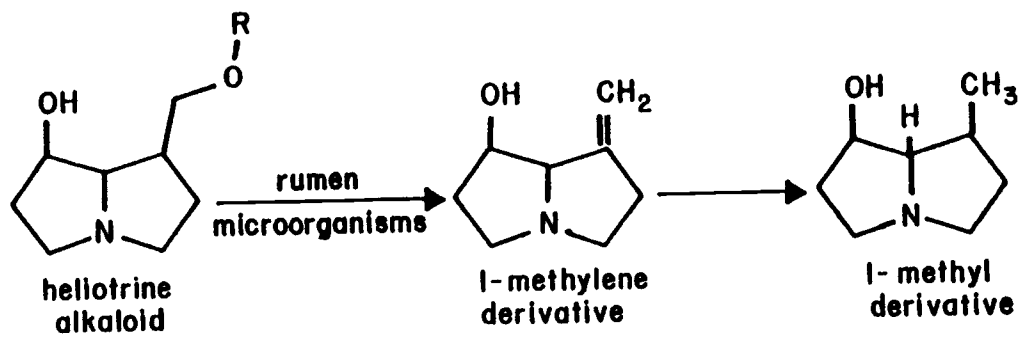


FIGURE 2. RUMEN METABOLISM OF HELIOTRINE

In the experiments conducted by Shull et al., the toxicity of tansy ragwort was not altered after incubation in ovine rumen fluid. These workers concluded that resistance of sheep to tansy ragwort is due to decreased liver activity and not rumen detoxification.

Gastrointestinal

Changes by intestinal microflora represent another way in which pyrrolizidine alkaloids might be metabolized. After passage through the liver, certain foreign compounds are excreted into the intestine via the bile. In some instances reabsorbtion and enterohepatic recycling may occur (Figure 3). While this phenomenon has not been studied with the pyrrolizidines, results from studies done with certain drugs suggest it could be important. Diethylstilbestrol, for example, a drug widely used for its estrogenic activity undergoes enterohepatic recycling. The drug undergoes hepatic glucuronidation, biliary excretion, intestinal hydrolysis of the glucuronides by bacteria and then is reabsorbed as the parent drug (Smith, 1978).

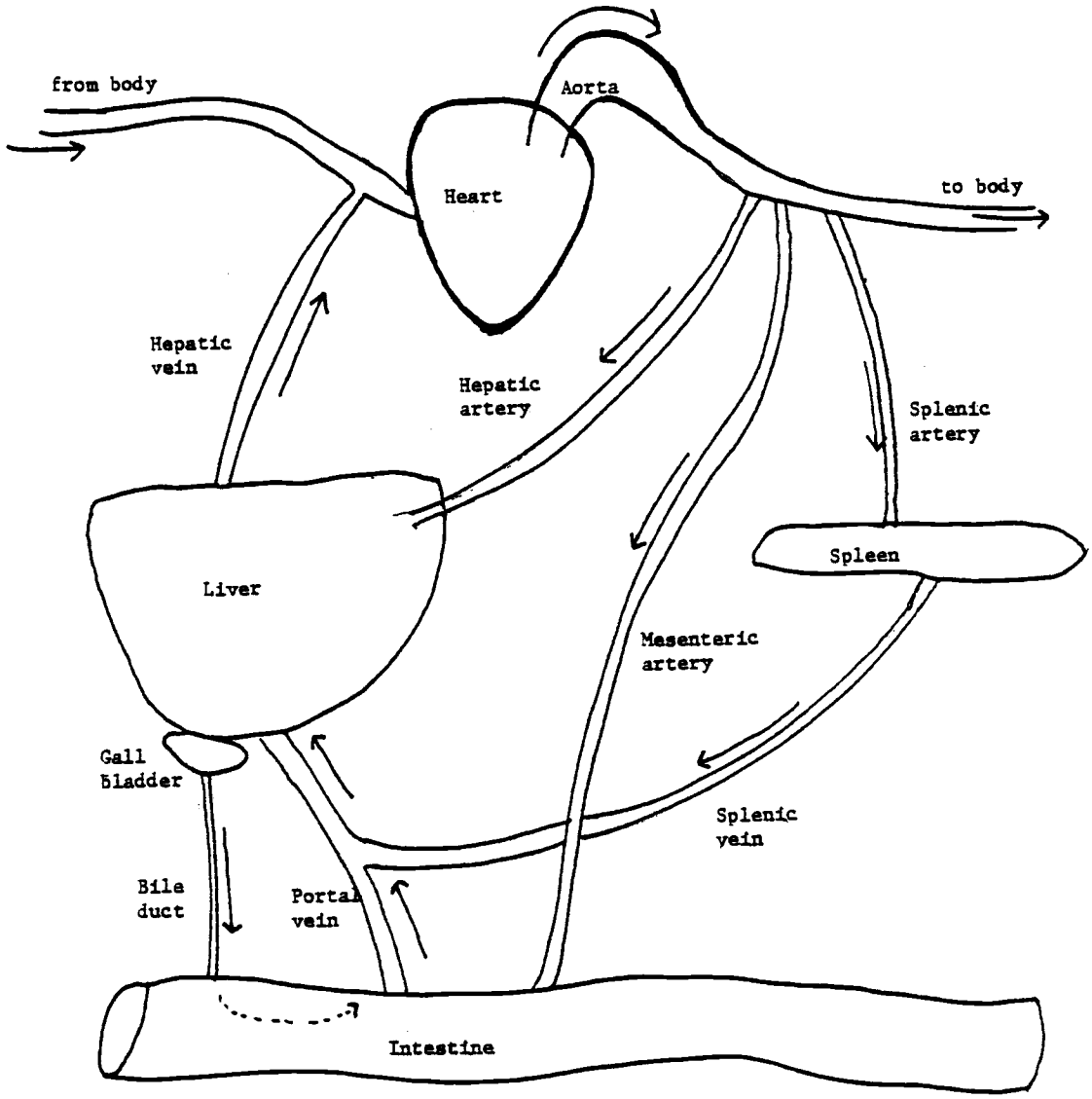


FIGURE 3. ENTEROHEPATIC CIRCULATION

Detection of Metabolites

Several methods have been used to detect pyrrolizidine alkaloids in the experiments discussed previously. The Ehrlich's test, developed by Mattocks (1967) is a spectrophotometric determination where reaction of pyrroles with dimethylaminobenzaldehyde (Ehrlich's reagent), produces a mauve color absorbing maximally at 562 mu. To form the pyrroles the alkaloids must first be N-oxidized with hydrogen peroxide and then be reduced by heating with acetic anhydride. By omitting steps, amounts of pyrrole, N-oxide and basic alkaloid can be determined from plant or animal tissue extracts.

Various chromatographic methods have also been employed. Gas chromatography is useful in detection and can give relative concentration of different alkaloids in a given sample. Paper and thin layer chromatography have also been used with success in rumen fermentation studies. 1-methylene derivatives can be detected on resolved chromatographs by developing with Dragendorff's reagent (bismuth subnitrate) which appears as a distinctive orange spot with a purple outer ring (Bull et al, 1968). Structure and conformation can be elucidated by use of nuclear magnetic resonance (N.M.R.), which signals proton shifts. Mass spectral, ultraviolet and infrared analyses have also been employed.

Dietary Factors Affecting Toxicity

Metabolism of pyrrolizidines can be significantly altered by non-nutrient and nutrient changes in the diets of animals.

Rumen

Increased rumen metabolism of heliotrine to 1-methylene and 1-methyl derivatives has been shown to occur in sheep by the addition of methane inhibitors (Lanigan, 1972). Compounds including chloral hydrate, halogenated methane derivatives (e.g. chloroform, iodoform, etc.), unsaturated fatty acids, trichlorethyl adipate, and certain anti-microbial agents such as monensin are known to elicit rumen methane inhibition (Prins and Seekles,1967; Quaghebeur and Oyaert,1970; Sawyer and Sniffen,1974; Clapperton, 1977). These compounds have a direct toxic effect on the methanogenic bacteria in the rumen (Van Nevel et al, 1969). During fermentation in the rumen, ingested carbohydrates are oxidized via the various pathways of different microorganisms, giving rise to CO_2, metabolic hydrogen and numerous volatile fatty acids (Hungate, 1966). Metabolic hydrogen, in the form of a reduced substrate acts as an electron donor or energy source for the methane bacteria, and CO_2 acts as the electron acceptor. As a result of bacterial metabolism, CO_2 is reduced to methane and the bacteria derives its energy for growth in the process. During methane inhibition, metabolic hydrogen increases in the rumen. The increased reduction potential favors the growth of other organisms which result in increased production of propionate and decreased production of acetate and CO_2. Growth of Peptococcus heliotrinreductans is enhanced by this treatment, as it utilizes metabolic hydrogen as an electron donor in reductive metabolism but is apparently not affected by methane inhibitors (Lanigan, 1976). Pyrrolizidine alkaloids along with nitrate, fumarate, and arginine may serve as electron acceptors and become reduced during metabolism. Enhanced growth

of this organism was found to occur *in vitro* by the addition of nitrate or arginine (Lanigan, 1976). This information may be of practical importance *in vivo* with ruminants if these compounds show protective effects against pyrrolizidine poisoning.

Changes in the nutrient composition of the diets of ruminants have also been shown to alter pyrrolizidine breakdown in the rumen. Lanigan (1970) found that sheep on pasture display much higher capacity for reductive heliotrine metabolism than sheep fed on chaff diets. Since pasture has higher concentrations of readily available carbohydrates (starches, sugars) which favor propionate production and increased metabolic hydrogen, growth of *P. heliotrinreductans* may be enhanced. Vitamin B_{12} (cyanocobalamin) also plays an important role in the reductive cleavage of pyrrolizidine alkaloid in rumen fluid. While *P. heliotrinreductans* does not have a growth requirement for B_{12}, the variable response to B_{12} observed with rumen contents indicates the possibility that more than one bacterial species in the rumen can metabolize heliotrine and that one or more of them may require B_{12} (Lanigan, 1970). In laboratory studies conducted by Wood *et al.* (1967) evidence was provided that methyl transfer reactions leading to the formation of methane are dependent on methyl cobalamin and thus reduced methyl transfer. This action of B_{12} in methane bacteria might account for the variability of B_{12} in inducing heliotrine reduction by other bacteria.

Liver

Metabolism of pyrrolizidine alkaloids in the liver can also be altered by dietary and nutritional factors, and ingestion of foreign chemicals.

Inducing Agents. The activities of liver microsomal enzymes are markedly increased when animals are treated with drugs, pesticides or carcinogens. The increase in activity appears to represent an increased concentration of enzyme protein and is referred to as "enzyme induction." More than 200 compounds are known to stimulate microsomal enzymes. The compounds are extremely diverse and there is no apparent relationship between their structure and inducing ability except that most are lipid soluble at physiological pH (Conney, 1967). Susceptibility of rats to acute lethal and hepatotoxic effects of injected lasiocarpine was prevented by oral pretreatment with P.C.N. (pregnenolone-16α-carbonitrile), spironolactone, ethylestrenol, phenobarbital and diphenylhydantoin. In contrast, similar pretreatment prior to monocrotaline or heliotrine administration resulted in marked aggravation of intoxication (Tuchweber et al., 1974). Since microsomal enzymes are responsible for formation of the active pyrrole which damages tissue, it follows that induction of these enzymes should increase toxicity. Tuchweber et al suggested that protection against lasiocarpine may have been due to increased bilary flow, especially in the P.C.N. treated rats, as this compound is noted for this action. The contrasting results obtained with the different alkaloids tested suggests different activation and conjugation pathways might exist and metabolites other than pyrroles could play an important role in toxicity. Mattocks and White (1971) found that

hepatic microsomes from male rats pretreated with phenobarbitone converted retrorsine to pyrrole metabolites *in vitro* much more rapidly than in controls. Formation of N-oxide was increased to a smaller extent. Pretreatment with D.D.T. had a similar effect, except that pyrrole formation was induced even more than N-oxides. 3-Methyl cholanthrene pretreatment had no significant effect, even though this polycyclic hydrocarbon has been shown to induce microsomal enzymes in other studies (Conney, 1967). In a later study, Mattocks (1972) showed that phenobarbitone pretreatment was capable of reducing the toxicity of i.p. injected retrorsine in male rats, but increased the susceptibility in females. It was concluded that when the rate of metabolism in the normal rat is low (retrorsine in female rats) pretreatment increases susceptibility, whereas when the normal rate of metabolism is high (retrorsine in male rats) pretreatment decreases susceptibility. It was suggested that pretreatment might accelerate conversion of labile primary metabolites to less toxic secondary metabolites. Cheeke and Garman (1974) demonstrated that pretreatment with D.D.T. in rats fed tansy ragwort increased growth performance in both sexes while survival time was increased only in the males. Jago (1971) also reported the effect of enzyme induction and sex on pyrrolizidine toxicity. Similar to the study by Tuchweber *et al.* (1974), Jago found that pretreatment with phenobarbitone protects against lasiocarpine toxicity but increases the toxicity of heliotrine. In considering structural differences of the alkaloids, it appears that different pathways of metabolism exist. Toxicity of lasiocarpine, an open diester containing a double bond in the esterified acid moiety

is lowered by pretreatment. Increased toxicity after pretreatment occurs with monocrotaline and heliotrine. Neither contain a double bond in the acid moiety, although the former is a cyclic diester and the latter an open monoester. Retrorsine, a cyclic diester containing a double bond, becomes less toxic to males and more toxic to females after pretreatment. Dietary tansy ragwort which contains a mixture of cyclic diesters with and without double bonds becomes less toxic to males after pretreatment while the females show no change. These structural differences may not only affect rate of metabolism to the pyrrole derivative, but may also be important in conjugation and excretion mechanisms.

Enzyme Inhibitors. Certain compounds exist which have the property of inhibiting microsomal enzyme activity. Certain enzyme inhibitors such as ethionine, puromycin, actinomycin D, and S.K.F.-525 (B-diethylaminoethyl diphenylpropylacetate) block protein synthesis resulting in reduced amounts of microsomal enzyme protein (Conney, 1967; Lee et al., 1972). Urea, deoxycholate, and some sulfhydryl reagents promote decomposition of cytochrome P-450 to P-420 with an associated loss of hydroxylase activity. Other types of inhibitors, such as metyrapone or dicoumarol effect microsomal electron transport (Christensen and Wissing, 1971). Pretreatment of rats with S.K.F.-525 (i.p. injection) was shown to have a protective effect against later (50 min) injection with retrorsine. As indicated by lower levels of bound pyrroles in the liver 2 hours past injection, S.K.F.-525 has the effect of reducing activity of pyrrole forming enzymes (Mattocks, 1972). Pyrrolizidine alkaloids themselves have also been reported to be enzyme inhibitors. The rate of in vitro pyrrole production has been shown to be reduced by

pretreatment with tansy ragwort extract (Shull et al., 1976) while concentration of liver P-450 is reduced by retrorsine pretreatment (White, 1976).

Protein. Level and quality of dietary protein, addition of certain polypeptides and amino acids, either in the diet or parenterally have also been shown to have a significant effect in altering the toxic action of xenobiotics. The feeding of a low protein diet almost abolishes the lethal and hepatoxic effects of carbon tetrachloride in rats, a compound which is normally activated to a toxic metabolite by microsomal enzymes (McLean, 1969). Kato et al. (1968) noted that low protein diets decrease liver microsomal activity and liver weight, while high protein diets have the opposite effect. It follows that since pyrrolizidine alkaloids are microsomally activated to toxic pyrroles, a reduction in dietary protein should reduce enzyme activity and thus reduce toxicity. Conversion of retrorsine to pyrroles in vitro was lower in liver microsomal fractions taken from rats which were starved or fed a protein free diet (Mattocks and White, 1971). Cheeke and Garman (1974) however found that high dietary protein (fish meal) had a protective effect against tansy ragwort toxicity. In this case, the high quantity of high quality protein may have enhanced detoxification by producing an ample pool of amino acids for pyrrole conjugation, and by increasing the amount and activity of enzymes responsible for conjugation reactions.

In other work, the effect of dietary protein quality on induction and inhibition of microsomal enzymes was studied. Baker and Street (1970) found a diet of low quality protein (soy α protein) to be superior in

supporting microsomal enzyme induction in rats given inducing agents (D.D.T.). Enzyme inhibitors (S.K.F.-525) however, were found to be not as effective in animals fed low quality protein (gluten) (Miranda and Webb, 1973). Since pyrrolizidine alkaloids are themselves inhibitors of pyrrole formation (Shull et al., 1976; White, 1976), a high protein quality diet may increase this effect thereby reducing toxicity. The amino acid cysteine and other thiol compounds such as mercaptoethanolamine (MEA) have been shown to be protective against pyrrolizidine alkaloids. Dosing with MEA or cysteine prolongs survival and improves the growth of rats which receive monocrotaline parenterally (Hayashi and Lalich, 1968). The protective effect of MEA has also been observed with lasiocarpine (Rogers and Newberne, 1971). Buckmaster et al. (1976) found that survival time of rats fed tansy ragwort could be increased by addition of one percent cysteine to the diet. Cysteine was presumed to be the active protective agent in a reaction of the sulfhydryl group with the pyrrole. This theory is supported by other work (Shull et al., 1976) where total liver sulfhydryls were reduced after alkaloid injection. Since cysteine has the effect of increasing the level of glutathione in liver tissue (White, 1976), a mercapturic acid conjugation is a likely detoxification mechanism because most compounds metabolized in this manner must first undergo enzyme catalyzed conjugation with glutathione (Chasseaud, 1973).

Carbohydrates. While the specific biochemical role of carbohydrates in the microsomal enzyme system has not been elucidated, several effects have been noted. High intakes of sugar, especially glucose, increase the duration of sleep induced by barbiturates in mice. This

seems to be correlated to decreased microsomal activity (Campbell and Hayes, 1974).

Rats fed a pure sucrose diet showed reduced microsomal pyrrole formation from retrorsine *in vitro*, and sucrose also had a protective effect *in vivo* (Mattocks, 1972). The effect *in vivo* was greater than that *in vitro* suggesting that protection not only arises because of reduced pyrrole formation, but also by protection against active metabolites. Glucuronidation of active metabolites as a detoxification mechanism would be implied in this study, since activity of this pathway is related to the general availability of glucose (LaDu, 1971). A study conducted by Thorpe and Ford (1968) seems to support this. These workers found depleted glycogen levels in calves being fed tansy ragwort in addition to considerable amounts of cytoplasmic lipids. Depleted glycogen levels would occur if glucose was being used for glucuronide formation.

Lipids. Changes in the activities of microsomal enzymes have also been attributed to variations in the lipid fraction of experimental diets. This might be expected since 30 to 55 percent dry weight of endoplasmic reticulum is lipid. Fat-free diets result in depressed cytochrome P-450 levels and reduced microsomal enzyme activity (Campbell and Hayes, 1974). Induction of microsomal enzymes by compounds such as phenobarbital is more effective when the diet contains fish oils or linoleic acid compared to coconut oil or beef fat (Marshall and McLean, 1971; Century, 1973).

Gefferth *et al.* (1977) observed a similar effect in rats fed different types of lipids. In this study, microsomal enzyme activity

(aniline hydroxylase and aminopyrine demethylase) was enhanced when diets high in essential fatty acids were fed (10% sunflower oil) compared to a standard laboratory diet (5% fat). The diets high in essential fatty acids resulted in changes in the fatty acid composition of the livers. These workers postulated that the change led to increased microsomal enzyme activity.

Vitamins. Levels of lipotropes in the diet may also have an effect on the fatty composition of liver tissue. Lipotropes are compounds which either function as methyl donors or assist in methyl transfer during synthesis of phospholipids which are important for triglyceride transport. Diets low in lipotropes, that is deficient in choline, vitamin B_{12} and methionine, induce deposition of fat in the liver. The reduced production of phospholipids, particularly phosphatidylcholine (lecithin) during lipotrope deficiency, causes a reduction in activity of the microsomal enzyme system. Strobel et al. (1970) identified phophatidycholine as the heat stable factor required for drug metabolism in microsomal systems containing cytochrome P-450. This lipid is essential for electron transfer to P-450. The effect of lipotrope level on toxicity of lasiocarpine was studied in pregnant rats by Newberne (1968). It was found that when the mother rats consumed a diet low in lipotropes, the alkaloid caused more severe damage in livers of the offspring. In a later study (Newberne et al., 1971) conducted with young male rats, diets low in lipotropes had a protective effect against monocrotaline pyrrole injected into the mesenteric vein. These workers concluded that dietary lipotrope deficiency decreased formation of pyrroles from alkaloids, but has no effect on the pyrrole once it is formed.

In addition to the lipotropic vitamins (B_{12}, choline) other vitamins have been indicated which affect liver metabolism. Vitamin A, a lipid soluble compound important in regulating the stability of biological membranes, has been shown to significantly increase liver microsomal proteins, RNA, and phosphatidyl choline when fed at high doses to rats (Ram and Misra, 1974). Young et al., (1974) reported decreased levels of stored vitamin A in the liver of rats when dietary D.D.T. (insecticide inducing agent) and/or methionine (lipotrope amino acid) was increased. Shull (1976) found that above normal levels of vitamin A increased pyrrole formation from Senecio alkaloids but the effect was not significant.

Deficiency of vitamin E reduced microsomal enzyme activity in rat liver because of impaired synthesis of heme and hemoproteins (Campbell and Hayes, 1974), while riboflavin deficiency causes reduced levels of liver proteins, important in the microsomal electron transport mechanism (Shargel and Mazel, 1973). The relationship between these vitamins and metabolism of the pyrrolizidine alkaloids has yet to be determined.

Addition of nicotinamide to rat liver microsomal preparations had the effect of consistently inhibiting pyrrole formation but not N-oxide formation from retrorsine (Mattocks and White, 1971). Nicotinamide is required for formation of pyridine nucleotides and has the effect of inhibiting pyridine nucleotidases and other enzymes, while in other cases it enhances enzymatic activity.

Minerals. A relationship between mineral metabolism and pyrrolizidine toxicity has been established.

Australian work with sheep by Bull et al. (1956) showed that liver damage produced by consumption of Heliotropium predisposed sheep to the

hemolytic crises of copper toxicity. Affected sheep in the study were jaundiced and exhibited high liver copper concentrations, 80 percent of which were over 1,000 ppm. Hemolysis is caused by the release of stored copper by the liver when necrosis begins to occur. The effect of excess copper on erythrocytes results in hexose monophosphate shunt stimulation. This is associated with a loss of intracellular reduced glutathione, leaving cells open to oxidative injury (Metz and Sagone, 1972).

Complex formation between copper and the pyrrolizidine alkaloids present in Heliotropium was investigated by Farrington and Gallagher, 1970) and found to occur in vitro. It was suggested that complex formation may result in the abnormally high levels of liver copper seen in sheep and rats subjected to pyrrolizidines. This appears unlikely since the optimum pH for complex formation is 10 to 12 and this is well above the physiological range. Only those alkaloids possessing a 1,2-glycol grouping in the esterifying acid form a complex, and this excludes senecionine and seneciphylline. The fact that pyrrolizidine alkaloids and their pyrrole metabolites are relatively short lived in the liver compared with chronically accumulating copper also suggests complex formation an unlikely factor in hepatic copper accumulation.

Since pyrrolizidine alkaloids are known to be mutagenic by their action on DNA (discussed earlier), a more likely explanation for increased copper accumulation would be an aberration of copper proteins. A mispairing of nucleotides in the portion of DNA coding for these proteins could change certain key amino acids thereby changing their structure or binding sites. This could cause an increase in the avidity for copper by copper thionein or other protein very similar to that noted in Wilson's

disease. In Wilson's disease, copper homeostasis is affected by the expression of an inborn error of metabolism. Copper accumulates in the liver, brain, kidneys and cornea together with low ceruloplasmin levels and high direct reacting serum copper levels (Underwood, 1977). In this disease, copper thionein has been shown to have a Cu-binding constant four times as great as in controls. In human patients with Wilson's disease, hemolytic crises often occur along with post necrotic cirrhosis (Lombeck and Bremer, 1977). Chelating agents such as D-penicillamine have been successful in increasing copper excretion.

With sheep, the condition of copper accumulation is acutely dangerous in that this species is more susceptible to copper poisoning than other farm animals (Ross, 1966). Sheep fed diets high in copper, such as those containing dried poultry waste were found to accumulate abnormally high levels of liver copper (Suttle et al., 1978). Toxins other than pyrrolizidines have also been noted to cause increased accumulation of hepatic copper storage. Gardiner (1966) found lupines to cause this effect when ingested by sheep. This effect is compounded in instances where molybdenum and sulfur compounds are deficient in the feed, since molybdenum mediates ruminal conversion of sulfur compounds to sulfides which can bind copper and prevent absorption (Huisingh et al., 1975). Such is the case with pastures containing high amounts of subterranean clover. This forage contains 10-15 ppm copper and extremely low molybdenum levels that rarely exceed 0.1 to 0.2 ppm (Underwood, 1977).

Diets containing brown seaweed were found to counteract copper accumulation in sheep (Herbert and Wiener, 1978). This seaweed contains alginates which are capable of divalent cation chelation. Phytates

found in soybean meal form very stable complexes with copper and reduce assimilation of this element (Underwood, 1977). Rations high in soybean meal may therefore be beneficial in controlling copper toxicity in sheep exposed to pyrrolizidine containing plants or otherwise high copper.

Experiments with the vervet monkey by Van der Watt et al. (1971) indicate that weekly injections of retrorsine caused, in addition to veno-occlusion, a ten fold increase in liver iron. Iron containing pigment was present in portal and fibrotic areas and within many abnormal megalocytes. The distribution of iron in the liver of retrorsine intoxicated monkeys closely resembled the hemosiderotic condition frequently found in African Bantu people. In rats fed tansy ragwort (Buckmaster et al., 1976) spleens were greatly enlarged and livers were darkly pigmented after poisoning was induced. It was hypothesized that following pyrrolizidine consumption, iron from accelerated erythrocyte destruction is initially concentrated in the liver and then transferred to the spleen. McLean (1970) reported in her review that hemosiderin deposits appear in the kidneys and liver (Kuppfer cells), along with anemia during the late effects of pyrrolizidine poisoning. Along with pyrrole damage, parenchymal iron overload may add to the effects of pyrrolizidine poisoning, since it too may produce fibrosis of the liver and hepatomegalocytosis (Powell, 1974).

Mineral deficiency may also be correlated with incidence of pyrrolizidine toxicity. Palfrey et al. (1967) in a survey conducted in Nova Scotia, found increased losses of cattle due to tansy ragwort intoxication on farms where owners invariably fed less hay, grain, legume and mineral supplements than those sustaining no losses. From

this survey it would be hard to conclude a direct interaction between mineral or other nutrient deficiency and tansy ragwort toxicity since the reported data was not obtained in a controlled experiment. It would appear likely, however, that those who fed mineral supplements were better managers and probably had less tansy ragwort in their pastures, thus explaining the reduced losses.

Deficiencies in dietary calcium, magnesium, iron, iodine, zinc, selenium and copper have all been reported to affect microsomal enzymes in varying degrees (Becking, 1972; Campbell, 1974). Shull et al. (1979) concluded that dietary selenium status was likely to influence metabolism of pyrrolizidine alkaloids. Mattocks (1971) found that addition of calcium (Ca^{2+}) and magnesium (Mg^{2+}) stimulated production of pyrrolic metabolites from retrorsine when added to rat microsomal preparations in vitro. Manganese (Mn^{2+}) potentiated the enzyme activity even in the presence of optimal concentrations of Mg^{2+} or Ca^{2+}. Since chemically reduced NADPH was used in the study, it was concluded that the ions were not acting on the NADPH generating system.

EXPERIMENTAL

The following three groups of experiments were designed to investigate some of the toxic actions of tansy ragwort. By studying the effects of different feeding regimes, the influence of rumen metabolism and the diet-toxin interrelationships, it was anticipated that recommendations could be devised to help alleviate the economic burden of tansy ragwort poisoning in livestock.

PART A:
FACTORS AFFECTING THE TOXICITY OF DIETARY TANSY RAGWORT: STORAGE TIME, HEAT TREATMENT, PLANT PART, LENGTH OF FEEDING

Reduction in alkaloid content of dried tansy ragwort has been reported to occur after prolonged storage in the dried finely ground form. Spectrophotometric analysis revealed the reduction to be from 0.16% to 0.012% (dry wt.) after 6 months storage (Dickinson and King, 1978). Decomposition of pyrrolizidine alkaloids during heating has also been reported (Mattocks, 1961). These factors are of importance where feeding trials are being conducted using stored or pelleted diets containing pyrrolizidine alkaloids. Differences in alkaloid distribution between various functional plant parts (i.e. flowers, leaves, roots, stems) are of interest to those attempting to extract the natural product or use the material for toxicological study.

Length of feeding period of tansy ragwort to cause mortality has importance when the chronic lethal dose is considered. An exposure of short duration may have the same effect as a long exposure if the lethal dose is consumed after a short period.

Objectives of this study were to determine the extent of decomposition of pyrrolizidine alkaloids present in tansy ragwort during prolonged storage and after pelleting, rank the various plant parts as to their toxicity and alkaloid distribution and to determine the chronic lethal dose by feeding rats the toxic material for various lengths of time.

Materials and Methods

The tansy ragwort used in all experiments was collected near Corvallis, Oregon, forced air-dried at 45 C, ground through a 1 mm screen in a Wiley mill and incorporated into rat diets. Toxicity was determined by length of survival of weanling Long-Evans hooded rats (Simonsen origin). All rats were fed the experimental diets and watered ad libitum, and housed individually in wire mesh cages to allow for measurement of feed intake and weight gain.

Experiment A-1: Storage Time

To determine the effect of prolonged periods of storage on the toxicity of tansy ragwort, 24 male rats of approximately 105 g initial body weight were divided randomly into four treatments and fed diets containing the following: (1) dehydrated suncured alfalfa, (2) tansy ragwort flowers stored 1 month, (3) tansy ragwort flowers stored 14 months, (4) tansy ragwort flowers stored 26 months (Table 1). The flowers were collected during different years (1976, 1977, 1978), stored in plastic bags, and kept at room temperature away from light. Differences in toxicity were assessed by survival time.

Experiment A-2: Heat Treatment

The effect of heat treatment during pelleting was determined by comparing survival times of 12 male rats with 120 g initial body weight divided into two treatments as follows: (1) diet of ground (through 0.5 mm screen) non-pelleted meal containing ten percent tansy ragwort, (2) same diet only pelleted (6 x 26 mm) before being ground (Table 1).

Experiment A-3: Plant Parts

To ascertain differences in the toxicity of various plant parts, 30 male rats of 105 g initial body weight were randomly divided into treatments of diets containing tansy ragwort flowers, stems, leaves and roots, and the control (Table 1). Plant parts were separated and dried immediately after collection. Dirt was removed from the roots with a cold water spray before drying. Rats were fed the diets for 180 days during which feed intake, weight gain, and survival were recorded.

Relative distribution of alkaloids within each plant part was determined by the following procedure:

(1) 40 g sample ground through a 30 mesh screen (Cyclone grinding apparatus).

(2) Moisten sample with 10 ml, 10% citric acid (W/V).

(3) Extract in Soxlet apparatus with 60 ml 100% ethanol for 6 hours. Temperature 83 C.

(4) Reduce extract to 100 ml by boiling. Cool.

(5) Acidify with 100 ml 1 N H_2SO_4. Cover flasks and refrigerate for approximately 36 hours.

(6) Filter twice through ethanol extracted wood pulp #40 filter paper. (Removes insoluble material.)

(7) Extract out tars with 50 ml chloroform three times. Combine extracts (150 ml) and wash with 50 ml 1 N H_2SO_4. Add washings back to aqueous sample.

(8) Adjust sample pH to 10 with concentrated NH_4OH. Refrigerate 5 hours.

(9) Extract alkaloids from the alkaline sample with 50 ml reagent chloroform three times.

(10) Reduce chloroform fraction by evaporation in vacuo with roto-evaporator, to an oily mass. Warm water bath (40 C) facilitates evaporation.

(11) Sample further dried in vacuum dessicator over NaOH, for 3 days away from light.

(12) Dry alkaloid sample dissolved in 15 ml absolute methanol, filtered through #41 ashless filter paper.

(13) Alkaloid solution injected into gas chromatograph with the following parameters:

Detector: flame ionization; 16 p.s.i. H_2,16 p.s.i. air; carrier gas N_2 at 18 p.s.i.; oven temperature, 250 C; column packing 3% OV-17 on chrome Q; chart speed 1.27 cm/minute.

Experiment A-4: Length of Feeding--Chronic Lethal Dose

Seventy male rats of approximately 88 g initial body weight were randomly divided into seven groups and fed dietary tansy ragwort for differing lengths of time (1,2,3,4,5,6 weeks) after which they were switched to OSU rat pellets. The seventh group was fed the tansy ragwort diet for the duration of the experiment (Table 1). Weight gain, diet intake and survival time were recorded. Survivors were terminated at 585 days. Chronic LD_{50} was determined using the data from the groups where no mortality occurred during exposure to tansy ragwort diet. The 90 day LD_{50} with a five percent confidence interval was obtained graphically using the procedure described by Litchfield and Wilcoxon (1948).

TABLE 1. PERCENT COMPOSITION OF RAT DIETS

Ingredient	Storage time	Heat treatment	Plant part[4]	Length feeding
Tansy ragowrt				
Flowers	5	--	(5)	--
Whole plant[1]	--	10	--	10
Roots	--	--	--	--
Leaves	--	--	(5)	--
Stems	--	--	(5)	--
Ground corn	53.5	48.5	53.5	48.5
Soy meal	30	30	30	30
Sucrose	5	5	5	5
Corn oil	3	3	3	3
Mineral mix[2]	3	3	3	3
Vitamin mix[3]	0.5	0.5	0.5	0.5

NOTE: Where positive (+) controls were used, suncured dehydrated alfalfa meal replaced tansy ragwort.

[1]Excluding root.
[2]Jones and Foster (1942).
[3]Cheeke et al. (1977).
[4]Parentheses mean diets contained one of the plant parts, not the others, in a given treatment.

Results and Discussion

Experiment A-1: Storage Time

The activity of the pyrrolizidine alkaloids in tansy ragwort flowers was not altered significantly ($P<.05$) after prolonged storage when measured using rats as biological assay organisms (Table 2). This is in contrast to previous work (Dickinson and King, 1978) where chemical analysis revealed a greater than ten fold reduction in alkaloid content after 6 months storage. Possibilities for this variation may include differences in storage regime or physical/chemical binding of the alkaloids during storage which renders them less detectable by chemical methods but to become unbound during the digestive process in animals. Further work needs to be done in this area to determine optimum storage conditions for experimental work and ways to increase pyrrolizidine alkaloid degradation in order to reduce livestock losses from contaminated hay.

Experiment A-2: Heat Treatment

Heat treatment during pelleting did not significantly increase ($P<.05$) survival time of rats consuming a tansy ragwort containing diet when compared to rats consuming the same diet unpelleted (Table 3). Mattocks (1961) reported that much alkaloid may decompose during heating. While heating may reduce the ease of extraction by formation of gummy polymers, it does not appear to alter toxicity when assayed in a biological system.

TABLE 2. EFFECT OF STORAGE TIME ON TANSY RAGWORT TOXICITY

Storage time (months)	Survival time (days)	Tansy ragwort intake (grams)	Feed intake (grams)	Average daily gain (first 14 days)
1	$39^{a} \pm 13$	25 ± 6	493 ± 119	2.1 ± 0.3
14	$53^{a} \pm 5$	22 ± 3	446 ± 60	2.0 ± 0.2
26	$57^{a} \pm 5$	31 ± 6	626 ± 127	2.2 ± 0.2
Control (alfalfa)+	---	---	3452 ± 136	5.4 ± 0.4

[a]Means ± SEM. Means followed by different superscript vertically are significantly different ($P<.05$) as determined by analysis of variance and protected l.s.d. test.
+Controls terminated at 150 days.

TABLE 3. EFFECT OF HEAT TREATMENT DURING PELLETING ON TANSY RAGWORT TOXICITY

Treatment	Survival time (days)	Tansy ragwort intake (grams)	Feed intake (grams)	Average daily gain (first 7 days)
Non-pelleted	$44^a \pm 6$	$46^a \pm 7$	$456^a \pm 69$	$2.06^a \pm 0.27$
Pelleted	$46^a \pm 4$	$50^a \pm 5$	$496^a \pm 52$	$2.03^a \pm 0.27$

[a]Means $\pm$ SEM. Means followed by different superscripts vertically are significantly different ($P<.05$) as analyzed by student's t test.

Experiment A-3: Plant Parts

Differences in toxicity of various plant parts were studied again using rats as biological assay organisms. In order of decreasing toxicity to rats, the various plant parts were ranked as follows: flowers > leaves > roots > stems (Table 4). Again, differences exist when these biological results are compared to the chemical results obtained in other studies. Dickinson and King (1978) measured the alkaloid content of tansy ragwort spectrophotometrically and found the plant parts to contain the following in order of decreasing total alkaloid content: flowers (0.37%), roots (0.33%), leaves (0.16%), stem (0.11%). This suggests differences in relative concentration of individual alkaloids among plant parts. A given amount of each of the six alkaloids present in tansy ragwort may give similar spectrophotometric values but differ significantly in toxicity to animals.

When extracts of each of the plant parts were subjected to gas chromatographic analysis, differences in relative alkaloid concentrations were found to exist (Figure 4). In order of decreasing concentration in the plant parts each alkaloid was present as follows: senecionine, flower > root > leaf > stem; seneciphylline, flower > leaf > root > stem; jacoline, leaf > flower > root > stem; jacobine, leaf > flower > root > stem; jacozine, leaf > flower > root > stem. These results seem to be in agreement with the rat LD_{50} values present in the literature (Bull, 1968). Senecionine and seneciphylline are the most toxic alkaloids in tansy ragwort, with values of 77 and 85 mg/kg LD_{50} respectively, while jacobine and jaconine are somewhat less toxic with values of 138 and 168 mg/kg LD_{50}. Flowers which are highest in

TABLE 4. DIFFERENCES IN TOXICITY OF PLANT PARTS

Plant part	Survival time (days)	Tansy ragwort intake (grams)	Feed intake (grams)	Average daily gain (first 14 days)
Flower	39[a] ± 13	25 ± 6	493 ± 119	2.1 ± 0.3
Leaf	51[a] ± 15	38 ± 12	762 ± 242	3.1 ± 0.2
Root	105[b] ± 18	81 ± 18	1610 ± 350	3.6 ± 1.5
Stem	+	161 ± 72	3217 ± 62	6.8 ± 0.3
Control (alfalfa)	+	---	3452 ± 136	5.4 ± 0.4

[a]Means ± SEM. Means followed by different superscripts vertically are significantly different ($P<.05$) as determined by analysis of variance and protected l.s.d. test.
+Terminated at 150 days. Necropsy showed no apparent gross lesions.

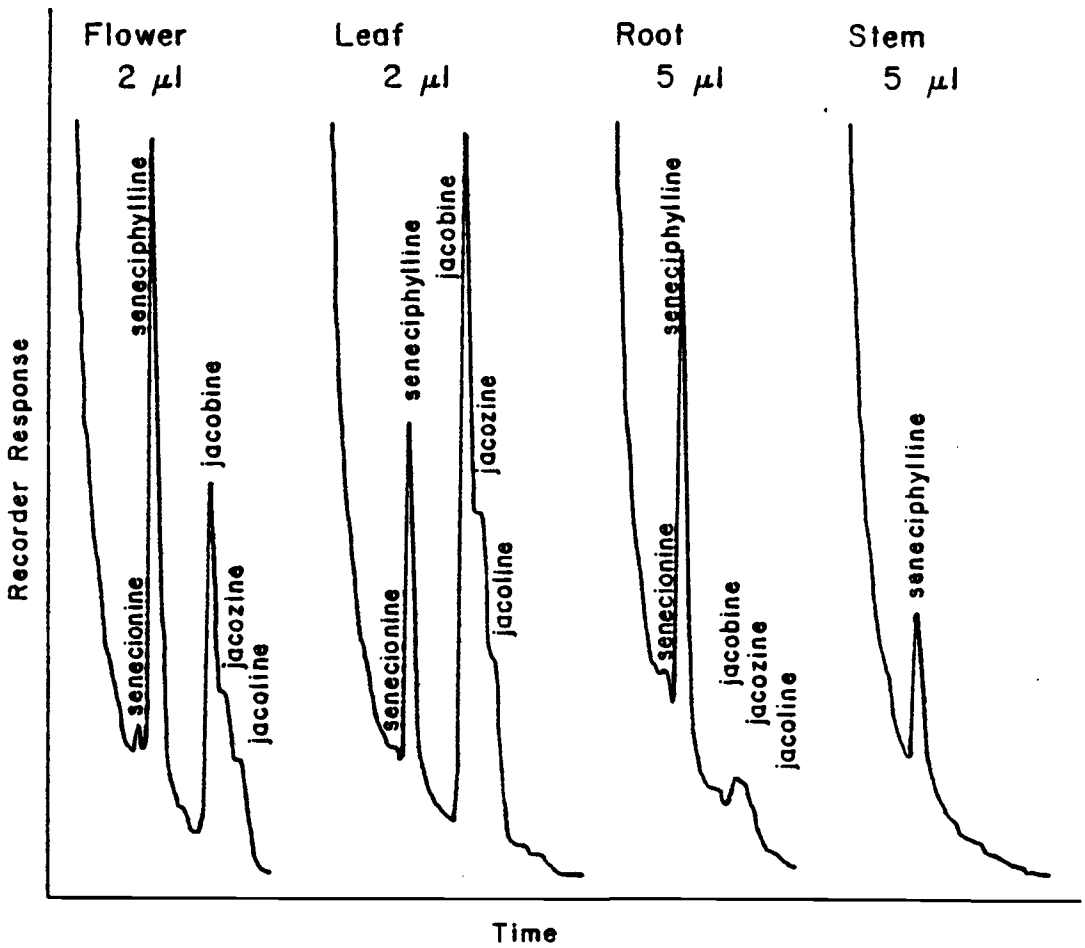


FIGURE 4. RELATIVE DISTRIBUTION OF PYRROLIZIDINE ALKALOIDS AMONG TANSY RAGWORT PLANT PARTS

senecionine and seneciphylline were indeed the most toxic plant part when fed to rats.

Experiment A-4: Length of Feeding--Chronic Lethal Dose

To determine the chronic lethal dose of tansy ragwort present in the diets of rats, survival time was compared in groups of rats fed tansy ragwort-containing diets for various lengths of time (Table 5). No significant difference occurred in survival time in the groups fed the toxic diets for 2, 3, 4, 5, and 6 weeks and ad libitum. The group fed the toxic diet for 1 week had a significantly longer survival time. Since none of the rats in the 1, 2 or 3 week groups died until after they were switched from the toxic tansy ragwort diet to OSU rat pellets, survival time data from these groups were used to calculate the chronic 90 day LD_{50}, which was 31 g of tansy ragwort per kg body weight with a confidence interval of 10--53 g/kg ($P<.05$). Several points of interest were noted in this experiment:

(1) A one-week feeding period of diet containing tansy ragwort is enough to cause mortality in some animals.

(2) A lag period exists between exposure and response.

(3) Mortalities tend to occur after periods of rapid weight gain in the groups when the unpalatable tansy ragwort diets were replaced with OSU rat pellets (Figure 5). This suggests that extra stress on the liver during compensatory growth may trigger mortality due to previous hepatic damage.

TABLE 5. EFFECT OF LENGTH OF FEEDING TANSY RAGWORT ON SURVIVAL

Length fed 10% tansy ragwort diet (weeks)	Survival time (days)	Tansy ragwort intake (grams)
1	$347^{a} \pm 84^{1}$	8 ± 1
2	$114^{b} \pm 50$	16 ± 2
3	$133^{b} \pm 48$	24 ± 2
4	$37^{b} \pm 5$	28 ± 3
5	$71^{b} \pm 17$	37 ± 5
6	$86^{b} \pm 22$	46 ± 5
Ad libitum	$50^{b} \pm 4$	51 ± 6

[a]Means ± SEM. Means followed by different superscripts vertically are significantly different ($P<.05$) as determined by analysis of variance and protected l.s.d. test.
[1]Four out of ten rats were still alive after 585 days.

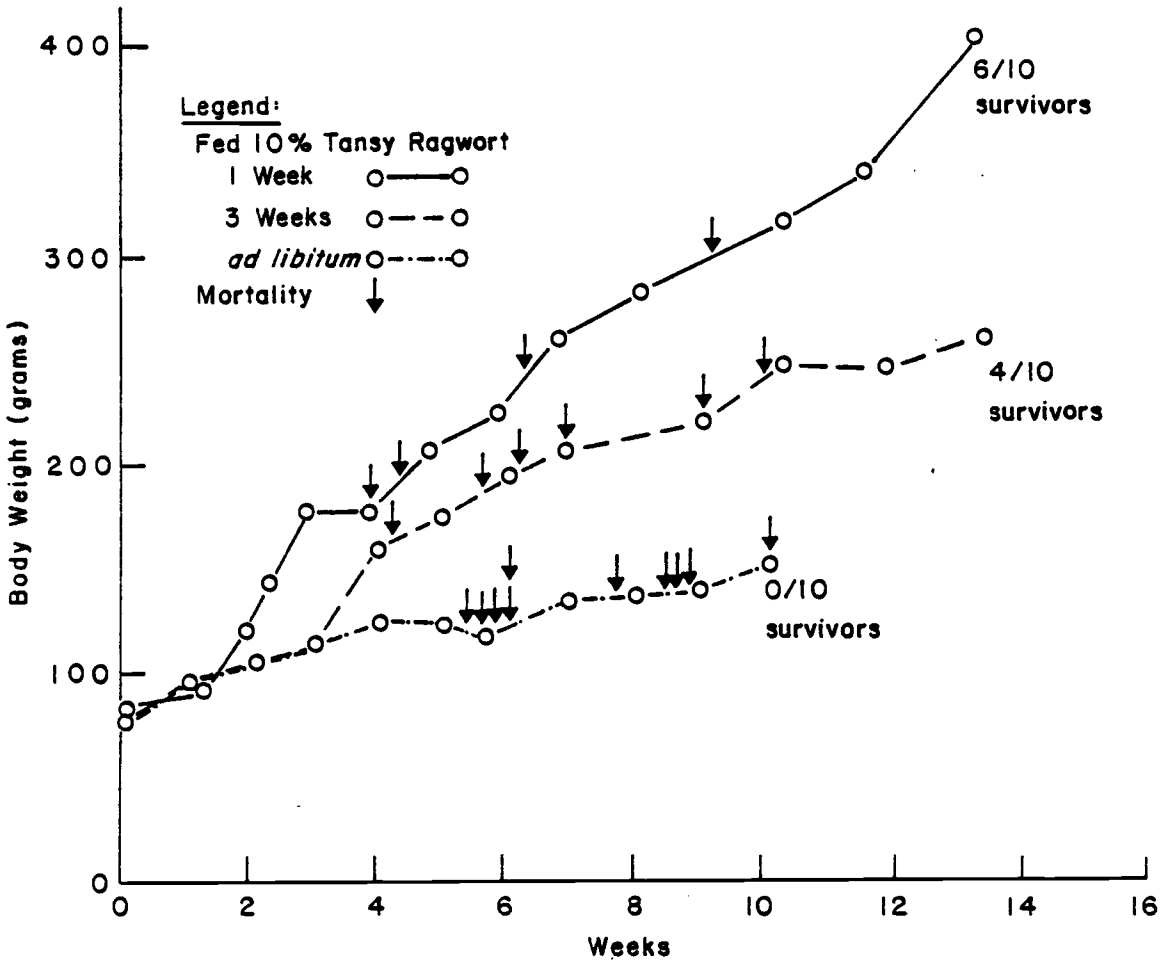


FIGURE 5. MORTALITY AND WEIGHT GAIN OF RATS CONSUMING TANSY RAGWORT FOR DIFFERENT LENGTHS OF TIME

PART B:
OVINE RUMEN METABOLISM OF TANSY RAGWORT

In susceptible animals, liver microsomal enzymes are responsible for metabolizing pyrrolizidine alkaloids into the active pyrrole which causes extensive tissue damage (Mattocks, 1968). In sheep, which are resistant to pyrrolizidine alkaloid poisoning it has been shown that the alkaloids in Heliotropium are metabolized in the rumen by a specific microorganism (Peptococcus heliotrinreductans) to non-toxic methylene and methyl derivatives (Lanigan, 1976). Growth of this microorganism is enhanced by pretreatment with Heliotropium and also addition of methane inhibitors (Lanigan, 1972). The objectives of this study were to determine if the pyrrolizidine alkaloids present in tansy ragwort undergo similar metabolism in the ovine rumen.

Materials and Methods

Experiment B-1: Ovine Rumen Fermentation--Rat Survival

To determine whether rumen microflora have the capability of detoxifying pyrrolizidine alkaloids, tansy ragwort was fermented in ovine rumen fluid, McDougalls buffer and water (Table 6), freeze dried and then incorporated into rat diets. Each batch was incubated at 37 C for 48 hours in two liter erlenmeyer flasks fitted with gas traps. CO_2 was bubbled through the mixture for 5 minutes prior to incubation. Survival time of the rats was used to assess if detoxification occurred. The rumen fluid was aspirated from a fistulated crossbred wether and transported to the lab in prewarmed vacuum bottles. In the normal and

autoclaved treatments, the wether was fed 1.0 kg/day basal ration (Table 6), and in the 50-1, 50-5, and 50-5+ iodoform treatments, the wether was fed 0.5 kg basal mixed with 0.5 kg whole plant tansy ragwort (excluding root) daily. The addition of tansy ragwort to the diet did not appear to reduce palatability. pH was measured with a Fisher 310 pH meter in the rumen fluid, and in all the incubation mixtures before and after fermentation. Likewise, total volatile fatty acid (VFA) μmole/ml and ratio of acetic to propionic acids (A:P) were determined via gas chromatography using a Varian Aerograph series 1200 equipped with a flame ionization detector. In the autocalved treatment, rumen fluid was subjected to autoclaving at 120 C at 1.04 kg/cm^2 for 1 hour prior to the addition of tansy ragwort, buffer, and water. In the 50-1 treatment rumen fluid was collected after 1 week pretreatment of the wether with 50 percent dietary tansy ragwort. In the 50-5 treatment rumen fluid was collected after 5 weeks pretreatment as it was in the 50-5+ iodoform treatment. The latter had iodoform added to the incubation mixture resulting in a concentration of 0.005 mM iodoform.

Fifty-six male rats of approximately 85 g initial body weight were randomly distributed into seven treatments. In the positive (+) control treatment, suncured alfalfa meal was substituted for tansy ragwort while the negative (-) control treatment contained unincubated tansy ragwort (Table 7). Differences in toxicity between the treatments were determined by length of survival.

TABLE 6. OVINE RUMEN FERMENTATION

COMPOSITION OF INCUBATION MIXTURE

Tansy ragwort	100 g
Rumen fluid (ovine)	500 ml
McDougalls Buffer[1]	400 ml
Distilled water	400 ml
Iodoform (50-5 + iodo treatment)	2.56 mg (0.005 mM)

[1]McDougall (1948).

[2]Leslie, Inc.

RATION FED TO RUMEN FLUID DONOR

Basal		
Ryegrass straw	30%	1 kg/day for normal and autoclaved treatments
P.N.W. Barley	26%	
Cottonseed meal	16%	
Alfalfa meal	10%	
Beet pulp	10%	
Molasses	7%	
Limestone flour	0.6%	
Trace mineralized salt[2]	0.4%	
Basal + tansy ragwort		
Basal	50%	1 kg/day for 50-1, 50-5 and 50-5 + iodo treatments
Tansy ragwort	50%	

TABLE 7. OVINE RUMEN FERMENTATION PERCENT COMPOSITION OF RAT DIETS

Suncured dehydrated alfalfa meal (+ control)	(5)
Tansy ragwort unincubated (- control)	(5)
Tansy ragwort incubated in:	
autocalved rumen fluid (autoclaved)	(5)
normal rumen fluid (normal)	(5)
1 week pretreated rumen fluid (50-1)	(5)
5 week pretreated rumen fluid (50-5)	(5)
5 week pretreated rumen fluid and .005 mM iodoform (50-5 + iodo)	(5)
Ground corn	48.5
Soybean meal	30
Sucrose	5
Corn oil	3
Mineral mix[1]	3
Vitamin mix[2]	0.5

[1]Jones and Foster (1942).

[2]Cheeke et al. (1977).

Parentheses mean diet contained one but not the other ingredients in a given treatment.

Experiment B-2: Detection of Non-Toxic Metabolites from Tansy Ragwort--Ovine Rumen Fluid Incubation Mixtures In Vitro

Two 30 g samples of tansy ragwort flowers were ground to pass through a 30 mesh screen. Samples were mixed separately with 150 ml ovine rumen fluid, 120 ml McDougalls buffer containing glucose and urea, and 120 ml of water. One batch was immediately frozen while the other was fermented in a two liter erlenmeyer flask for 48 hours at 37 C after initially bubbling CO_2 through the mixture to remove air. After incubation, samples were freeze dried. Once dry, all samples were ethanol extracted in a Soxlet apparatus and further purified with chloroform as described in experiment A-3. Final extracts were subjected to gas chromatographic analysis (described in A-3) to determine if degradation of alkaloids or formation of metabolites was occurring.

Results and Discussion

Experiment B-1: Ovine Rumen Fermentation--Rat Survival

Lanigan (1974) has shown that certain microorganisms in the rumen of sheep (Peptococcus heliotrinreductans) have the capability of metabolizing the pyrrolizidine alkaloids present in Heliotropium to nontoxic 1-methylene and 1-methyl derivatives by reduction of the 1-2 double bond in the pyrrolizidine nucleus and cleavage of the ester. It was indicated that metabolic hydrogen donors such as reduced coenzymes were essential for heliotrine metabolism. Increases in these hydrogen donors by diet changes or by inhibition of methane bacteria which use hydrogen to reduce CO_2 to CH_4 were shown to increase the rate

of formation of nontoxic metabolites. Pretreatment of the rumen fluid donor with Heliotropium also had this response when the rate of alkaloid metabolism was measured in vitro.

In this experiment, incubation of tansy ragwort with ovine rumen fluid did not show detoxification when rat survival time was used to assay toxicity (Table 8). Detoxification was not significantly ($P<.05$) increased by pretreatment of the wether with 50 percent dietary tansy ragwort for 1 week, 5 weeks or by the addition of iodoform as a methane inhibitor to the incubation media. To determine if ingestion of tansy ragwort had any adverse effects on rumen fermentation in vivo, total VFA's, A:P ratio and pH were measured in the collected rumen fluid. Results appear in Table 9. A drop in total VFA's and an increase in A:P ratio after 1 week pretreatment with 50 percent tansy ragwort in the wether indicated that the rumen microflora were not yet fully adapted to the change in diet. A relative decrease in propionate production suggests an increase in C_1 intermediates used by methanogenic bacteria and a decrease in feed efficiency since propionate is a more direct precursor to glucose than acetate in the ruminant. After 5 weeks pretreatment, total VFA production appeared to rise toward normal values, while the A:P ratio dropped below normal. It was hypothesized that pyrrolizidine metabolizing organisms were successfully competing with methanogenic bacteria for available metabolic hydrogen and the resulting C_1 intermediates were metabolized to such other end products as propionate. The pH data indicate that tansy ragwort consumption would not result in rumen acidosis.

TABLE 8. EFFECT OF *IN VITRO* OVINE RUMEN FERMENTATION ON TANSY RAGWORT TOXICITY TO RATS

Treatment	Survival Time (days)	Tansy ragwort intake (grams)	Feed intake (grams)	Average daily gain (first 18 days)
+ Control	No mortality	---	1817 ± 38	5.69 ± 0.43
- Control	43[a] ± 6	52 ± 10	514 ± 97	2.46 ± 0.17
Autoclaved	55[a] ± 7	71 ± 10	710 ± 96	3.56 ± 0.31
Normal	53[a] ± 10	77 ± 19	768 ± 194	3.19 ± 0.16
50-1	44[a] ± 3	53 ± 5	525 ± 45	3.37 ± 0.18
50-5	56[a] ± 8	72 ± 15	721 ± 137	3.23 ± 0.22
50-5 + Iodoform	44[a] ± 8	75 ± 14	571 ± 135	3.23 ± 0.26

[a]Means ± SEM. Means followed by different superscripts vertically are significantly different ($P<.05$) as determined by analysis of variance and protected l.s.d. test.

TABLE 9. VOLATILE FATTY ACID (V.F.A.) ANALYSIS AND pH OF RUMEN FLUID AND INCUBATION MIXTURES

Treatment	Rumen fluid			Mixture before incubation		Mixture after incubation	
	Total V.F.A. umol/ml	A:P[a]	pH	A:P	pH	A:P	pH
Normal	89 ± 7	3.4 ± 0.2	6.0 ± 0.2	3.6 ± 0.4	6.4 ± 0.05	4.2 ± 0.9	4.4 ± 0.01
Autoclaved	---	---	---	3.1 ± 0.3	6.7 ± 0.1	7.3 ± 2	4.3 ± 0.1
50-1	77 ± 3	3.7 ± 0.1	6.4 ± 0.1	3.6 ± 0.1	6.5 ± 0.02	2.7 ± 0.1	4.6 ± 0.02
50-5	85 ± 4	3.1 ± 0.2	6.4 ± 0.2	3.5 ± 0.3	6.4 ± 0.1	2.6 ± 0.4	4.5 ± 0.04
50-5 + iodo	---	---	---	3.7 ± 0.3	6.4 ± 0.1	2.4 ± 0.3	4.5 ± 0.03

Means ± SEM.

[a]A:P = acetate/propionate.

A:P ratio and pH were also measured during the in vitro phase of the experiment before and after incubation of the tansy ragwort, rumen fluid and buffer mixtures (Table 9). It was noted that as the length of pretreatment increased, the A:P ratio decreased. When iodoform was added to the incubation mixture to inhibit methanogenisis, the lowest A:P ratio was observed. To determine whether any compounds were present in rumen fluid which might alter toxicity without microbial action a treatment where rumen fluid was autoclaved prior to incubation was included. Fermentation still occurred presumably due to contamination by organisms such as wild yeasts present on the plant material. A:P ratio and final pH values indicated the presence of a totally different microflora in this treatment.

While pretreatment and addition of iodoform had a positive effect on altering the microflora in favor of pyrrolizidine detoxication as indicated by shifts in the A:P ratios, the rat survival time data showed no significant effect when incubated tansy ragwort was compared to un-incubated tansy ragwort. This is contrary to the Australian work with Heliotropium. Several factors may account for this difference. First, all the alkaloids studied in Heliotropium are open esters whereas the alkaloids present in tansy ragwort are all macrocyclic closed esters. Secondly, in the Australian work, the metabolites were not assayed for toxicity in a biological system. Further work needs to be done to determine if the metabolites are in fact non-toxic.

The results of this experiment support previous research by Shull et al. (1976) where the toxicity of tansy ragwort to rats was not altered after incubation in ovine rumen fluid. These workers found

sheep to have decreased hepatic microsomal enzyme capacity to metabolize pyrrolizidine alkaloids into the damaging pyrrole metabolite when compared to susceptible species. It was concluded that the resistance of sheep to tansy ragwort intoxication was due to decreased liver activity and not rumen detoxification.

Experiment B-2: Detection of Non-Toxic Metabolites from Tansy Ragwort--Ovine Rumen Fluid Mixtures

Figure 6 shows the chromatograms of extracts of tansy ragwort samples incubated in ovine rumen fluid and not incubated, but mixed with ovine rumen fluid. The results indicate neither degradation of alkaloids or formation of metabolites. The pattern of alkaloid distribution is essentially the same in each sample. The unincubated sample appears to have less total alkaloid present than the incubated sample. If degradation were occurring the opposite would be expected. These results further support the hypothesis that tansy ragwort is not detoxified in rumen of sheep. The fact that more total alkaloid was found to be present in the sample which was incubated suggests that rumen fermentation may actually be responsible for the breakdown of plant material to release more of the alkaloids present.

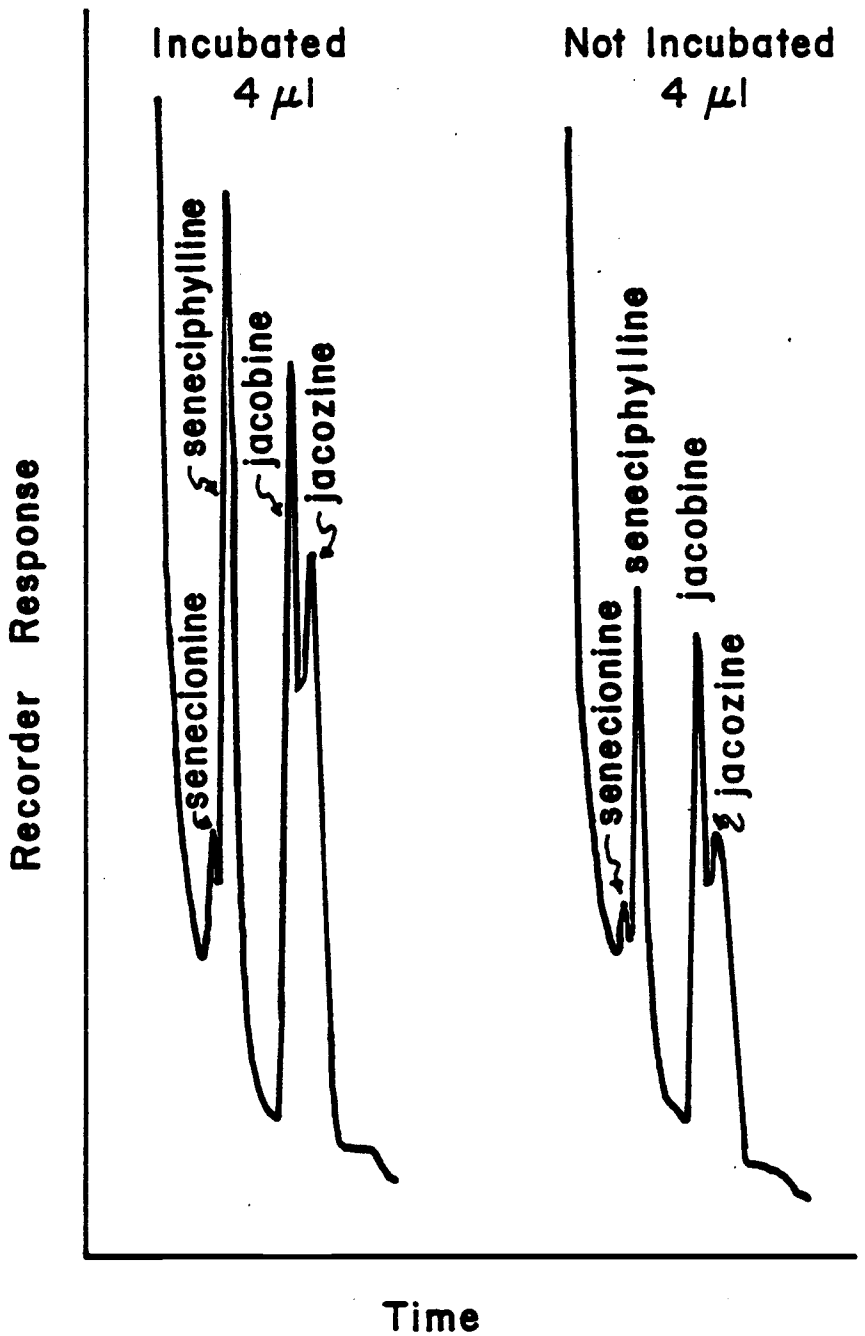


FIGURE 6. EFFECT OF RUMEN FERMENTATION ON THE DISTRIBUTION OF ALKALOIDS IN TANSY RAGWORT

PART C:
DIET AND TOXIN INTERRELATIONSHIPS: NUTRIENTS AND OTHER DIETARY FACTORS INFLUENCING TANSY RAGWORT TOXICITY

The activity of the liver microsomal enzyme system is dependent on the type, quantity and quality of nutrients and other factors present in the diet. Since the alkaloids contained in tansy ragwort are converted to active forms by the liver, it follows that dietary alterations might be beneficial to animals inadvertently consuming tansy ragwort. The objectives of the following experiments were several fold. In the first experiment, phenothiazine, a commonly used livestock anthelmintic with microsomal enzyme inducing properties (Wattenburg and Leong, 1965), was assessed with and without cysteine for protective activity. Cysteine is important in conjugation-detoxification mechanisms. In the second experiment, the effect of lipid level and sex on the toxicity of tansy ragwort was examined. The integrity of the membranes which comprise the smooth endoplasmic reticulum or microsomes is dependent on lipid availability. In addition, steroid hormones which require lipids for their formation are dependent on lipid level and sex. These factors were examined with regard to toxicity. The objective of the third experiment was to determine the effect of protein level and source on the toxicity of dietary tansy ragwort. Conflicting reports are found in the literature pertaining to protein level and the toxicity of liver activated toxins. Lethal effects of carbon tetrachloride were abolished by feeding low protein diets to rats (McLean, 1968), whereas high protein diets were protective to rats consuming tansy ragwort (Cheeke and Garman, 1974). Different sources of protein were examined due to their different amino acid composition which might effect detoxification pathways.

The objectives of the fourth and fifth experiments were to determine if the alkaloids present in tansy ragwort cause abberations in mineral metabolism. Such effects were noted in Australia with the pyrrolizidine alkaloid containing plant Heliotropium. This plant was found to induce copper toxicity in sheep. By determining the extent in which tansy ragwort is active in this regard, recommendations might be made to ranchers who use sheep as biological control agents.

Materials and Methods

Experiment C-1: Effect of Phenothiazine and Cysteine

Sixty male rats of approximately 105 g initial body weight were randomly distributed into six treatments of ten rats each. Additions to the basal diet were as follows: control, five percent alfalfa; phenothiazine, five percent alfalfa plus 0.15 percent phenothiazine; tansy ragwort, 5% tansy ragwort flowers; tansy ragwort plus cysteine, 5% tansy ragwort flowers with 1% cysteine; tansy ragwort plus cysteine and phenothizaine, 5% tansy ragwort flowers, 1% cysteine and 0.15% phenothizaine. Protective activity of the additions to the tansy ragwort containing diets was assessed by comparing survival time means of these treatments to the controls. Percent composition of the diets appear in Table 10. In addition, average daily gain, feed intake and tansy ragwort intake were compared for the treatments.

Experiment C-2: Effect of Lipid Level and Sex

Forty male and forty female rats of approximately 120 g initial body weight were randomly assigned with regard to sex into eight

TABLE 10. EFFECT OF PHENOTHIAZINE AND CYSTEINE ON THE TOXICITY OF FIVE PERCENT DIETARY TANSY RAGWORT: PERCENT COMPOSITION OF RAT DIETS

BASAL RATION

Ingredient	Percent of diet
Ground corn[1]	53.5
Soybean meal[1]	30
Sucrose	5
Corn oil	3
Mineral mix[2]	3
Vitamin mix[3]	0.5

ADDITIONS TO BASAL

Treatment	Ingredient	Percentage
Control	Dehydrated suncured alfalfa	5
Phenothiazine	Dehydrated suncured alfalfa	5
	Phenothiazine	0.15
Tansy ragwort	Tansy ragwort flowers	5
Tansy ragwort + phenothiazine	Tansy ragwort flowers	5
	Phenothiazine	0.15
Tansy ragwort + cysteine	Tansy ragwort flowers	5
	Cysteine	1
Tansy ragwort + cysteine + phenothiazine	Tansy ragwort flowers	5
	Cysteine	1
	Phenothiazine	0.15

[1] In those treatments wehre cysteine and phenothiazine were added, ground corn was substituted.
[2] Jones and Foster (1942).
[3] Cheeke et al. (1977).
[4] 94% phenothiazine (Stocklin Supply, Portland, OR).

treatments with ten rats each. Treatments were arranged in a 2x2x2 factorial design. The factors were: lipid level, 1 or 15%; sex, male or female; tansy ragwort flowers, present or not present in the diet. Treatment effects were assessed by differences in survival time, tansy ragwort intake, feed intake and average daily gain. Percent composition of the experimental diets appear in Table 11. Both diets were isonitrogenous, and isocaloric. In the high lipid diet, the butter used contained salt, and was first melted over low heat before slowly mixing into the rest of the diet. The diets were stored under refrigeration to prevent rancidity.

Experiment C-3: Effect of Protein Level and Source

Eighty male rats of approximately 102 g initial body weight were randomly assigned into ten treatments with eight rats each. Treatments were arranged in a 2x5 factorial design. Factors were: dietary protein level, 12 or 25%; protein source, fish meal (herring), soybean meal, cottonseed meal, casein or gelatin. Calcium carbonate and/or dicalcium phosphate were added to the rations as needed, to bring the levels of calcium and phosphorus up to the requirements for the growing rat (N.R.C., 1972). In the gelatin treatments, DL tryptophan was added at a level corresponding with three-fourths of the requirement, since this protein source is completely lacking in this amino acid. Sucrose was substituted in place of the protein source where necessary in order to formulate each diet at either 12 or 25% crude protein. Diet formulations appear in Table 12. The effect of protein on toxicity was assessed by survival time and tansy ragwort intake. Average daily gain was used as an indicator of general health and vigor.

TABLE 11. EFFECT OF LIPID LEVEL AND SEX ON THE TOXICITY OF FIVE PERCENT DIETARY TANSY RAGWORT FLOWERS: PERCENT COMPOSITION OF DIETS

Ingredient	Percent of diet	
	High lipid	Low lipid
Tansy ragwort flowers	(5)[1]	(5)
Alfalfa (controls)	(5)	(5)
Corn starch	34	65.5
Casein	23	23
α cellulose	17.5	0
Butter	14	0
Corn oil	1	1
Mineral mix[2]	4	4
Vitamin mix[3]	1.5	1.5

[1]Parentheses mean that one ingredient but not the other was used in a given diet.
[2]Jones and Foster (1942).
[3]Cheeke et al., (1977).

TABLE 12. EFFECT OF PROTEIN LEVEL ANO SOURCE ON TOXICITY OF FIVE PERCENT TANSY RAGWORT FLOWERS: PERCENT DIET COMPOSITIONS

Ingredient	Diet protein level									
	Herring		Soybean meal		Cottonseed meal		Casein		Gelatin	
	12%	25%	12%	25%	12%	25%	12%	25%	12%	25%
Herring meal	16.7	34.7	----	----	----	----	----	----	----	----
Soybean meal	----	----	26.7	55.6	----	----	----	----	----	----
Cottonseed meal	----	----	----	----	29.3	61.0	----	----	----	----
Casein meal	----	----	----	----	----	----	14.7	30.6	----	----
Gelatin	----	----	----	----	----	----	----	----	12.6	26.3
Sucrose	67.8	50.8	56.9	28.7	54.6	23.3	68.9	53.8	70.2	56.5
Tansy ragwort flowers	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Corn oil	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
α cellulose	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0
Vitamin mix[1]	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Trace mineral salt[2]	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Dicalcium phosphate	1.01	----	1.47	0.44	0.66	----	1.63	0.37	2.60	2.60
Calcium carbonate	----	----	0.40	0.74	0.93	1.17	0.28	0.73	----	----
DL tryptophan[3]	----	----	----	----	----	----	----	----	0.13	0.13

[1]Cheeke et al. (1977).
[2]Leslie Corp.
[3]To provide 3/4 of growth requirement.

Experiment C-4: Accumulation of Liver and Spleen Copper, Iron and Zinc in Rats Intoxicated by Tansy Ragwort

Seventy-two male rats of approximately 125 g initial body weight were randomly assigned into twelve treatments with six rats per treatment. Treatments were arranged in a 3x4 factorial design. Factors were: dietary copper level, 0, 50, 250 ppm; dietary tansy ragwort flowers, 0, 1, 2.5, 5 percent. Copper was added as copper sulfate (anhydrous) to the diets. Tansy ragwort flowers were substituted for dehydrated alfalfa meal in those diets containing the toxic plant. The rations (Table 13) were essentially a corn-soy based natural diet containing approximately 18 percent crude protein. Rats were fed and watered ad libitum for 28 days and then anesthetized with ether and exsanguished by heart puncture. Collected blood was treated with calcium oxalate (0.5 ml/10 ml) to prevent clotting. Necropsy was performed to obtain organs for mineral analysis. Blotted weights of liver, spleen and kidney were measured from each rat. Organs were then frozen in HCl washed, distilled water rinsed glass bottles. Fractions of whole blood and blood plasma were also frozen for further analysis.

To determine the mineral content of tissues, samples were prepared and assayed as follows:

1) Approximately 1.5 g wet tissue was weighed into HCl washed, glass distilled water-rinsed 50 ml oven-dried tared erlenmeyer flasks.

2) Samples were dried in oven for 48 hours at 100 C and then cooled in a dessicator and reweighed.

3) 10 ml, 69.7 percent concentrated reagent grade nitric acid was added to each sample.

4) Samples were warmed on hot plate (85 C) for 48 hours until clear yellow with no undissolved traces.

5) 2 ml concentrated reagent grade perchloric acid was added to each sample.

6) Samples were heated at high heat (185 C) until dry with white powder remaining in flask.

7) Exactly 10 ml 0.1 N HCl was added to each to dissolve residue.

8) Samples were aspirated into Jarrel-Ash flame atomic absorption spectrophotometer equipped with a recorder (range 0.1-5 ppm Cu; 1-20 ppm Fe; 0.1-4 ppm Zn).

9) Appropriate dilutions were made for samples off scale with 0.1 N HCl.

10) Standard curve was prepared directly from recorder response signals of standard solutions and was used for determination of samples.

11) ppm dry weight tissue = (dilution factor x ppm of aspirated sample) divided by dry weight sample.

Experiment C-5: Effect of Diet on Liver Copper Levels of Rats Consuming a High Copper Diet

Lower than expected liver copper levels in Experiment C-4 suggested the possibility that copper absorption was being impaired by phytate or other compounds in the corn-soybean meal diet. To test this possibility, this experiment was conducted to compare liver copper levels when rats were fed diets with either casein or soybean meal as the protein supplement.

Sixteen male rats of approximately 96 g initial body weight were radnomly assigned into two groups with eight rats each. Group one was fed a casein based diet while group two was fed a corn-soy based diet (Table 14). Diets were isoproteinaceous and isocaloric for swine

TABLE 13. ACCUMULATION OF LIVER AND SPLEEN COPPER, IRON, AND ZINC: PERCENT COMPOSITION OF DIETS

Ingredient	Percent
Corn meal	53.5
Soybean meal	30
Alfalfa meal[1]	(5) (4) (3.5) (0)
Tansy ragwort flowers[1]	(0) (1) (2.5) (5)
Sucrose	5
Corn oil	3
Mineral mix[2]	3
Vitamin mix[3]	0.5
Cu[4]	(0) (50) (250) ppm

[1]Depending on treatment, tansy ragwort was substituted with alfalfa meal for a combined total of 5 percent.
[2]Jones and Foster (1942).
[3]Cheeke et al. (1977).
[4]Depending on treatment, anhydrous $CuSO_4$ was added to result in either 0, 50, or 250 ppm added copper.

TABLE 14. EFFECT OF DIET ON LIVER COPPER LEVELS: PERCENT DIET COMPOSITION

Ingredient	Percent of diet	
	Corn-Soy based	Casein based
Corn meal	53.5	----
Soybean meal	30	----
Alfalfa meal	5	----
Casein	----	23
Corn starch	----	30
α cellulose	----	10
Sucrose	5	29
Corn oil	3	3
Mineral mix[1]	3	4
Vitamin mix[2]	0.5	1
Copper[3]	250 ppm	250 ppm

[1]Jones and Foster (1942).
[2]Cheeke et al. (1977).
[3]Copper added as anhydrous $CuSO_4$ (0.63 g $CuSO_4$ per kg diet).

NOTE: Each diet contains 18.5 percent crude protein and 3330 kcal/kg swine digestible energy.

digestible energy. Copper was added to each diet at a level of 250 ppm (anhydrous $CuSO_4$). Rats were fed and watered ad libitum for 28 days and then sacrificed by suffocation in carbon dioxide. Livers were carefully removed, blotted, weighed, and placed in HCl washed, distilled water rinsed plastic containers. Aliquots of each liver (approximately 1.5 g wet) were subjected to copper analysis exactly as described in Experiment C-4.

Results and Discussion

Experiment C-1: Effect of Phenothiazine and Cysteine

Phenothiazine appeared to have a detrimental effect in altering the toxicity of tansy ragwort. Although not statistically significant ($P<.05$), survival time was decreased by 4 days in rats fed tansy ragwort with added phenothiazine (Table 15); in all cases, addition of phenothiazine reduced feed intake and average daily gain. Cysteine, however, when added to the diet showed protective activity against tansy ragwort toxicity. Survival time was significantly increased ($P<.05$) from 50 to 62 days even though total tansy ragwort intake was significatntly greater ($P<.05$) in the cysteine treatment. In all cases, cysteine increased average daily gain. When cysteine and phenothiazine were both added to the tansy ragwort diet, survival time was greater than tansy ragwort alone, but less than tansy ragwort plus cysteine. These effects were nonsignificant ($P<.05$).

Phenothiazine has been identified as having a significant capacity to induce benz-α-pyrene hydroxylase, a detoxification enzyme present in rat microsomal tissue (Wattenburg and Leong, 1965). This hydroxylase

TABLE 15. EFFECT OF PHENOTHIAZINE AND CYSTEINE ON THE TOXICITY OF 5% DIETARY TANSY RAGWORT FLOWERS

Treatment	Survival time (days)	Tansy ragwort intake (grams)	Feed intake (grams)	Average daily gain (first 14 days)
Control	----	----	$1700^{c} \pm 29$	$7.17^{d} \pm 0.17$
Phenothiazine	----	----	$1598^{c} \pm 47$	$5.95^{c} \pm 0.26$
5% Tansy ragwort flowers	$50^{a} \pm 3$	$27.0^{a} \pm 2.0$	$541^{a} \pm 39$	$2.10^{ab} \pm 0.14$
5% Tansy ragwort flowers + 0.15% phenothiazine	$46^{a} \pm 3$	$22.8^{a} \pm 3.1$	$456^{a} \pm 62$	$1.89^{a} \pm 0.18$
5% Tansy ragwort flowers + 1% cysteine	$62^{b} \pm 3$	$34.1^{b} \pm 2.4$	$682^{b} \pm 47$	$2.79^{b} \pm 0.19$
5% Tansy ragwort flowers + 0.15% phenothiazine + 1% cysteine	$55^{ab} \pm 4$	$28.7^{ab} \pm 2.1$	$574^{ab} \pm 41$	$2.85^{b} \pm 0.18$

[1] Means ± SEM. Means followed by different superscripts vertically are significantly different ($p<.05$) as determined by analysis of variance and protected l.s.d. test.

enzyme converts the potent carcinogen benz-α-pyrene into inactive hydroxy derivatives readily excretable due to elevated water solubility. Induction of microsomal enzymes by phenothiazine has also been shown to be effective in reducing the incidence of carcinomas in animals ingesting braken fern (Pteris aquilina), a poisonous plant with an undetermined toxin (Pamukcu et al., 1971). Because of these noted effects, it appeared worthwhile to investigate the interaction of phenothiazine with tansy ragwort. The results of this experiment indicate that induction of N-oxidation or conjugation pathways by phenothiazine is unlikely since toxicity of tansy ragwort was not reduced. Mattocks (1971) suggested separate pathways for N-oxidation and pyrrole formation and that N-oxides are likely detoxification products being highly water soluble. If these pathways are in fact separate, then it would appear from the results that phenothiazine is mildly active in inducing pyrrole formation. Since the protective effect of cysteine is not enhanced by phenothiazine, increased rate of pyrrole formation does not appear to cause an increase in non-toxic thiol conjugates even when sulfhydryls are readily available. From this, it can be hypothesized that cysteine may exert its protective effect before pyrrole formation or conversely, metabolites other than pyrroles may be responsible for alkylation to physiologically important sulfhydryl containing macromolecules.

Experiment C-2: Effect of Dietary Lipid Level and Sex

Both male and female rats fed tansy ragwort diets containing additional lipids (14% as butterfat) had significantly lowered survival

TABLE 16. EFFECT OF LIPID LEVEL AND SEX ON THE TOXICITY OF 5% DIETARY TANSY RAGWORT FLOWERS

Lipid level	Sex	Tansy ragwort flowers	Survival time (days)	Tansy ragwort intake (grams)	Feed intake (grams)	Average daily gain (first 14 days)
1%	M	+	$44^{b} \pm 3$	$20^{a} \pm 2$	$403^{a} \pm 40$	$1.59^{c} \pm 0.14$
	M	-		0	$1118^{d} \pm 49$	$4.70^{e} \pm 0.29$
	F	+	$47^{c} \pm 3$	$19^{a} \pm 2$	$388^{d} \pm 40$	$0.74^{a} \pm 0.11$
	F	-		0	$836^{b} \pm 45$	$3.07^{d} \pm 0.22$
15%	M	+	$36^{a} \pm 2$	$18^{a} \pm 1$	$362^{a} \pm 19$	$1.30^{bc} \pm 0.09$
	M	-		0	$977^{c} \pm 54$	$4.51^{e} \pm 0.27$
	F	+	$38^{a} \pm 3$	$16^{a} \pm 2$	$327^{a} \pm 39$	$0.80^{ab} \pm 0.12$
	F	-		0	$863^{bc} \pm 35$	$3.38^{d} \pm 0.22$

^{abcde}Means $\pm$ SEM. Means followed by different superscripts vertically are significantly different ($p<.05$) as determined by analysis of variance and protected l.s.d. test.

times (Table 16). In both the low lipid and high lipid treatments, females had longer survival times than males. This effect was significant ($P<.05$) in the low lipid, but not the high lipid groups. The interaction between lipid level and sex was not significant ($P<.05$), indicating that changes in survival times across lipid levels were equivalent in both males and females. There was no significant difference ($P<.05$) in tansy ragwort intake for any group fed the toxic diets. When compared to the control treatments, it was clear that addition of tansy ragwort to the diet significantly ($P<.05$) decreased feed intake and average daily gain. In male rats, increased lipid level decreased feed intake and average daily gain, while in female rats, intake and average daily gain were increased. This effect was significant for the males ($P<.05$) but not the females. The effect of lipid level on survival time may be due to several factors. Alterations in the activity of the microsomal enzymes which are responsible for pyrrole formation may be enhanced by increases in dietary lipids. Increased activity of certain microsomal enzymes has been reported to occur in rats fed high levels of lipids (Gefferth and Blaskovits, 1977). While essential fatty acids appear to be most effective in this regard, it could be possible that non-essential fatty acids might also be active through a sparing effect. Another explanation for decreased survival time with high lipid diets would be increased absorption of pyrrolizidine alkaloids from tansy ragwort in the gut. Increased levels of liver fat may also be responsible for the increased toxicity in the high lipid groups. Newberne (1968) found that diets low in lipotropes enhanced the toxic effects of the alkaloid lasiocarpine. Because lipotropes are responsible for hastening removal

of fat from the liver, a diet high in fat would have the same effect as a diet low in lipotropes. Sex effects in this experiment may have been due to differences in steroid hormone production. Since females had longer survival times than males, it would appear likely that estrogenic compounds could have protective activity.

Experiment C-3: Effect of Protein Level and Source

Survival time of rats consuming five percent tansy ragwort flowers ad libitum was significantly altered ($P<.05$) by changes in the level or source of dietary protein. Likewise, there was a significant interaction ($P<.05$) bewteen protein level and source with regard to survival time (Table 17). When total intake of tansy ragwort was taken into account the protective activity of increased protein level became even more apparent for some of the sources. For both soybean meal and casein, rats consumed 39 percent more tansy ragwort as the protein was increased from 12 to 25 percent. For each of these sources, the increase in tansy ragwort intake was significant ($P<.05$), as was the increase in survival time. Rats consuming soybean meal or casein lived 12 or 15 days longer respectively when the level of protein was increased. For both levels of protein, rats showed the greatest response to fish meal with regard to average daily gain. The worst response in terms of average daily gain was observed with gelatin, an incomplete low-quality protein. In both the high and low level gelatin treatments, the rats were unable to maintain initial body weight. Feed and tansy ragwort intake were not significantly different ($P<.05$) in the low level gelatin treatment when compared to other low level protein sources. The fact that the low level gelatin rate showed the greatest length of survival when compared to other low

TABLE 17. EFFECT OF PROTEIN LEVEL AND SOURCE ON TOXICITY OF 5% DIETARY TANSY RAGWORT FLOWERS

Protein level	Protein source	Survival time (days)	Tansy ragwort flower intake (grams)	Feed intake (grams)	Average daily gain (first 14 days)
12%	Fish meal	$32^{ab} \pm 2$	$12.1^{ab} \pm 1.4$	$242^{ab} \pm 28$	1.18 ± 0.13
	Soybean meal	$28^{a} \pm 2$	$13.0^{ab} \pm 2.4$	$260^{ab} \pm 48$	0.97 ± 0.30
	Cottonseed meal	$35^{ab} \pm 1$	$14.5^{abc} \pm 0.7$	$290^{abc} \pm 13$	0.30 ± 0.19
	Casein	$38^{bc} \pm 3$	$14.5^{abc} \pm 1.7$	$289^{abc} \pm 33$	0.30 ± 0.22
	Gelatin + 0.2% DL tryptophan	$46^{cde} \pm 2$	$12^{ab} \pm 0.7$	$240^{ab} \pm 14$	-1.65 ± 0.10
25%	Fish meal	$39^{bcd} \pm 3$	$19.6^{cd} \pm 2.2$	$392^{cd} \pm 43$	2.49 ± 0.46
	Soybean meal	$40^{bcd} \pm 4$	$21.3^{de} \pm 3.2$	$426^{de} \pm 63$	2.10 ± 0.33
	Cottonseed meal	$22^{ab} \pm 4$	$16.6^{bcd} \pm 2.3$	$332^{bcd} \pm 46$	1.58 ± 0.24
	Casein	$53^{e} \pm 4$	$23.9^{d} \pm 2.3$	$447^{e} \pm 45$	1.42 ± 0.11
	Gelatin + 0.2% DL tryptophan	$47^{de} \pm 2$	$10.8^{a} \pm 0.8$	$215^{a} \pm 15$	-1.19 ± 0.08

^{abcde}Mean $\pm$ SEM. Means followed by different superscripts are significantly different ($P<.05$) as determined by analysis of variance and protected l.s.d. test.

level sources indicates protective activity of this protein source. This effect was not as extreme when gelatin was compared to other sources in the high level treatments. In the high level treatments, feed intake and thus tansy ragwort intake was significantly lower ($P<.05$) for gelatin when compared to the other sources. Casein showed greatest protective activity of the protein sources in the high level treatments. Rats consumed significantly more ($P<.05$) of the casein diets compared to other sources except soybean meal in the high level treatments.

From these results it is clear that a simple relationship does not exist between level and source of protein and toxicity of tansy ragwort. In the low level treatments, the lowest quality protein was most protective, while in the high level treatments, the highest quality protein was most protective. The effect of the low level, low quality gelatin diet may be explained in several ways. Reduction of microsomal protein and thus reduced activity of pyrrole forming enzymes in this treatment may be responsible for decreased toxicity. When the amino acid profile of gelatin is taken into account (Table 18) it becomes apparent that this protein source contains a large percentage of glycine. Reduction in toxicity due to conjugation of pyrrolizidine alkaloids with glycine may be significant. Further work needs to be performed to test this hypothesis. The significantly ($P<.05$) longer length of survival despite high tansy ragwort intake in the high level casein treatment may be due to conjugation with sulfur amino acids. Casein contains a relatively high level of methionine although it is somewhat low in cystine. Casein also contains relatively high levels of

TABLE 18. AMINO ACID CONTENT OF DIETS WITH VARIOUS SOURCES AND LEVELS OF PROTEIN

Protein Source	% Crude protein	% Protein in diet	Percent amino acids												
			Arg	Lys	Met	Cys	Trp	Gly	His	Leu	Ile	Phe	Tyr	Thr	Val
Herring meal	71	--	5.51	6.23	2.08	1.19	.72	5.10	1.73	5.56	3.64	2.75	2.00	3.07	4.16
	--	12	.92	1.04	.35	.20	.12	.85	.29	.93	.61	.46	.33	.51	.69
	--	25	1.91	2.16	.72	.41	.25	1.77	.60	1.93	1.26	1.26	.69	1.07	1.44
Soybean meal	45	--	3.20	2.84	.66	.67	.63	2.69	1.10	3.60	2.63	2.20	1.40	1.79	2.40
	--	12	1.11	.99	.23	.23	.17	.93	.38	1.25	.91	.76	.45	.62	.83
	--	25	1.78	1.58	.37	.37	.35	1.50	.61	2.00	1.46	1.22	.78	1.00	1.33
Cottonseed meal	41	--	4.24	1.68	.61	.87	.57	2.22	1.11	2.38	1.66	2.73	1.10	1.36	1.99
	--	12	1.24	.49	.18	.25	.17	.65	.33	.70	.49	.65	.32	.40	.58
	--	25	2.59	1.02	.37	.53	.35	1.35	.68	1.45	1.0.	1.36	.67	.83	1.21
Casein	82	--	3.40	.70	2.70	.30	1.00	1.50	2.50	8.60	5.70	4.60	4.70	3.80	6.80
	--	12	.50	1.03	.40	.04	.15	.22	.37	1.26	.84	.68	.69	.56	1.00
	--	25	1.04	2.14	.83	.09	.31	.46	.77	2.63	1.74	1.41	1.44	1.16	2.08
Gelatin	95	--	7.60	4.40	.74	.10	0	22.4	.95	3.40	1.80	2.20	.19	1.70	2.80
	--	12	.96	.55	.09	.01	0(.13)[1]	2.82	.12	.43	.23	.28	.02	.21	.35
	--	25	2.00	1.16	.19	.03	0(.13)[1]	5.89	.25	.89	.47	.58	.05	.45	.74

[1]Adapted from Titus and Fritz (1971).
[1]dl Tryptophan was added to the diet at a level of 0.2% to result in 0.13% available tryptophan.

lysine, leucine, tryptophan and valine. These amino acids may not only be important in direct or indirect (through sparing effect) conjugation reactions, but could be necessary for detoxification enzyme formation. From these results it would appear that activity of pyrrole forming enzymes is dependant on a low protein threshold, whereas activity of N-oxide and conjugation enzymes is dependant on greater quantity of protein and available amino acids in the diet.

Experiment C-4: Accumulation of Liver and Spleen Copper, Iron and Zinc in Rats Intoxicated by Tansy Ragwort

Retention of copper in rat hepatic tissue was increased as levels of copper sulfate and/or tansy ragwort were increased in the diet.

At 0, 50 and 250 ppm added dietary copper, liver levels of copper increased greater than 4, 18 and 21 fold as the level of tansy ragwort was increased from zero to five percent in the diets (Table 19). These increases were significant ($P<.05$) in the 50 and 250 ppm added copper groups but not in the zero ppm added copper group. At lower additions of tansy ragwort (eg. 1 and 2.5 percent) increases in liver copper were also observed for all dietary copper levels, however the effect was significant ($P<.05$) only in the 2.5-250 treatment. Additions of tansy ragwort and copper sulfate had no significant effect ($P<.05$) on altering hepatic levels of zinc in rats (Table 19). When liver tissue was assayed for iron, the highest level was observed in the 2.5-50 treatment. The level of iron in this treatment was significantly greater than all other treatments. The general trend for iron levels was to increase with an increase in tansy ragwort and to be greatest when copper was added at 50 ppm (Table 19).

For spleen tissue, copper levels were not altered significantly ($P<.05$) except in the high tansy ragwort, high copper treatments (5-50, 5-250). The 5-50 treatment showed a greater than three fold significant increase in spleen copper over the 0-50 treatment while the 5-250 treatment showed a slightly less than three fold increase in spleen copper over the 0-250 treatment (Table 20). Spleen zinc was not affected significantly ($P<.05$) in any of the treatments. Spleen iron levels were significantly higher ($P<.05$) in the 2.5-50 treatment compared to all other treatments and significantly lower ($P<.05$) in the 5-50 treatment. There did not appear to be any definite trend for spleen iron levels to change in relation to dietary copper or tansy ragwort.

The organ weight data (Table 21) indicated that additions of dietary tansy ragwort tend to decrease liver weights, and increase spleen and kidney weights. Expressed as percent of total body weight, liver weights were significantly lower ($P<.05$) in the five percent tansy ragwort fed treatments when compared to the one percent tansy ragwort level treatments. Spleen weights were significantly higher ($P<.05$) in the five percent tansy ragwort fed treatments compared to either the zero or one percent tansy treatments. Kidney weight was significantly higher ($P<.05$) only in 5-50 and 5-250 treatments when compared to 0-50 or 1-50 and 0-250 and 1-250.

The results of this experiment demonstrate clearly that pyrrolizidine intoxication is directly related to mineral metabolism. With copper, the relationship is most apparent. Pyrrolizidine alkaloids increase liver retention of copper. It is not known whether this excess copper comes from increased intestinal absorption or at the expense of other

copper containing compounds in the body. The biochemical site of accumulation is also unknown. It may be hypothesized that the accumulation site is copper thionein, a sulfhydryl rich storage protein. The mutagenic properties of pyrrolizidine alkaloids may cause the avidity for copper by this protein to increase. If this is the case, then it would also seem possible that at least some if not all of this copper could come from endogenous sources. The function of some important copper containing enzymes might then be affected. Superoxide dismutase for example, which contains Cu^{2+} and Zn^{2+} (Lehninger, 1976) and is important in the reduction of the toxic superoxide anion could be adversely affected. Hemolysis of red blood cells and destruction of tissue would ensue. This is in fact what is observed in certain cases of pyrrolizidine alkaloidosis. Loss of activity of ceruloplasm could account for abnormal iron metabolism since this enzyme catalyzes the oxidation of Iron II to Iron III. In certain cases hemosiderin deposits have been noted in spleen tissue (see appendix).

Other copper containing enzymes which might be affected include polyphenol oxidase important in melanin formation, keratin oxidase, uricase and dopamine-B-hydroxylase, an enzyme involved in norepinephrine biosynthesis. To observe all of these effects, a long enough period for depletion of copper from these enzymes would be necessary. Further work involving various species at toxic and deficient levels of copper are necessary to determine the role of minerals in pyrrolizidine toxicity.

In examining the feed intake and weight gain data it was interesting to note that at all levels of tansy ragwort, additions of copper sulfate to the diet appeared to increase palatability and average daily gain (Table 22).

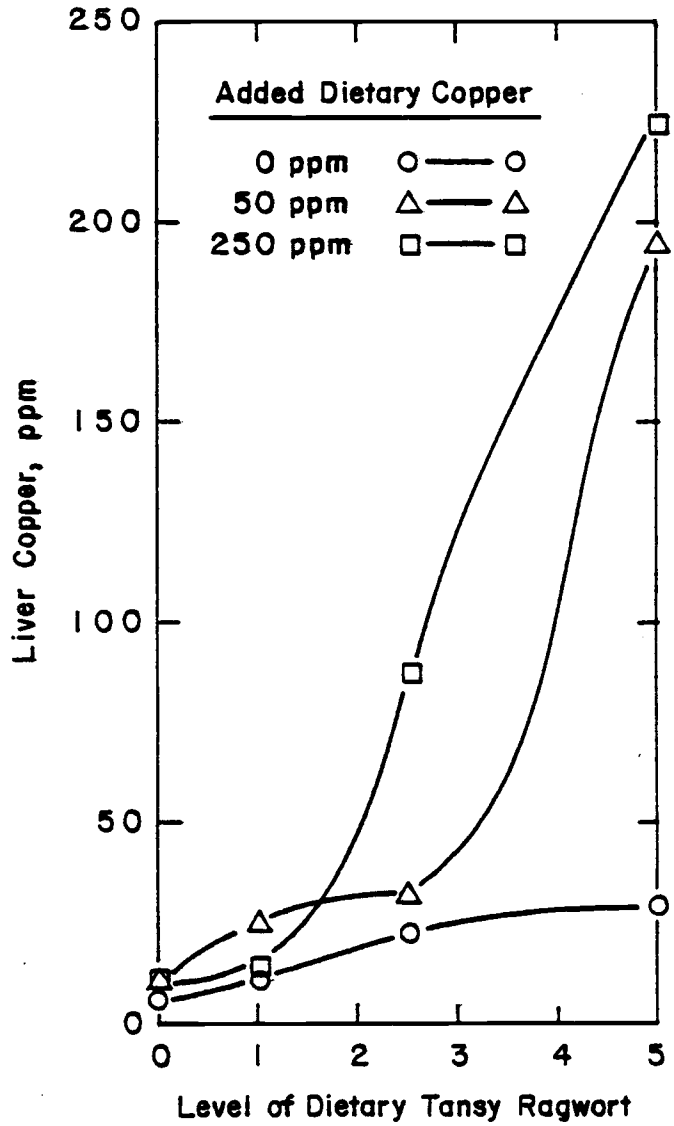


FIGURE 7. LIVER COPPER VS. LEVEL OF DIETARY TANSY RAGWORT

TABLE 19. LIVER LEVELS OF COPPER, ZINC AND IRON FROM RATS CONSUMING TANSY RAGWORT AND ADDED COPPER

Percent dietary tansy ragwort	Liver Cu (ppm dry wt.) ppm added dietary copper			Liver Zn (ppm dry wt.) ppm added dietary copper			Liver Fe (ppm dry wt.) ppm added dietary copper		
	0	50	250	0	50	250	0	50	250
0	$6.5^{a}\pm 1.1$	$10.6^{a}\pm 3.7$	$10.6^{a}\pm 1.5$	$51^{a}\pm 12$	$60^{a}\pm 16$	$67^{a}\pm 10$	$166^{ab}\pm 23$	$192^{b}\pm 56$	$198^{bc}\pm 20$
1	$9.1^{a}\pm 1.0$	$23.8^{a}\pm 10.1$	$11.7^{a}\pm 2.5$	$75^{a}\pm 7$	$79^{a}\pm 11$	$60^{a}\pm 14$	$109^{a}\pm 11$	$188^{b}\pm 32$	$160^{ab}\pm 24$
2.5	$20.5^{a}\pm 3.4$	$31.3^{a}\pm 10.2$	$92.3^{b}\pm 28.9$	$82^{a}\pm 12$	$87^{a}\pm 10$	$77^{a}\pm 6$	$281^{d}\pm 84$	$434^{e}\pm 89$	$236^{bc}\pm 56$
5	$27.5^{a}\pm 10.2$	$194.4^{c}\pm 36.1$	$223.4^{c}\pm 70.8$	$43^{a}\pm 15$	$71^{a}\pm 9$	$86^{a}\pm 10$	$182^{abc}\pm 35$	$341^{d}\pm 76$	$271^{cd}\pm 41$

^{abcd}Means $\pm$ SEM. Means with different superscripts under a given mineral are significantly different ($P<.05$) as analyzed by analysis of variance and protected l.s.d. test.

TABLE 20. SPLEEN LEVELS OF COPPER, ZINC AND IRON FROM RATS CONSUMING TANSY RAGWORT AND ADDED COPPER

Percent dietary tansy ragwort	Spleen Cu (ppm dry wt.) ppm added dietary copper			Spleen Zn (ppm dry wt.) ppm added dietary copper			Spleen Fe (ppm dry wt.) ppm added dietary copper		
	0	50	250	0	50	250	0	50	250
1	$7.0^{a}\pm1.0$	$6.9^{a}\pm1.0$	$5.9^{a}\pm0.4$	$71^{a}\pm6$	$65^{a}\pm4$	$54^{a}\pm6$	$794^{bcd}\pm126$	$715^{bd}\pm109$	$671^{b}\pm49$
2	$5.8^{a}\pm0.5$	$6.4^{a}\pm0.8$	$7.1^{a}\pm1.1$	$66^{a}\pm6$	$68^{a}\pm12$	$80^{a}\pm7$	$822^{cd}\pm65$	$989^{ef}\pm101$	$975^{ef}\pm142$
2.5	$8.1^{a}\pm0.9$	$7.9^{a}\pm1.2$	$7.2^{a}\pm1.2$	$75^{a}\pm4$	$72^{a}\pm8$	$62^{a}\pm8$	$811^{cd}\pm86$	$1103^{f}\pm147$	$747^{bc}\pm79$
5	$7.0^{a}\pm1.0$	$21.3^{c}\pm4.0$	$16.9^{b}\pm6.5$	$81^{a}\pm3$	$57^{a}\pm11$	$84^{a}\pm3$	$925^{de}\pm110$	$476^{a}\pm52$	$767^{bc}\pm55$

^{abcdef}Means $\pm$ SEM. Means with different superscripts under a given mineral are significantly different ($P<.05$) as analyzed by analysis of variance and protected l.s.d. test.

TABLE 21. ORGAN WEIGHTS OF RATS CONSUMING TANSY RAGWORT AND ADDED DIETARY COPPER

Tansy ragwort flowers (% of diet)	Dietary Copper mg/kg	Organ weights[1] (percent of body weight)		
		Liver	Spleen	Kidney
0	0	$4.76^{bcd} \pm 0.16$	$0.20^{a} \pm 0.02$	$0.77^{ab} \pm 0.03$
0	50	$4.86^{bd} \pm 0.11$	$0.22^{a} \pm 0.02$	$0.78^{abc} \pm 0.04$
0	250	$4.69^{bcd} \pm 0.24$	$0.21^{a} \pm 0.01$	$0.77^{ab} \pm 0.03$
1	0	$4.89^{d} \pm 0.12$	$0.27^{a} \pm 0.04$	$0.77^{ab} \pm 0.02$
1	50	$4.96^{d} \pm 0.38$	$0.30^{ab} \pm 0.02$	$0.83^{abcd} \pm 0.06$
1	250	$4.91^{d} \pm 0.07$	$0.32^{abc} \pm 0.04$	$0.76^{a} \pm 0.02$
2.5	0	$4.87^{d} \pm 0.32$	$0.57^{ef} \pm 0.03$	$0.89^{cde} \pm 0.03$
2.5	50	$4.82^{bcd} \pm 0.36$	$0.44^{cd} \pm 0.06$	$0.82^{abcd} \pm 0.02$
2.5	250	$4.81^{bd} \pm 0.24$	$0.53^{def} \pm 0.08$	$0.90^{de} \pm 0.02$
5	0	$3.99^{ab} \pm 0.57$	$0.43^{bcde} \pm 0.05$	$0.88^{bcde} \pm 0.05$
5	50	$3.50^{a} \pm 0.26$	$0.58^{ef} \pm 0.05$	$1.11^{f} \pm 0.06$
5	250	$4.11^{abc} \pm 0.19$	$0.63^{f} \pm 0.04$	$0.98^{e} \pm 0.05$

[1]Computed from blotted wet tissue.

^{abcde}Means ± SEM. Means with different superscripts vertically are significantly different ($P<.05$) as analyzed by protected l.s.d. test.

TABLE 22. DIET INTAKE AND AVERAGE DAILY GAIN OF RATS CONSUMING TANSY RAGWORT AND ADDED COPPER

Tansy ragwort flowers (% of diet)	Dietary copper (mg/kg)	Tansy ragwort flower consumption (g)	Copper consumption (mg)	Feed consumption (g)	Average gain daily (first 14 days)
0	0	0	4.7 ± 0.4	499 ± 98	4.67 ± 0.82
0	50	0	32.8 ± 2.1	551 ± 35	5.20 ± 0.40
0	250	0	153.1 ± 7.3	590 ± 28	6.32 ± 1.07
1	0	4.9 ± 0.2	4.7 ± 0.2	494 ± 24	4.07 ± 0.40
1	50	4.9 ± 0.1	29.3 ± 0.6	492 ± 10	3.24 ± 0.22
1	250	5.3 ± 0.3	138.3 ± 7.6	533 ± 29	4.51 ± 0.35
2.5	0	8.7 ± 0.4	3.3 ± 0.1	347 ± 15	3.49 ± 1.15
2.5	50	9.0 ± 0.5	21.5 ± 1.1	361 ± 19	3.25 ± 0.52
2.5	250	9.2 ± 0.8	95.8 ± 8.2	369 ± 32	3.06 ± 0.68
5	0	18.3 ± 1.3	3.5 ± 0.2	365 ± 25	2.25 ± 0.02
5	50	16.4 ± 1.3	19.5 ± 1.5	328 ± 25	2.27 ± 0.39
5	250	19.4 ± 2.0	102.1 ± 9.6	387 ± 39	2.66 ± 0.27

In the tansy ragwort free treatments, average daily feed intake was 17.8, 19.7 and 21.1 g for the 0, 50 and 250 ppm added copper diets respectively. Average daily gain was 4.67, 5.20 and 6.32 for 0, 50 and 250 ppm added copper, respectively.

This is in direct disagreement with research by Milne and Weswig (1965). These researchers found average daily feed intake to be 12.7, 9.1 and 5.9 for 0, 50 and 200 ppm added dietary copper, respectively, and average daily gain to be 3.43, 2.39 and 1.09 for 0, 50 and 200 ppm added copper. Differences in levels of accumulated liver copper were also noted bewteen the tansy ragwort free rats and the data collected by Milne and Weswig (1965). For the tansy ragwort free rats, liver copper (ppm dry weight) were 6.5, 10.6 and 10.6 respectively, for 0, 50 and 250 ppm added dietary copper, while in the data reported by Milne and Weswig, the levels were 8.7, 18.2 and 68.5 (ppm dry weight liver Cu), respectively, for 0, 50 and 200 ppm added dietary copper. Differences in strains of rats used and basal diet differences may account for the disagreement between the studies. This was examined in the next experiment.

Experiment C-5: Effect of Diet on Liver Copper Levles of Rats Consuming a High Copper Diet

Retention of liver copper was significantly greater ($P<.01$) in rats fed a casein based diet when compared to a corn-soy diet. At the level of 250 ppm added copper used in this experiment, a greater than four fold increase in hepatic copper was found in rats fed casein based rations as opposed to corn-soy based rations (Table 23). The effect was further magnified by the fact that the casein fed rats ingested 22 percent

TABLE 23. CHELATING EFFECT OF SOYBEAN MEAL: DIET CONSUMPTION AND LIVER COPPER

Diet	Feed consumption (g)	Average daily gain (first 14 days)	Copper intake (mg)	Liver copper (mg/kg dry)	Liver weight (wet g)	Liver weight (% of body wt.)
Casein	$462^{a}\pm14$	$6.21^{a}\pm0.22$	$115.5^{a}\pm3.5$	$37.0^{a}\pm8.9$	$14.0^{a}\pm0.7$	$5.8^{a}\pm0.2$
Soybean meal	$589^{b}\pm13$	$8.03^{b}\pm0.25$	$147.3^{b}\pm3.3$	$7.9^{b}\pm0.5$	$16.1^{a}\pm0.8$	$5.5^{a}\pm0.2$

^{ab}Means + SEM. Means with different superscripts vertically are significantly different ($P<.01$) as analyzed by Student's t test.

less total copper than the corn-soy rats. Feed consumption and thus copper intake were significantly lower ($P<.01$) in the casein treatment. Average daily gain was also significantly lower ($P<.01$) in the corn-soy treatment. There was no significant difference ($P<.01$) in liver weights between treatments when expressed as either whole wet liver or as a percentage of body weight.

Phytates form very stable complexes with copper and reduce assimilation of this element (Underwood, 1977). Since soybean meal contains high levels of phytic acid, reduced absorption of copper might be expected. This effect was clearly observed in this experiment. In the previous experiment, pyrrolizidine alkaloids increased liver retention of copper, but only when relatively high levels of tansy ragwort and copper were fed simultaneously. Because the basal ration contained soybean meal and thus phytic acid, the effect of pyrrolizidine alkaloids on copper accumulation was not as great as expected. From the results of these experiments, it would be recommended that in further work dealing with the interaction of pyrrolizidines on mineral metabolism, casein based diets should be used.

CONCLUSIONS AND SUGGESTIONS FOR FUTURE RESEARCH

A definite relationship between diet and toxicity of Senecio jacobaea has been established. The results obtained in Part A of this investigation demonstrate the stable nature and problems associated with the chronic toxicity of the closed ester pyrrolizidine alkaloids contained in tansy ragwort. Since the alkaloids are resistant to decomposition over time and by heat, curing and storage of hay made from infested pastures would not be expected to alter toxicity. Further study would be beneficial in this area to determine if commercial feed preservatives or other compounds could be added during processing to reduce toxicity of contaminated feeds.

Differences in relative alkaloid distribution were noted between the various anatomical parts of the tansy ragwort plant. Tansy ragwort contains six separate alkaloids each with a different level of toxicity. From the feeding study with various plant parts, it appears that the alkaloid jacobine is less toxic than seneciphylline. The effect of soil amendments on the biotransformation of these alkaloids within the plant would be worthwhile information in that certain amendments could alter toxicity.

As expected, a lag period was found to exist between exposure of animals to tansy ragwort and symptoms of toxicity. A one week period of exposure was enough to cause mortality months later in laboratory rats.

In Part B of the study, ovine rumen fermentation was determined to not have an effect on the toxicity of the pyrrolizidine alkaloids contained in tansy ragwort. While pretreatment of sheep with tansy ragwort and additions of methane inhibitors enhanced the conditions *in vitro* for

rumen detoxification organisms, alterations in pyrrolizidine toxicity were not observed. It was concluded that the resistance of sheep to pyrrolizidine alkaloidosis must be due to either hepatic detoxification or lack of gastrointestinal absorption. Determination of the mechanism of detoxification in sheep would be useful in that it might be applied to economically important susceptible species. Suggestions for future research in this area would include toxicity trials with sheep after hepatic enzyme induction.

In Part C, nutritional factors affecting pyrrolizidine toxicity were examined. Because dietary addition of phenothiazine increased toxicity of tansy ragwort, this compound was determined to increase pyrrole production. Since the protective effects of dietary cysteine were not enhanced by phenothiazine, it was concluded that cysteine may exert its protective effect before pyrrole formation. The increased toxicity of tansy ragwort by additions of dietary lipids may have been due to increased levels of liver fat, or increased absorption of pyrrolizidine alkaloids from the gut. The interaction between protein level and source on tansy ragwort toxicity indicated the possible beneficial effects of dietary alterations. Enzymes responsible for toxic pyrrole production appeared to be deactivated only when animals are extremely deficient in essential amino acids. Enzymes involved in detoxification of pyrrolizidines require high levels of essential amino acids. By determining which amino acids are required most by the enzymes in each pathway, progress could be made in increasing detoxification through alteration in dietary protein.

Induction of hepatic copper accumulation was observed in animals fed tansy ragwort. Changes in iron metabolism but not zinc metabolism were also indicated during intoxication. This effect is economically important to the sheep industry. While sheep are resistant to pyrrolizidine alkaloids they are sensitive to copper toxicity. Since sheep have been advocated as biological agents to control the spread of tansy ragwort, further research needs to be conducted to determine ways to reduce the possibility of copper toxicity in these animals. Further beneficial research would include the isolation of some copper containing proteins from livers of pyrrolizidine intoxicated animals to determine if alterations in amino acid composition were occurring. This could be likely since pyrrolizidine alkaloids have been found to be mutagenic. Studies involving copper, molybdenum and sulfur interactions with regard to pyrrolizidine intoxication would also be beneficial. Not only would such research aid in alleviating the probelms associated with pyrrolizidine toxicity but would also permit a better understanding of human metabolic diseases such as Wilson's disease. By studying the mutagenic properties of pyrrolizidines and other xenobiotics with regard to mineral metabolism, advances might also be made in the field of cancer research.

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APPENDICES

PLATE A. Rat liver - normal.

PLATE B. Rat liver - showing effect of pyrrolizidine toxicosis (bilary, hyperplasia, necrosis, megalocytosis).

Magnification: 100X
Stain: hematoxylin-eosin
Reproduction: color Xerox

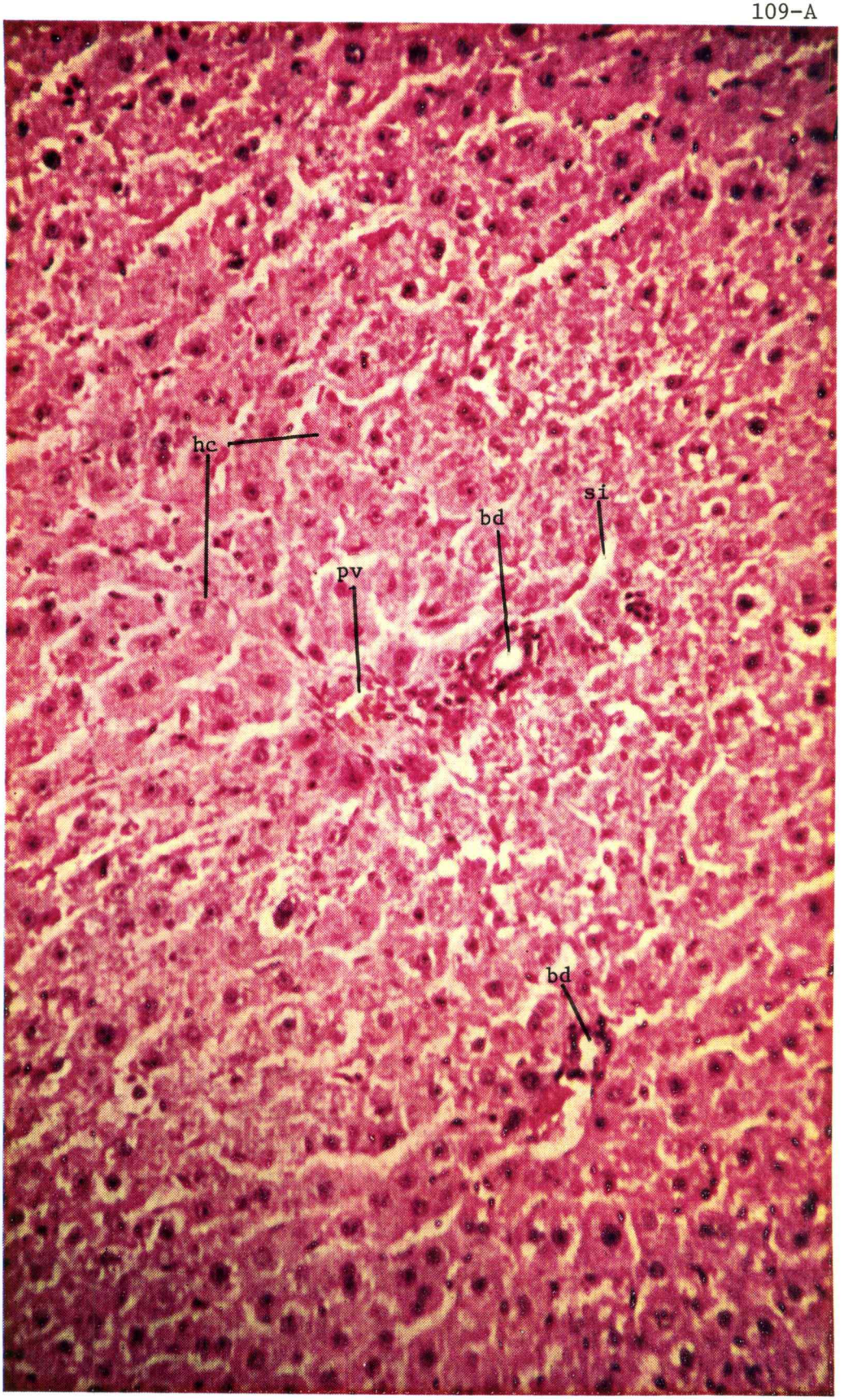
pv - portal vein
bd - bile duct
si - sinusoid
hc - hepatocyte
mg - megalocyte
nc - area of necrosis
mv - major venous vessel

PLATE C. Rat spleen - normal.

PLATE D. Rat spleen - showing hemosiderin deposits in macrophages.

Magnification: 400X
Stain: hematoxylin-eosin
Reproduction: color Xerox

rbc - red blood cell
mp - macrophages
hs - hemosiderin deposits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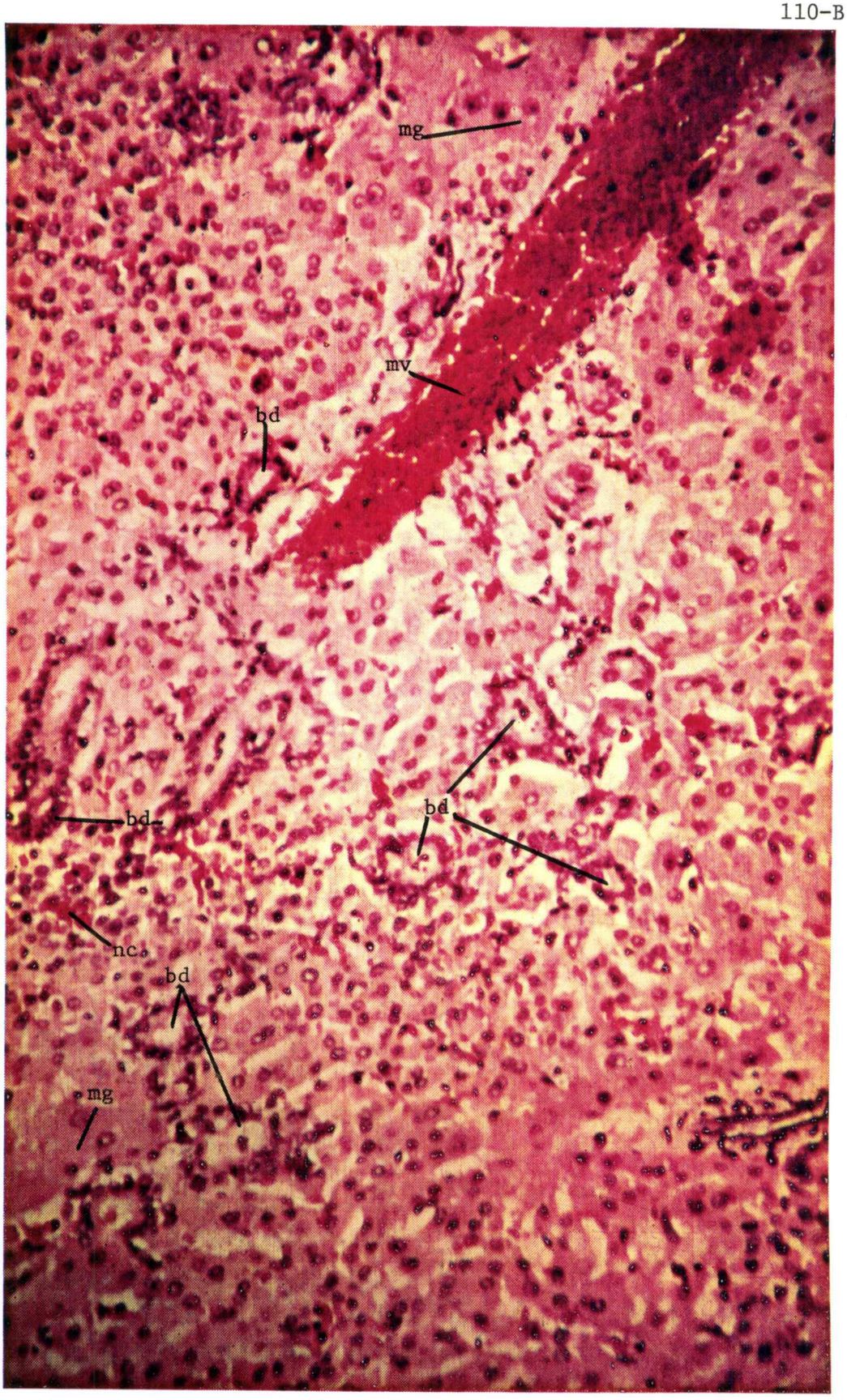
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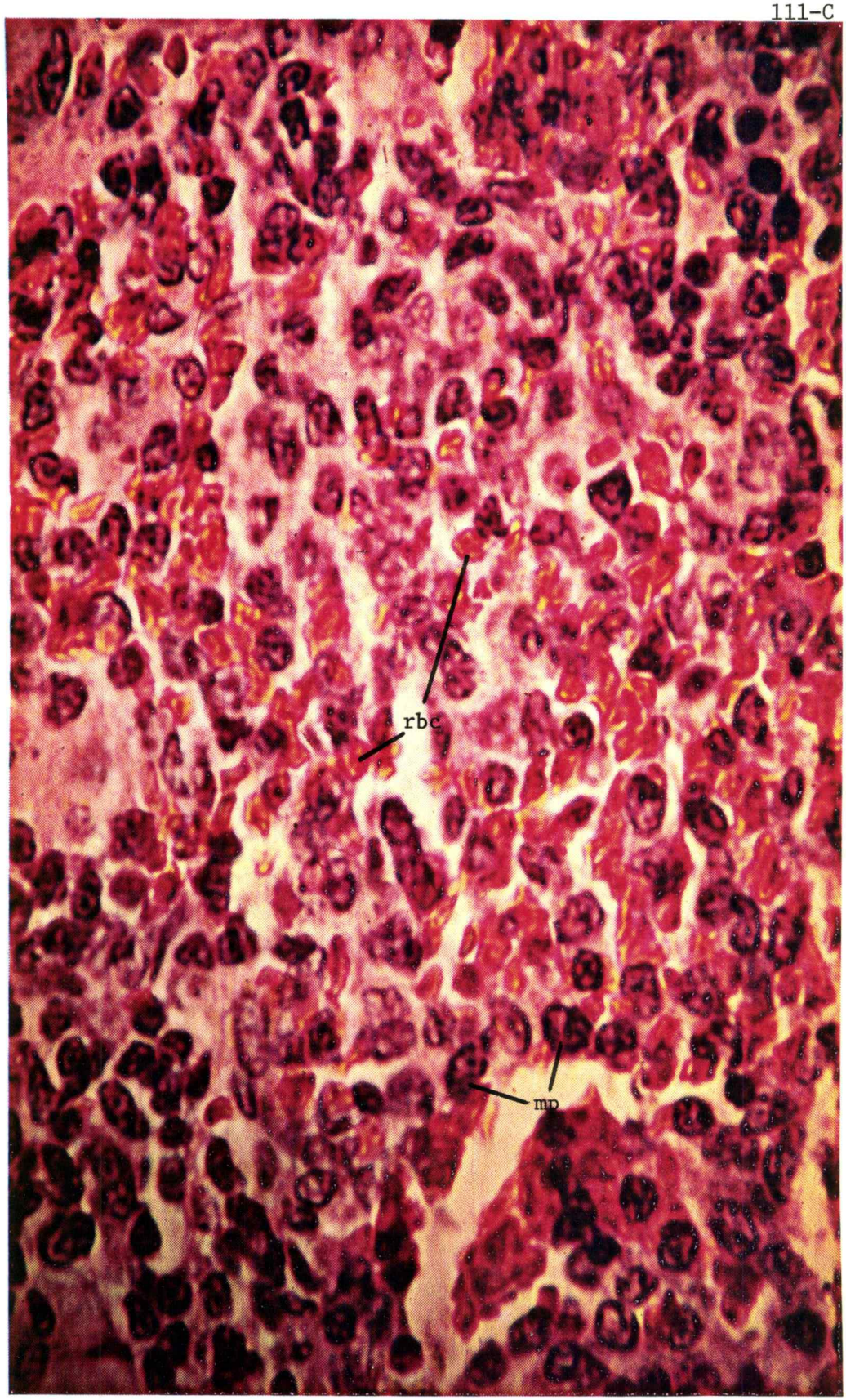
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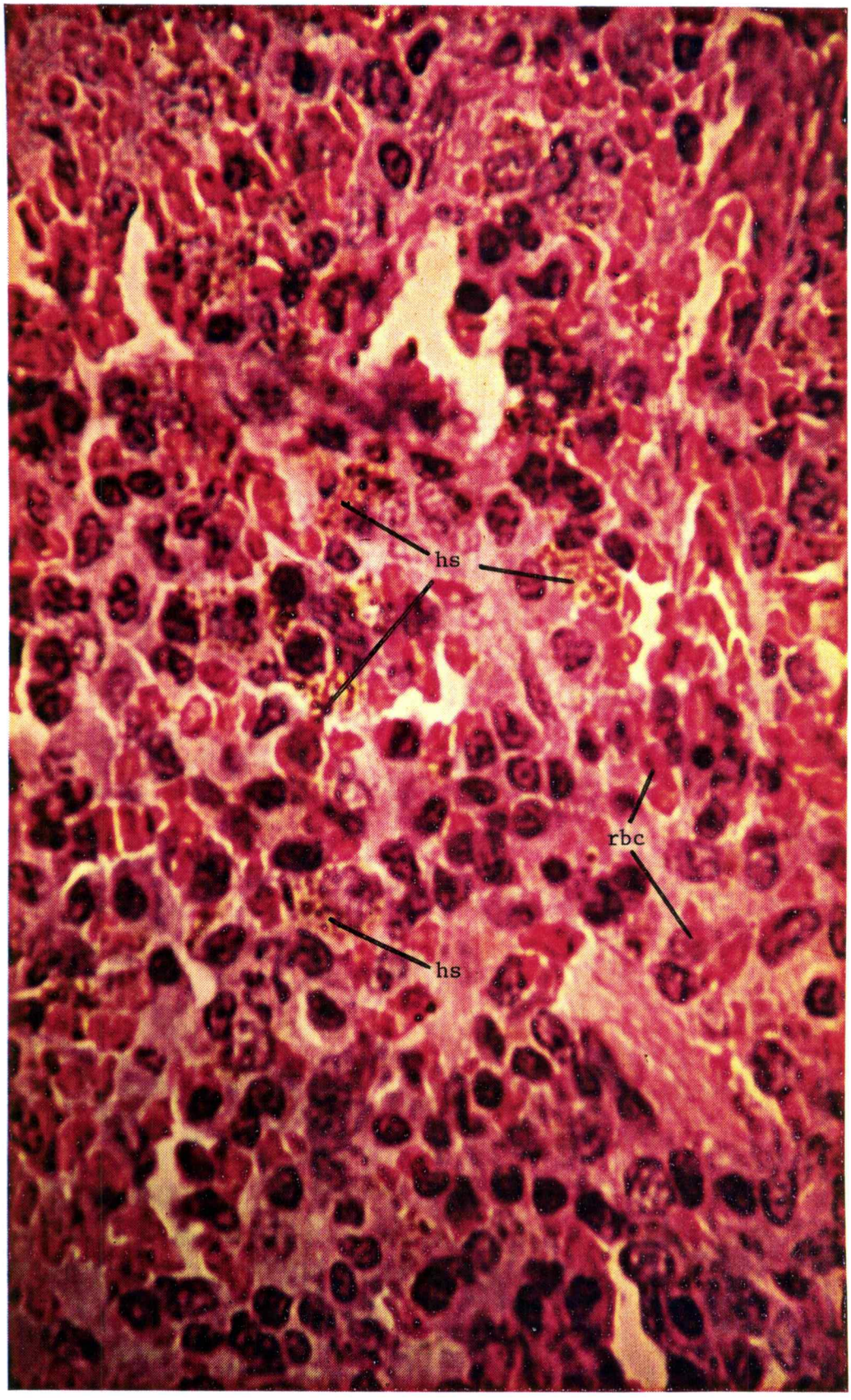
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