AN ABSTRACT OF THE THESIS OF

Randy D. White for the degree of Doctor of Philosophy

in Animal Science presented on August 18, 1982

Title: Mutagenic and Toxicologic Implications of Pyrrolizidine (Senecio) Alkaloids

Abstract approved: Redacted for privacy

P. R. Cheeke

The Salmonella/mammalian-microsome mutagenicity test was used to evaluate Senecio jacobaea (SJ) plant, pyrrolizidine alkaloids (PA) and metabolites in goat milk, to evaluate response differences with microsomes from several animal species and to compare SJ plant responses to other plants. Bioassessment experiments were conducted to evaluate PA toxicity changes associated with copper (Cu) and molybdenum (Mo) ingestion in sheep and esterase inhibition, microsomal enzyme induction and cysteine supplement in rats.

Acetone extracts of SJ caused negative or toxic mutagenic responses in the absence of liver microsomes and positive responses after microsomal activation. Senecio PA and monocrotaline gave only negative responses. Milk from SJ-fed goats produced positive mutagenic responses after microsomal activation. Only slight species microsomal differences were observed. Acetone extracts from alfalfa, lettuce and thread-leaf groundsel gave only negative mutagenic responses. Comfrey extracts produced toxic responses that were abolished by the microsomal system. Bracken produced a positive response after bioactivation with liver microsomes.

The dietary addition of Cu or Cu and Mo to SJ diets fed to lambs caused only marginal effects on liver Cu levels and no apparent hemolytic

effects. Lambs died after eating about 100% of their body weight in dried SJ with lambs fed the SJ+Cu+Mo diet dying about four wks before lambs fed the SJ+Cu diet.

Dietary exposure to esterase inhibiting organophosphates and carbamate resulted in increased PA toxicity. Increased toxicity was demonstrated by decreased PA (Senecio longilobus) LD_{50} values after dietary exposure to organophosphates (coumaphos, tri-o-cresyl phosphate and malathion) or the carbamate (carbaryl). Rats fed a SJ diet with tri-o-cresyl phosphate (TOCP) had decreased ($P<.05$) weight gain while rats fed a SJ diet with 2-pyridine aldoxime methiodide (2-PAM) had similar weight gain to those fed just the SJ diet. Rats fed SJ, SJ+TOCP or SJ+2-PAM diets had similar survival times (approx 60d).

Induction of microsomal enzymes by phenobarbital (PB) or dietary eucalyptus leaves caused only marginal effects on the acute LD_{50} for a PA (Senecio longilobus) mixture. Dietary phenothiazine (PT) did not alter SJ toxicity but did cause reduced feed intake and weight gain. Rats fed SJ with cysteine had increased ($P<.05$) survival time and SJ ingestion and slightly increased weight gain. Rats fed SJ+cysteine+PT had increased ($P<.05$) survival time over SJ and SJ+PT groups with a slight decrease in survival time compared to the SJ+cysteine group.

MUTAGENIC AND TOXICOLOGIC IMPLICATIONS OF PYRROLIZIDINE (SENECIO) ALKALOIDS

by

Randy Dee White

A Thesis

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of
Doctor of Philosophy

Completed August 18, 1982

Commencement June 1983

APPROVED:

Redacted for privacy

Professor of Animal Science in charge of major

Redacted for privacy

Head of Department of Animal Science

Redacted for privacy

Dean of Graduate School

Date thesis is presented August 18, 1982

Typed by Sue Ferdig for Randy Dee White

ACKNOWLEDGEMENTS

The encouragement and support of several people has made the completion of this research possible. I would like to first express a sincere appreciation to Dr. P. R. Cheeke for his council, guidance and professional assistance as an outstanding major professor, instructor and researcher. I would like to thank the Department of Animal Science and Agricultural Chemistry for financial support. In addition, I would like to thank the members of my graduate committee, Dr. D. R. Buhler, Dr. P. H. Krumperman, Dr. J. E. Oldfield, Dr. D. W. Weber and Dr. I. J. Tinsley for their time and service. Special thanks is also extended to Dr. P. H. Krumperman for direct professional assistance that made portions of this research possible.

I would also like to acknowledge and thank Betsy Garrett, Dr. Ralph Reed, Karen Robinson and Dr. Robert Swick for their excellent technical assistance. Gratitude is also extended to the entire staff of the Animal Science Department at Oregon State University for their friendship, patience and assistance.

Sincere appreciation is extended to my parents for their continual support and encouragement in my pursuit of additional education. I also thank Melanie's parents for their added support. Heartfelt gratitude is extended to my wife, Melanie, for her never-ending encouragement and understanding and to Elizabeth for her joyful diversions.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

MUTAGENIC AND TOXICOLOGIC IMPLICATIONS OF PYRROLIZIDINE (SENECIO) ALKALOIDS

INTRODUCTION

Considerable livestock losses in the U.S. Pacific Northwest and other temperate regions of the world are directly related to the consumption of plants that contain pyrrolizidine alkaloids (PA) (Mattocks, 1968; Muth, 1968; Duby, 1975). The predominant effect associated with this PA consumption is that of irreversible tissue damage that can result in chronic lethal effects. Extensive reviews are available on the chemistry, metabolism and toxicology of PA (Bull et al., 1968; Schoental, 1968; Warren, 1970; McLean, 1970; Mattocks, 1972b). The parent PA are relatively non-toxic but mixed function oxidase (MFO) dehydrogenation of the 1, 2-dehydropyrrolizidine ring forming pyrrole (dihydropyrrolizidine) metabolites results in dramatic increases in toxicity (Mattocks, 1968; Culvenor et al., 1969). Induction of the MFO system by drugs, such as phenobarbital, DDT and 3-methylcholanthrene, cause increased conversion of alkaloids to pyrrole metabolites in vitro (Mattocks and White, 1971; Allen et al., 1972; White et al., 1973 Chesney et al., 1974). Allen et al. (1972) demonstrated significant correlation between phenobarbital-induced MFO activity and increased PA toxicity, in rats, whereas, chloramphenicol inhibited enzyme activity reduced the production of metabolic pyrrole and decreased the toxicity. The classic pathological condition associated with PA intoxication is that of hepatic damage exhibited by necrosis, megalocytosis, fibrosis, biliary hyperplasia and veno-occlusion (McLean, 1970). Susceptibility to PA intoxication varies among species with rats, cattle and horses (Bull et al., 1968) being

highly susceptible while guinea pigs (Hopkirk and Cunningham, 1936), rabbits (Pierson et al., 1977), goats (Goeger et al., 1982) and sheep (Bull, 1956; Hooper, 1972) are relatively resistant. This relative resistance of sheep to PA poisoning has resulted in the promotion of sheep as biological agents for pastoral control of the PA-containing plant Senecio jacobaea (SJ), commonly called tansy ragwort (Mosher, 1979). Bull et al. (1956), however, demonstrated that long term ingestion of the PA-containing plant Heliotropium europaeum predisposed sheep to toxic, hepatic accumulation of copper. Swick (1981) reported accumulation of liver copper in sheep fed SJ when MFO were induced by phenobarbital. Miranda et al. (1981b) and Swick et al. (1982) reported substantially increased hepatic copper levels in rats fed SJ with supplemental copper. The complete divergence of PA-induced copper accumulation is yet to be resolved.

Recent reports concerning accidental contamination of foodstuffs or deliberate use of PA-containing plants in herbal preparations have substantiated that PA poisoning is a public health problem in many areas of the world (Watt and Breyer-Brandjwijk, 1962; McLean, 1970; Mohabbat et al., 1976; Tandon et al., 1976; Huxtable, 1979). In the United States, tea made from the PA-containing plant Senecio longilobus (SL), commonly called thread-leafed groundsel, has been implicated in the deaths of several individuals (Stillman et al., 1977; Fox et al., 1978; Huxtable, 1979). It has also been suggested that PA might be transferred to the milk of dairy animals consuming SJ- contaminated pastures, hay or silage (Muth, 1968; Dickinson, 1974;

Johnson, 1976). Schoental (1959) demonstrated that PA or their toxic metabolites are passed into the milk in sufficient quantities to produce liver lesions and death in rats suckling dams treated with PA even though the lactating rats were unaffected. Dickinson (1974) reported that adverse effects did not occur in young mice or rats fed milk from SJ-fed cows and Johnson (1976) reported similar results in mice, rats and calves. The potential for human hazard with respect to PA occurrence in milk, however, is probably greater with goat milk than cow milk as a single goat dairy often supplies the entire milk supply for a small number of people. Dickinson and King (1978) reported the presence of PA in milk from SJ-fed goats and Goeger et al. (1979) reported mild hepatic changes in calves and rats consuming milk from SJ-fed goats. The hazard from PA-contaminated milk is, as yet, unresolved.

In addition to the potential for pathogenic action in animals, Culvenor et al. (1969) and Mattocks (1969) indicated that the metabolic pyrrole derivatives of PA are capable of producing alkylating groups at the site of both ester linkages. Bifunctional alkylating agents of this type can be compared to nitrogen mustards and mitomycin C, which are believed to inhibit mitosis by linking themselves across the two strands of DNA. It would seem logical, then, that there might exist a potential mutagenic response associated with PA and their metabolites. This in fact has been the case as several PA have been shown to produce mutagenic (Clark, 1959; Clark, 1960; Brink, 1966; Alderson and Clark, 1966; Koletsky et al., 1978; Wehner et al., 1979; Yamanaka et al., 1979) and carcinogenic (Bull et al., 1968; Schoental, 1968; Schoental

et al., 1970; Newberne and Rogers, 1973; Allen et al., 1975) responses.

Dietary supplementation is beginning to show promise as a deterrent to PA toxicosis. Agents such as the amino acids cysteine (Buckmaster et al., 1976; Swick, 1980) and methionine (Retief, 1962; McLean, 1970), the vitamins B_6 and B_{12} (McLean, 1970) and the antioxidants butylated hydroxyanisole (Miranda et al. 1981c; Kim and Jones, 1982) and ethoxyquin (Miranda et al., 1981a; Kim and Jones, 1982) have demonstrated protective activity against PA poisoning. The development of a protective dietary supplement could result in a substantial economic contribution to agriculture.

The objectives of this study were to: 1) further investigate livestock and human hazards inherent with PA-containing plants by assessing the potential mutagenic activity associated with Senecio plant, PA and metabolites in goats milk and compare the mutagenic response of SJ plant extract with that of other plant extracts; and 2) assess possible changes in PA toxicity due to copper and molybdenum supplementation, esterase inhibition, MFO induction and cysteine supplementation.

Mutagenic Responses of Tansy Ragwort (Senecio jacobaea) Plant, Pyrrolizidine Alkaloids and Metabolites in Goat Milk with the Salmonella/Mammalian-Microsome Mutagenicity Test[1,2]

R.D. White,[3] P.H. Krumperman,[4] P.R. Cheeke,[3]
M.L. Deinzer[5] and D.R. Buhler[5]

Oregon State University
Corvallis, OR 97331

[1]Oregon Agricultural Experiment Station Technical Paper No. 6350
[2]This project was supported in part by NIH Grant No. CA-22524.
[3]Dept. of Animal Science
[4]Dept. of Food Science and Technology
[5]Dept. of Agricultural Chemistry

(The authors gratefully acknowledge the assistance of B. Arbogast for technical assistance in pyrrolizidine alkaloid determination in goat milk.)

Mutagenic Responses of Tansy Ragwort (Senecio jacobaea) Plant, Pyrrolizidine Alkaloids and Metabolites in Goat Milk with the Salmonella/Mammalian-Microsome Mutagenicity Test

R.D. White, P.H. Krumperman, P.R. Cheeke, M.L. Deinzer and D.R. Buhler

SUMMARY

Tansy ragwort (Senecio jacobaea) was evaluated for animal and human health hazard using the Salmonella/mammalian-microsome mutagenicity test. An acetone extract of tansy ragwort (TR) produced a negative mutagenic response for bacterial tester strains TA1535 and TA100 and a toxic response in tester strains TA1537 and TA98. Assay of this extract in the presence of mammalian liver microsomes resulted in positive mutagenic responses in tester strains TA1535, TA1537, TA98 and TA100. Species differences were evaluated by use of liver microsome preparations from induced rat and uninduced sheep, beef, hamster, trout and rat. Only a slight species difference was demonstrated. A mixture of pyrrolizidine alkaloids (PA), extracted from TR flowers, produced a negative response in tester strains TA1535, TA1537, TA98 and TA100. A negative response was also demonstrated when this PA mixture assayed with the Salmonella tester strains and induced rat liver microsomes (IRLM). A mixture of PA extracted from Senecio longilobus also produced a negative response. The major PA present in TR, jacobine, produced a negative response without and with IRLM exposure in tester strains TA1535, TA1537, TA98, and TA100. Another similar PA, monocrotaline, found in various Crotalaria species also gave a negative response. Milk from TR-fed goats was evaluated for mutagenic response. Milk from goats not receiving TR and from goats

receiving TR at a level of 1% of their body weight per day via rumen cannula produced a negative response without liver microsomes present. Milk from TR-fed goats, however yielded both negative and marginally positive responses for different combinations of tester strains and liver microsome preparations.

(Key Words:) Mutagenicity, Tansy ragwort (Senecio jacobaea) Pyrrolizidine Alkaloid, Goat Milk.

INTRODUCTION

Senecio jacobaea, commonly called tansy ragwort, is well known in the Pacific Northwest as a major toxic plant to livestock and is responsible for numerous deaths of cattle and horses each year (Anon, 1971; Duby, 1975; Muth, 1968). This plant is one of many species of Senecio that contain pyrrolizidine alkaloids (PA). The primary concern with plants containing PA is hepatotoxicity from the PA or a toxic metabolite. A carcinogenic response has also been demonstrated in laboratory animals following exposure to PA (Allen et al., 1975; Bull et al., 1968; Newberne and Rogers, 1973; Schoental, 1968; Schoental et al., 1970).

The widespread infestation of tansy ragwort (TR) throughout the Pacific Northwest has prompted public concern that a health hazard might exist. It has been suggested that PA might be transferred to the milk of animals consuming tansy-contaminated pastures, hay or silage (Muth, 1968; Dickinson, 1974; Johnson, 1976; Schoental, 1959). Schoental (1959) demonstrated that PA or their toxic metabolites are passed into the milk in sufficient quantities to produce liver lesions and death in rats suckling dams treated with PA even though the lactating rats were unaffected. Dickinson (1974) and Johnson (1976) reported that liver lesions and increased mortality did not occur in either young mice or rats receiving milk from cows fed TR. Johnson (1976) also reported no histopathologic changes in calves consuming milk from cows fed TR even though the cows eventually died and demonstrated lesions typical of PA toxicity.

Dickinson and King (1976) pointed out that human hazard, with respect to PA occurrences in milk, could be potentially greater with goat than with cow milk as dairy goat milk is generally not produced on a large enough scale to require pooling of milk from a number of sources. Also, the greater resistance of the goat to the toxic action of PA (Goeger et al., 1979; Goeger et al., 1982) would permit a lactating goat to consume considerable quantities of TR before symptoms would warrant milk disposal. Dickinson and King (1978) and Dickinson (1980) reported PA contents of 223 µg and 38.1 µg per 100 ml milk, respectively, from lactating does receiving TR at a level of 1% of their body weight per day. Goeger et al. (1979) reported mild hepatic changes in calves and rats consuming milk from goats fed TR but felt that the transfer of PA or toxic metabolites was not at a level which would cause short term toxic responses in susceptible species consuming the milk.

The development of the Salmonella/mammalian microsome mutagenicity test by Ames et al. (1975) has enhanced the efforts to study potentially hazardous agents. This system is widely used as a short-term predictive screening test to detect possible carcinogens. Significant correlation between mutagenic activity in the Ames system and carcinogens has been demonstrated (McCann et al., 1975). The versatility of the Ames test has been demonstrated by previous evaluations of milk (Green et al., 1980) and other complex mixtures (Kier et al., 1974; Commoner et al., 1978; Aeschbacker and Wurzner, 1980). The incorporation of mammalian microsomes has allowed assimilation of in vivo metabolism of xenobiotics (Ames et al., 1973) and microsomal

preparations can be made from various tissues of different animals. Muller et al. (1980) demonstrated species-specific differences in metabolizing activity and also differences from one substance to another by comparing the activity of liver microsomal fractions from several species with known mutagens in the Salmonella/mammalian-microsome mutagenicity test. Yamanaka et al. (1979) indicated that species differences exist with PA.

The objective of this study was to further investigate the potential livestock and human hazards of TR by: (1) assessing the mutagenic activity of TR plant, PA, and milk from TR-fed goats, (2) evaluating possible species differences associated with any mutagenic activity and (3) determining the PA content of TR-fed goat milk.

MATERIALS

Plant. The TR plant material, consisting of flowers in full bloom and leaves, was collected in the vicinity of Corvallis, OR. The TR was forced air dried at 50°C for 48 hr, chopped in a garden mulcher, and stored in air-tight containers prior to use.

Plant extracts. The dried TR plant used for acetone extracts was finely ground in a Wiley mill. Acetone extracts were prepared from 60 g of ground dried plant material shaken in 300 ml distilled acetone for 48 hrs. The subsequent solution was filter sterilized using a sterile Seitz cellulose filter. Test solutions were prepared each day of mutagenicity testing from the refrigerated stock extract solution. Initial volumes of stock extract were rotary evaporated to dryness and then reconstituted to a new volume with filter sterilized

distilled acetone to provide samples of original concentration or increased concentration of plant material. The reconstitution scheme was 21 ml stock solution evaporated and reconstituted to new volumes of 21 ml, 12 ml, 6 ml, or 3 ml new volume which is 100, 57.1, 28.6, and 14.3% of the original volume or 1, 2, 3 and 7 times the original concentration of plant material. These were expressed as relative concentrations of 1, 2, 3 and 7, respectively. The relative concentration of 0 was used as the revertant control and consisted of an initial volume of filter sterilized distilled acetone rotary evaporated and reconstituted to its original volume with filter sterilized distilled acetone.

Pyrrolizidine Alkaloids. Monocrotaline was supplied by Trans World Chemicals, Washington D.C. Mixed TR alkaloids were extracted from TR flowers and prepared by recrystallization after reduction of the N-oxides (Ramsdell and Buhler, 1981). Jacobine was isolated and collected from the recrystallized mixed TR alkaloid preparation by reversed-phase high pressure liquid chromatography (Ramsdell and Buhler, 1981). Mixed alkaloids from Senecio longilobus were extracted from whole plant (excluding roots) by a modified method of Campbell (1956) using ethanol instead of methanol. The S. longilobus was collected in the vicinity of Tucson, AZ, and dried and ground similarly to the TR. The PA preparations were dissolved in absolute ethanol for plating with ethanol placed on the revertant control plates.

Animal and Diets. Pregnant 3 year old Nubian goats were used. The control group consisted of 2 does and the TR treatment group

consisted initially of 3 does. Each group received an alfalfa-barley based pelleted diet at the rate of 1.5 kg per goat per day. The composition of this maintenance diet is shown in Table 1. The control group also received grass and hay ad lib. The TR treatment group had rumen cannulas (McCann et al., 1973) surgically inserted nine days prior to the beginning of TR feeding. The TR group received TR in the place of grass hay at the rate of 250 g dried, chopped TR administered twice daily as a slurry (3L water/250 g TR) directly into the rumen via the cannula. The total daily consumption per goat was 500 g dried, chopped TR which was about 1% of their body weight. TR feeding began approximately four weeks prior to parturition and continued through the duration of the milk mutagenicity screening.

Milk. Goats, after parturition, were milked twice daily. Milk samples for mutagenicity screening were aseptically collected into sterile (autoclaved) screwcap jars (250 ml capacity) at the evening milking prior to the day of mutagenicity testing. The sample collection was accomplished by wiping the udder with a clean, water-moistened cloth, squeezing and discarding several streams of milk from each teat, and holding the jar to the side of the animal and alternately squeezing each teat to evacuate milk, filling the jar with approximately equal volumes from each teat. The samples were then sealed and stored overnight under refrigeration (3.7^{o}C). On the day of mutagenicity screening, portions (20 ml) of each sample were placed on ice in sterile flasks with the remaining volumes resealed and frozen. Milk from control-fed and TR-fed goats was assayed for mutagenic activity with bacterial tester strains alone used as revertant

controls.

Bacterial Tester Strains. Four strains of histidine (his) auxotrophs of Salmonella typhimurium (supplied by B.N. Ames) were used. Strains TA1535 and TA100 were used to detect mutagens causing base pair substitutions and strains TA1537 and TA98 were used to detect various kinds of frameshift mutagens. The overnight cultures were adjusted to 30 percent transmission at 560 nm by the addition of sterile distilled water prior to use for plating.

Microsomal Fractions. Liver microsomal fractions or S-9 fractions were prepared from uninduced rat (male), hamster (male), trout (sex undetermined), beef (steer), and sheep (wether) and Arochlor 1254 induced rat (male) as described by Ames et al. (1975). The protein content of each S-9 fraction was determined by the Micro-Kjeldahl method (AOAC, 1980) with the content for induced rat and uninduced rat, hamster, trout, beef and sheep being 29.5, 25.4, 27.0, 20.7, 30.7, 29.7 mg N/ml respectively. Each S-9 component utilized in the direct plating technique was adjusted on the day of testing to contain 21.0 mg N/ml S-9 fraction. This was done in an attempt to prevent possible changes in the S-9 component just due to increased protein content which could suppress marginal species-specific differences that might exist.

METHODS

The Salmonella/mammalian mutagenicity test as outlined by Ames et al. (1975) was employed for assessment of mutagenic activities of TR plant extracts, pyrrolizidine alkaloids (monocrotaline, jacobine,

mixed TR PA, mixed S. longilobus PA) and milk from goats consuming the control or TR diet. All assays were carried out using 0.1 ml adjusted overnight culture and in the absence or presence of liver microsomes (S-9 mix) at a rate of 0.5 ml per plate. Triplicate plates were used for each determination and all positive (test revertant number of at least twice the revertant control number) or toxic (test revertant number of 0.5 or less than that of the revertant control number) responses were repeated at least twice.

TR plant extracts were assayed at relative concentrations of 0, 1, 2, 3 and 7 utilizing 0.1 ml prepared solution per plate. Plant extracts were tested in the absence and presence of induced rat and uninduced rat, hamster, trout, beef, and sheep liver microsomal preparations.

The PA were tested at concentrations of 0, 5, 50, and 500 µg per plate in 0.1 ml ethanol solution. PA were assayed in the absence and presence of induced rat liver S-9.

Milk samples from both control-fed and TR-fed goats were assayed at relative concentrations of 1, 2 and 4 with relative concentration 1 being 0.1 ml milk per plate, relative concentration 2 being 0.2 ml milk per plate and relative concentration 4 being 0.4 ml milk per plate. Milk samples were tested in the absence and presence of induced rat, and uninduced rat, hamster, trout, beef and sheep liver S-9. Control milk samples tested were collected at 3, 5, 6, 7, 17, 120 and 128 days post parturition. TR milk samples tested were collected at 2, 9, 30, 36, 42 and 56 days post parturition. Milk samples from individual goats as well as samples composed of equal volumes from two goats were

assayed. The PA content of the milk was also determined (Deinzer, et al., 1982). The milk samples were hydrolyzed to convert the PA to the common base, retronecine. After cleanup by column chromatography and derivatization, the PA content was determined by gas chromatography. The recovery of PA introduced into control milk was 82.5 percent for this analytical method.

RESULTS

Plant extract. The mutagenic responses produced by the TR plant acetone extract are summarized in Table 2. Metabolic activation was required to produce positive responses in each of the bacterial tester strains. In the absence of microsomal S-9 the extracts produced either negative (response similar to that of revertant controls) or toxic (test solution produced less than half the number of control revertants) responses depending upon which bacterial tester strain was used. These negative and toxic responses were consistent with the general mutational property of base pair or frameshift mutagens as demonstrated in Figure 1. In the absence of S-9 the plant extract produced negative responses with base pair mutagens, TA1535 and TA100, and toxic responses with frameshift mutagens TA1537 and TA98. Metabolic activation with S-9 produced both base pair and frameshift mutations for each species S-9 used. Minimal variations were produced between species S-9, within species S-9 and between bacterial tester strains within species S-9 but no real trends occurred as shown in Figure 2.

Pyrrolizidine Alkaloids (PA). The ratio of test revertants to

control revertants for the PA tested is shown in Table 3. A ratio number of at least 2 was required for a response to be determined positive. Mixed Senecio jacobaea PA, jacobine, mixed Senecio longilobus PA, and monocrotaline all produced negative responses.

Milk. The mutagenic responses produced by milk from TR-fed goats are summarized in Table 4. The results are average mutagenic responses for two separate tests on pooled (equal volumes from two goats) milk samples collected 9 and 30 days post-parturition. Control-fed goat milk (collected 5 and 17 days post parturition) produced a negative response in the absence and presence of S-9 and TR-fed goat milk produced a response similar to the control-fed goat milk in the absence of S-9. Bioactivation of TR-fed goat milk with S-9, however, produced marginal mutagenic responses only in TA1535 and TA1537 and species differences for S-9 bioactivation were minimal with no obvious trends exhibited as shown in Figure 3. The PA content of milk samples collected from TR-fed goats is shown in Table 5. It was interesting to note the decrease in PA content from about .8 ppm to about .4 ppm between days two and nine post-parturition.

DISCUSSION

It is well accepted that the principal toxicologic mechanism associated with TR is that of irreversible liver damage following the metabolism in the liver of the PA inherent to the plant forming highly reactive pyrrole metabolites (Bull, et al., 1968; Mattocks, 1968; Jago et al., 1970; Mattocks and White, 1971). In contrast, however, only PA not found in TR have been shown to be mutagenic to Drosophila

(Brink, 1955; Brink, 1969; Clark, 1959; Clark, 1969), Aspergillus nidulans (Alderson and Clark, 1966) and Salmonella typhimurium (Koletsky, 1978; Wehner et al., 1979). It has been suggested that PA mutagenic activity might be related to the necine base type of the PA tested. Yamanaka et al. (1979) reported that the PA senecionine and seneciphylline, which are found in TR and are of the retronecine base type, produced a negative response in the Salmonella/mammalian-microsome mutagenicity test but that PA of the heliotridine and otonecine base types produced positive responses. Some necine base structures and their relationship to mutagenic response with the Salmonella/mammalian microsome test are shown in Table 6. The PA tested in the present study were predominantly of the retronecine base type and produced negative responses.

The positive mutagenic responses demonstrated by the gross TR acetone extract and the marginal mutagenic responses demonstrated by the milk from TR-fed goats suggest that biological hazards, in addition to hepatotoxicity, may be associated with another factor in the plant independent from that of their inherent PA. Attempts were not made to isolate the mutagenic fraction from the TR acetone extract at this time. The test results also suggest that the marginal mutagenic responses produced by the milk from TR-fed goats may also be related to this unidentified factor or its metabolites. Mutagenicity screening in our laboratory of acetone extracts of S. longilobus, alfalfa, and lettuce, in contrast to TR acetone extracts, have produced negative responses (unpublished data). Acetone extracts of bracken fern (Pteridium aquilinum) which has been shown to contain a carcinogenic

factor (Evans, 1979), have also been screened in our laboratory and produced a marginal positive mutagenic response (unpublished data). The positive response of TR acetone extract in the Salmonella/ mammalian microsome mutagenicity test has been so consistent that we have used it on several occasions as the positive control in conjunction with tests on other compounds.

A hazard in livestock related to mutagenicity that may be associated with TR consumption has not been evident to date. This is probably due to the fact that susceptible animals like cattle and horses either die or are sent to market prior to the occurrence of detrimental intoxication. A possible hazard, however, may exist for animals like goats and sheep that are somewhat resistant to the acute hepatotoxic effects of TR. It is still not clear to what extent the hazard of consuming milk from TR-fed animals might be.

TABLE 1. PERCENT COMPOSITION OF GOAT MAINTENANCE DIET

Ingredient	Percentage
Alfalfa	52.0
Barley	30.0
Soybean Meal	10.0
Molasses	5.2
Bentonite	2.0
Trace mineral salt	.3
Dicalcium phosphate	.5

TABLE 2. MUTAGENICITY WITH THE SALMONELLA/MAMMALIAN-MICROSOME TEST OF AN ACETONE EXTRACT FROM TANSY RAGWORT (SENECIO JACOBAEA) WITH TESTER STRAINS TA1535, TA1537, TA98, AND TA100 WITHOUT LIVER MICROSOMES PRESENT AND IN THE PRESENCE OF INDUCED[1] RAT AND UNINDUCED RAT, HAMSTER, TROUT, BEEF, AND SHEEP LIVER MICROSOME S-9

S-9	TA1535	TA1537	TA98	TA100
Beef	+[2]	+	+	±[3]
Sheep	+	+	+	+
Hamster	+	+	+	-
Trout	+	+	+	-
Rat	+	+	+	+
Induced Rat	+	+	+	+
Without S-9	-[4]	toxic[5]	toxic	-

[1]Rats were induced with Arochlor 1254 and liver S-9 fraction prepared as described by Ames et al. (1975).

[2]+ indicates a positive response; test solution produced at least twice the number revertants produced on control plates and demonstrated a dose response.

[3]± indicates a positive trend.

[4]- indicates a negative response; test solution did not produce twice the number of control revertants.

[5]toxic indicates that test solution produced less than half the number of control revertants.

TABLE 3. PYRROLIZIDINE ALKALOID RESPONSE IN THE SALMONELLA/MAMMALIAN-MICROSOME MUTAGENICITY TEST WITH TESTER STRAINS TA1535,TA1537, TA98, AND TA100 IN THE ABSENCE AND PRESENCE OF INDUCED RAT LIVER MICROSOME S-9[1].

Pyrrolizidine Alkaloid Type	Amount Alkaloid Per Plate (g)	Ratio of Test Revertants/Control Revertants[2]							
		Without S-9				With Induced Rat S-9			
		TA1535	TA1537	TA98	TA100	TA1535	TA1537	TA98	TA100
Mixed *Senecio jacobaea*	5	1.7	.9	1.0	.9	1.3	1.1	1.1	.9
	50	1.4	1.3	1.0	1.0	.9	1.1	1.1	.9
	500	1.3	.9	1.0	1.0	1.2	.8	1.0	1.1
Jacobine	5	.9	.6	.8	.9	1.0	1.5	1.1	1.1
	50	1.1	.8	.9	.9	1.5	1.8	1.2	1.0
	500	1.0	.7	.8	.8	1.7	1.6	1.4	i.4
Mixed *Senecio longilobus*	5	1.2	.8	1.1	1.0	1.2	.9	1.1	1.0
	50	1.4	1.0	1.2	1.0	1.3	1.0	1.2	1.1
	500	1.2	1.0	1.1	.9	1.3	1.0	1.1	1.0
Monocrotaline	5	1.0	.9	.9	1.0	1.4	.8	.9	1.0
	50	1.1	1.2	.9	.8	1.2	1.0	1.0	1.0
	500	.9	.8	.8	.9	1.4	.7	1.0	1.0

[1]Rats were induced with Arochlor 1254 and liver S-9 fraction prepared as described by Ames et al. (1975).

[2]Ratio number = the average number of revertant colonies produced by the test solution divided by the average number of normal revertants produced on control plates; positive mutagenic response is for a ratio number of 2 or greater as described by Ames et al. (1975).

TABLE 4. MUTAGENICITY WITH THE SALMONELLA/MAMMALIAN-MICROSOME TEST OF MILK FROM GOATS RECEIVING TANSY RAGWORT (SENECIO JACOBAEA) USING TESTER STRAINS TA1535, TA1537, TA98, and TA100 WITHOUT LIVER MICROSOMES PRESENT AND IN THE PRESENCE OF INDUCED RAT[1] AND UNINDUCED RAT, HAMSTER, TROUT, BEEF, AND SHEEP LIVER MICROSOME S-9

S-9	TA1535	TA1537	TA98	TA100
Beef	+[2]	-[3]	-	-
Sheep	±[4]	±	-	-
Hamster	+	+	-	-
Trout	+	-	-	-
Rat	-	+	-	-
Induced Rat	+	±	-	-
Without S-9	-	-	-	-

[1]Rats were induced with Arochlor 1254 and liver S-9 fraction prepared as described by Ames et al. (1975).

[2]+ indicates a positive response; test solution produced at least twice the number revertants produced on control plates and demonstrated a dose response.

[3]- indicates a negative response; test solution did not produce twice the number of control revertants.

[4]± indicates a positive trend.

TABLE 5. PYRROLIZIDINE ALKALOID CONTENT OF MILK FROM GOATS[1] RECEIVING DIETARY TANSY RAGWORT (SENECIO JACOBAEA) DAILY

Goat	Number Days Post-Parturition	Pyrrolizidine Alkaloid Content (ppm± std dev)[2]
A[3]	2	.77±.03
V	2	.81±.07
K	9	.41±.06
A+V[4]	9	.42±.00
A+K	30	.37±.03
A+K	36	.50±.04
A+K	42	.38±.02
A+K	56	.33±.01

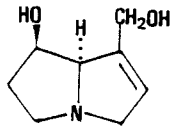
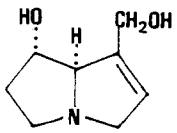
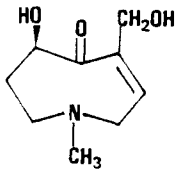
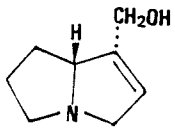
[1]Goats received 500 g dried, chopped tansy ragwort per day via rumen cannula in a water slurry.

[2]ppm indicates average (n=3) parts per million of pyrrolizidine alkaloid in milk corrected for the percent recovery of pyrrolizidine alkaloid (jacobine) introduced into samples of control milk; percent recovery = 82.5%.

[3]A,V,K indicates samples from individual goats.

[4]A+V, A+K indicates pooled samples of equal volumes from two goats.

TABLE 6. RELATIONSHIP BETWEEN NECINE BASE STRUCTURE AND MUTAGENIC RESPONSE WITH THE SALMONELLA/MAMMALIAN-MICROSOME MUTAGENICITY TEST

Necine base structure	Alkaloid name	Mutagenic Response[a]	
		Present study[b]	Previous Studeis[c,d]
	Jacobine	-	
	Lycopsamine		-
	Mixed S. jacobaea	-	
	Mixed S. longilobus[e]	-	
	Monocrotaline	-	-
	Retronecine		-
Retronecine	Retrorsine		-
	Senecionine		-
	Seneciphylline		-
	Heliotrine		+
	Lasiocarpine		+
Heliotridine			
	Clivorine		+
	Fukirotoxin		+
	Ligularidine		+
Otonecine	Senkirkine[e]		+
	Lindelofine		-
Lindelofine			

[a] Response indicated by - means a negative response and + means a positive response.
[b] Responses demonstrated in the current study.
[c,d] Responses demonstrated by either Yamanaka et al. (1979) or Wehner et al. (1979).
[e] Senecio longilobus has been shown to contain senkirkine as well as several retronecine-type PA (Roitman et al., 1979).

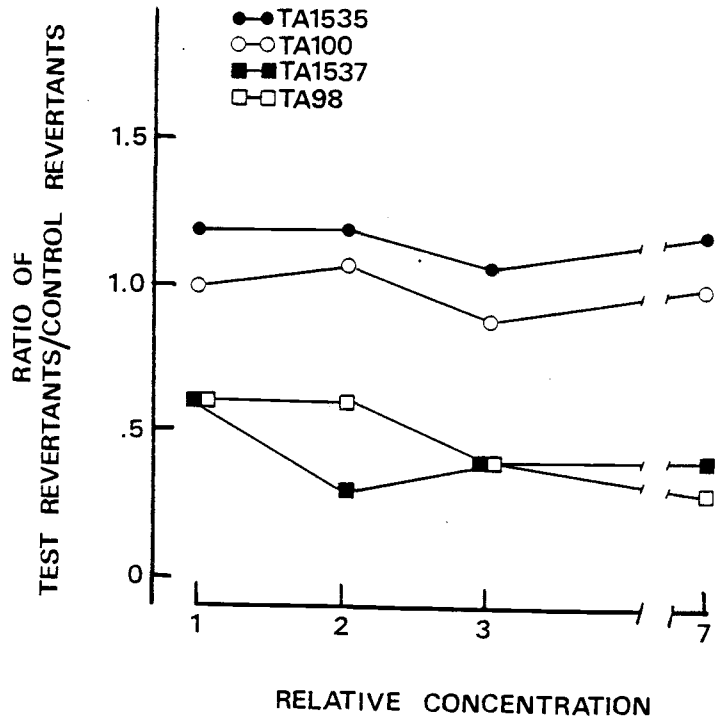


FIGURE 1. Dose response curve for acetone extract of tansy ragwort (Senecio jacobaea) tested with the Salmonella/mammalian-microsome mutagenicity test using tester strains TA1535, TA1537, TA98 and TA100 in the absence of microsomes.

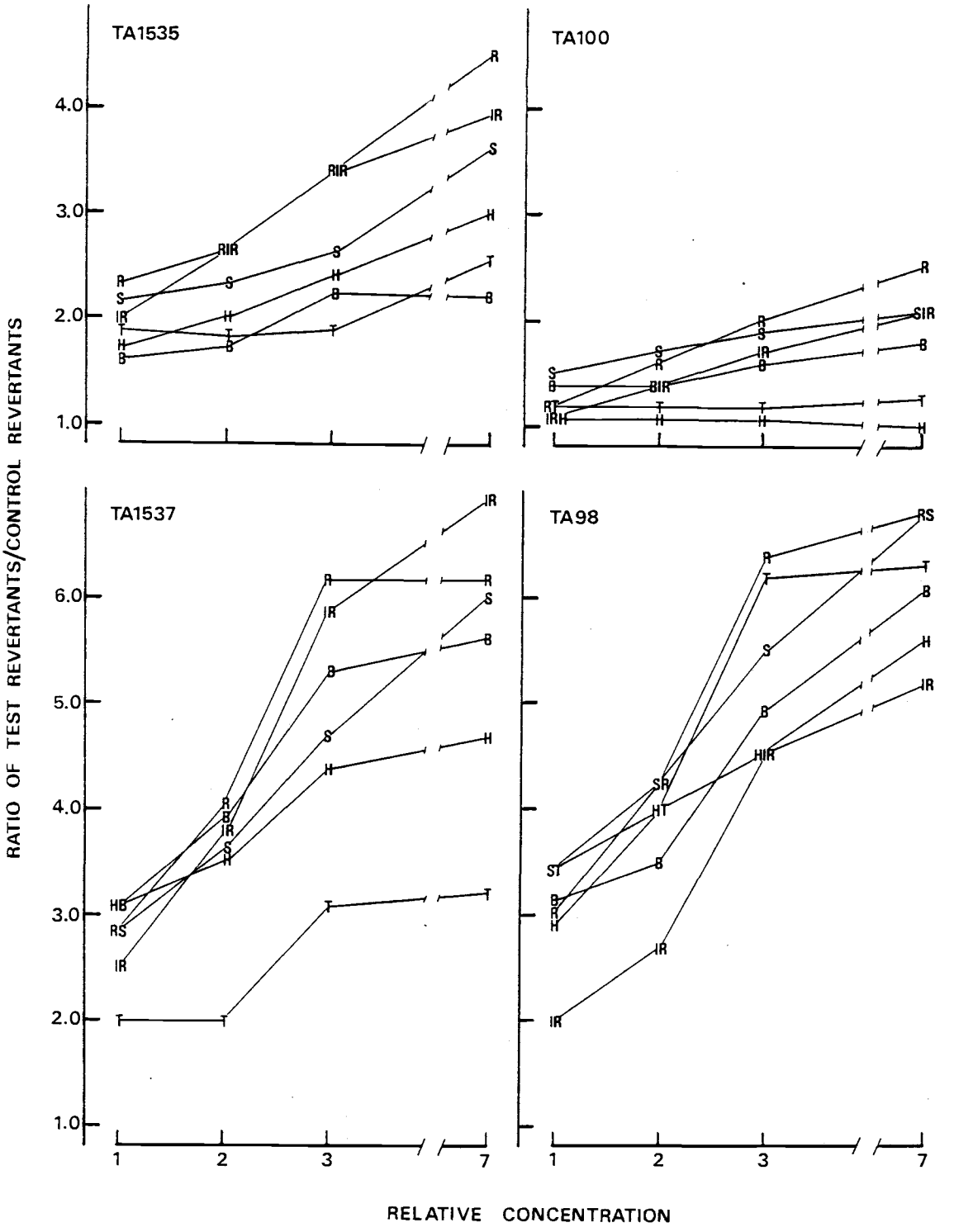


Figure 2. Dose response curves for acetone extracts of tansy ragwort (Senecio jacobaea) tested with the Salmonella/mammalian-microsome mutagenicity test using tester strains TA1535, TA1537, TA98 and TA100 in the presence of liver S-9 microsome preparations from Arochlor induced rat (IR) and uninduced rat (R), hamster (H), trout (T), beef (B) and sheep (S).

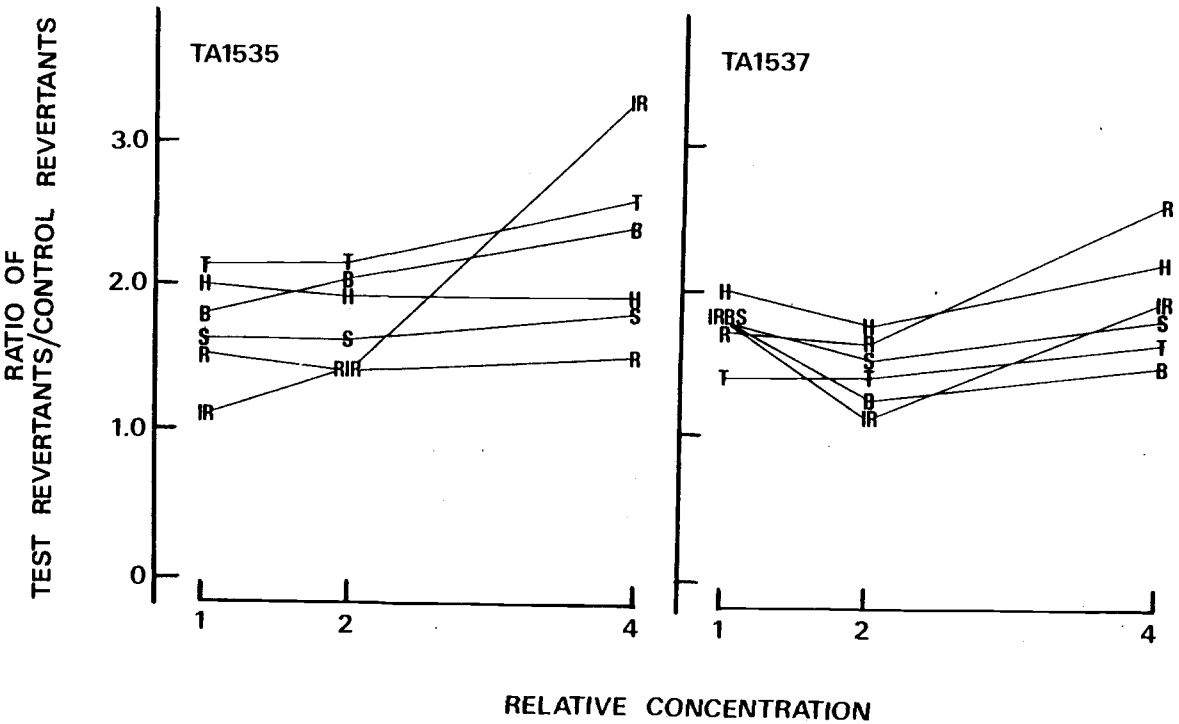


Figure 3. Dose response curves for milk from tansy ragwort (Senecio jacobaea)-fed goats tested with the Salmonella/mammalian-microsome mutagenicity test using tester strains TA1535 and TA1537 in the presence of liver S-9 microsome preparations from Arochlor induced rat (IR) and uninduced rat (R), hamster (H), trout (T), beef (B) and sheep (S).

An Evaluation of Acetone Extracts From Six Plants in the Ames Mutagenicity Test

Randy D. White [1], Paul H. Krumperman [2], Peter R. Cheeke [3] and Donald R. Buhler [1]

Oregon State University
Corvallis, OR 97331

[1]Dept. of Animal Science
[2]Dept. of Food Science and Technology
[3]Dept. of Agricultural Chemistry

An Evaluation of Acetone Extracts From Six Plants in the Ames Mutagenicity Test

Randy D. White, Paul H. Krumperman, Peter R. Cheeke and Donald R. Buhler

SUMMARY

Acetone extracts from six plants were evaluated for mutagenic activity with the Salmonella/mammalian-microsome mutagenicity test (Ames) utilizing tester strains TA98 and TA100 and in the presence and absence of induced rat liver microsomes. Extracts from alfalfa (Medicago sativa), lettuce (Lactuca sativa), and thread-leaf groundsel (Senecio longilobus) produced only negative responses. Comfrey (Symphytum officinale) and tansy ragwort (Senecio jacobaea) extracts produced toxic responses that were abolished in the presence of the microsomal bioactivation system. Bracken (Pteridium aquilinum) and tansy ragwort extracts produced positive responses following bioactivation with the liver microsomal system. The results suggest that the Ames mutagenicity test may be of some value in initial evaluations for potential toxic effects of plants consumed by animals and humans.

INTRODUCTION

Recent development of the Salmonella/mammalian microsomal mutagenicity test by Ames et al. (1975) has greatly enhanced efforts to examine and evaluate many dietary and environmental factors which have become suspect as carcinogens (Wynder, 1975; Hoover and Fraumeni, 1975). Many of these agents occur in the form of complex mixtures that have been difficult to deal with in the past. The Ames test has made in vitro testing a reality and provides a quick and relatively inexpensive means to assess the mutagenic potential of many agents that have been previously impossible to evaluate. Significant correlation has been demonstrated between the mutagenic response measured by the Ames test and known carcinogenic compounds (McCann et al., 1975; McCann et al., 1976). The versatility of the test has also been demonstrated with such complex mixtures as smoke condensates (Kier et al., 1974), beef extracts (Commoner et al., 1978), milk (Green et al., 1980), and coffee (Aeschbacher and Wurzner, 1980).

The purpose of this study was to use the Ames test as a screening procedure for evaluating potential health hazards associated with several plants that contain non-toxic or known carcinogenic compounds that are frequently consumed by animals and humans. The plants selected for this assessment were alfalfa (Medicago sativa), bracken (Pteridium aquilium), comfrey (Symphytum officinale), lettuce (Lactuca sativa capitata), tansy ragwort (Senecio jacobaea), and thread-leaf groundsel (Senecio longilobus). Alfalfa is commonly consumed by livestock and known to contain inhibitors such as saponins (Cheeke, 1977). Bracken, which is occasionally consumed by livestock

and humans, contains at least two toxic factors (Evans, 1976) and causes bladder and intestinal tumors (Evans, 1979). Comfrey, occasionally consumed by humans and livestock, contains pyrrolizidine alkaloids (Furuya and Araki, 1968) and has been shown to be carcinogenic (Hirono et al., 1978). Lettuce was selected for use as a non-toxic, commonly comsumed plant. Tansy ragwort contains pyrrolizidine alkaloids which cause irreversible liver damage in animals consuming the plant and has demonstrated a carcinogenic response in laboratory animals (Bull et al., 1968). Thread-leaf groundsel also occasionally consumed by livestock and humans (Huxtable, 1979) contains pyrrolizidine alkaloids similar to those found in tansy ragwort (Roitman et al., 1979).

MATERIALS AND METHODS

The plant material was taken from a variety of sources. Bracken, comfrey, and tansy ragwort were collected in the vicinity of Corvallis, OR., and thread-leaf groundsel was from the vicinity of Tucson, AZ. The bracken was harvested at the fiddle-head stage before the fronds were completely open; comfrey at the prebloom vegetative stage; and tansy ragwort in full bloom (mostly flower tops and leaves). Dehydrated alfalfa and lettuce were purchased from commercial sources. The plants, with the exception of alfalfa, were dried at 50^{o}C, finely ground in a Wiley mill, and stored at room temperature. Acetone extracts of each plant were prepared from 60 g ground dried plant material shaken in 300 ml distilled acetone for 48 hrs. The liquid portion was filter sterilized using a Seitz cellulose filter and stored under refrigeration until used.

Two strains of histidine requiring (his^-) auxotrophs of Salmonella typhimurium (supplied by B. N. Ames) were used. These strains were TA98 and TA100 which are both deficient in excision repair of DNA damage (uvrB) with strain TA98 susceptible to frameshift mutagens and strain TA100 susceptible to base-pair substitution mutagens. Both have an ampicillin-resistant R factor.

The solutions of acetone plant extracts were assayed by the plate incorporation procedure described by Ames et al. (1975). Test solutions were prepared each day of mutagenicity testing from the refrigerated stock extract solutions. Initial volumes of stock solution were rotary evaporated to dryness and then reconstituted to a new volume with filter sterilized distilled acetone to provide samples of original concentration or increased concentration of plant material. The reconstitution scheme was 21 ml stock solution evaporated to dryness and reconstituted to new volumes of 21, 12, 6 or 3 ml which is 100, 57.1, 28.6 and 14.3% of the original volume or 1, 2, 3 and 7 times the original concentration of plant material. These were expressed as relative concentrations of 1, 2, 3 and 7 respectively. The procedure was carried out under aseptic conditions. A relative concentration of zero consisted of an initial volume of sterile acetone rotary evaporated and reconstituted to its original volume with sterile acetone. This "reconstituted" acetone was used for the revertant control to detect the existence of any impurities in the acetone that might influence the mutation rate of the cultures. A consistent inoculum was maintained throughout the study by adjusting 15 hr cultures to 30% transmission at 560 nm using sterile distilled water. All

assays were carried out using 0.1 ml of prepared test solution, 0.1 ml adjusted overnight culture, and in the presence or absence of 0.5 ml Aroclor 1254 induced rat-liver microsomes (S-9 mix) per plate. Triplicate plates were used for each determination and all positive (revertant number of at least twice the revertant control number) or toxic (revertant number of .5 or less than that of the revertant control number) responses were repeated at least twice.

RESULTS and DISCUSSION

The mutagenic responses of each plant is summarized in Table 7. The natural mutation rates of the unexposed cultures were 14 and 74 per plate for TA98 and TA100 without the microsomal mix and 20 and 73 with the mix. These numbers are typical of the natural mutation rate for these cultures. Ames et al. (1975) considers the test to be positive if the number of revertants induced by exposure to the test compound becomes as great or greater than twice the natural spontaneous mutation rate. Most positive mutagens should also demonstrate a dose response where the number of induced revertants increase with an increase in concentration of the mutagen. An important component of the system is the in vitro addition of microsomes from homogenized liver (Ames et al., 1973) which contains the enzyme systems necessary to simulate the in vivo biotransformation of the test substance into the active mutagenic metabolite. Bracken and tansy ragwort extracts produced positive responses in strain TA98 when liver microsomes were added to the system. The tansy ragwort extract also produced positive response in TA100 in the presence of liver

microsomes. These results are depicted in Figure 4. All other plant extracts were negative in this testing procedure.

The comfrey and tansy ragwort extracts produced another interesting biotransformation response aside from bioactivation. Both comfrey and tansy ragwort extracts in the absence of the liver microsomes produced a substantial decrease in the spontaneous mutagenic rate in the culture TA98, indicating a toxic response of the extracts to this culture. In the presence of the S-9 mix, the extracts of both plants were detoxified and in the case of tansy ragwort extract bioactivation occurred as well as causing an increase of the mutation rate. Figure 5 depicts the toxic response for the comfrey and tansy ragwort extracts. The true nature of the toxic mechanism was undetermined here. Taylor and Taylor (1963), however, demonstrated that water extracts of comfrey leaves produced increased survival times of mice bearing spontaneous tumors and decreased tumor growth of transplanted tumors in mice which suggest antimutagenic activity.

The bioactivation of gross extracts from bracken and tansy ragwort and the detoxification of the comfrey extract, again, demonstrate the versatility of this test system. The fact that bracken and tansy ragwort have been shown to produce carcinogenic responses supports the favorable correlation between mutagenic activity and carcinogenicity previously reported by McCann et al. (1975). Based on these results, it appears that the Salmonella/mammalian microsome test has application for initial evaluation of potential health hazards associated with the consumption of plants by animals and humans.

ACKNOWLEDGEMENTS

This research was supported by Project 156 of the Oregon Agricultural Experiment Station and NIH Grant No. CA-22524. The manuscript was issued as Oregon Agricultural Experiment Station Technical Paper No. 6399.

TABLE 7

MUTAGENICITY WITH THE SALMONELLA/MAMMALIAN-MICROSOME TEST OF ACETONE EXTRACTS[1] FROM SIX PLANTS WITH TESTER STRAINS TA98 AND TA100 WITHOUT LIVER MICROSOMES PRESENT AND IN THE PRESENCE OF S-9 MICROSOME PREPARATION FROM INDUCED RAT LIVER[2]

Plant	TA98		TA100	
	without S-9	with S-9	without S-9	with S-9
Alfalfa (*Medicago sativa*)	–[3]	–	–	–
Bracken (*Pteridium aquilinum*)	–	+[4]	–	–
Comfrey (*Symphytum officinale*)	toxic[5]	–	–	–
Lettuce (*Lactuca sativa*)	–	–	–	–
Tansy ragwort (*Senecio jacobaea*)	toxic	+	–	+
Thread-leaf groundsel (*Senecio longilobus*)	–	–	–	–

[1] Acetone extracts were prepared from 60g of dried plant material, shaken in 300 ml acetone for 48 hrs, filter sterilized by passing through a sterile Seitz filter then stored under refrigeration until used.

[2] Rats were induced with Arochlor 1254 and S-9 fraction prepared as described by Ames *et al.* (1975).

[3] – indicates a negative response; test solution did not produce twice the number of control revertants.

[4] + indicates a positive response; test solution produced at least twice the number of control revertants and demonstrated a dose response.

[5] toxic indicates that the test solution produced less than half the number of control revertants.

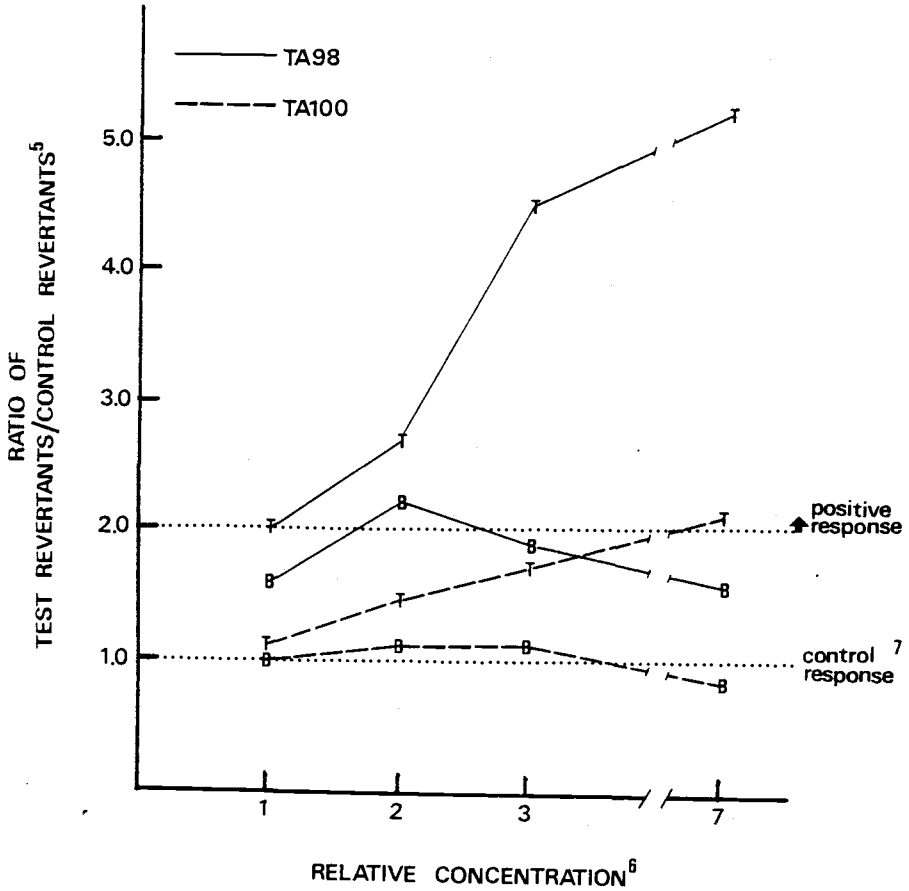


Figure 4. Dose response[1] curve for acetone extracts of bracken[2] (Pteridium aquilinum) and tansy ragwort[3] (Senecio jacobaea) tested with the Salmonella/mammalian-microsome mutagenicity test using tester strains TA98 and TA100 in the presence of S-9 microsome preparation from induced rat liver[4]

[1]Dose response calculated as averages of two separate tests

[2]B = bracken

[3]T = tansy ragwort

[4]Rats were induced with Arochlor 1254 and S-9 fraction prepared as described by Ames et al. (1975).

[5]Ratio number = the average number of revertant colonies produced by test solution divided by the average number of normal revertants produced on control plates; positive response is for ratio number of 2 or greater as described by Ames et al. (1975).

[6]Relative concentration was determined by the initial volume of extract rotary evaporated to dryness and then reconstituted to a new volume consisting of 0, 1, 2, 3 or 7 times the original concentration of extracted plant material.

[7]Control revertant number averaged 20 for TA98 and 73 for TA100

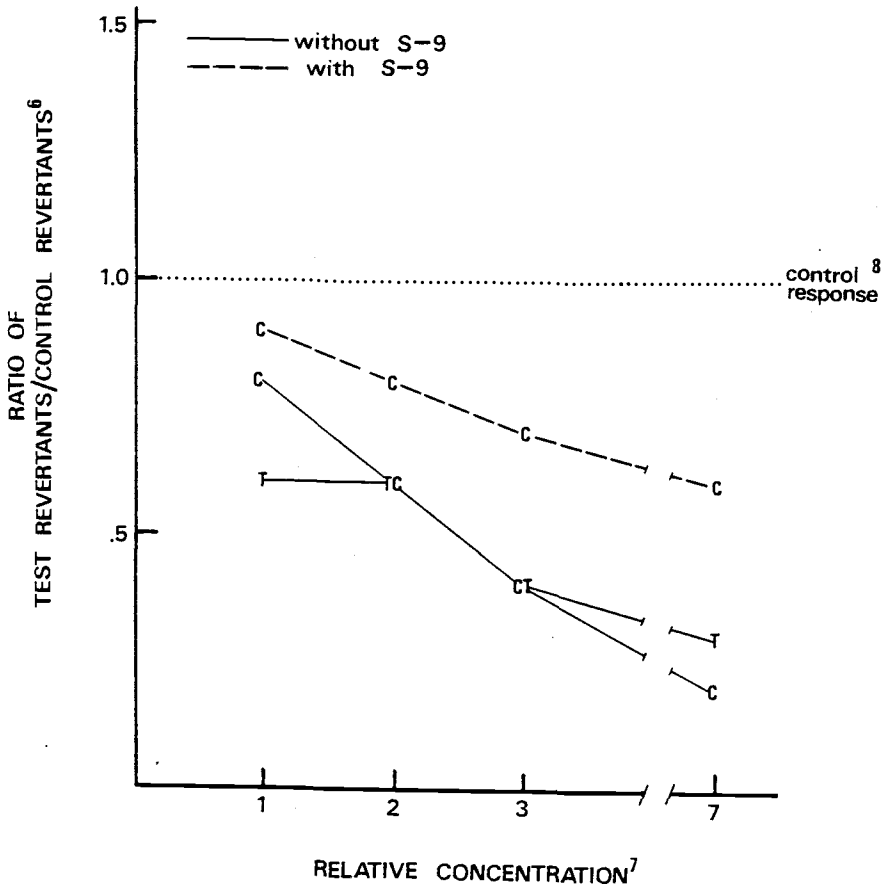


Figure 5. Toxic[1] dose[2] response curves for extracts of comfrey[3] (Symphytum officinale) and tansy ragwort[4] (Senecio jacobaea) tested with the Salmonella/mammalian-microsome mutagenicity test using tester strain TA98 without liver microsomes present and in the presence of S-9 microsome preparation from induced rat liver[5]

[1]Toxic indicates that the test solution produced less than half the number of control revertants

[2]Dose response calculated as averages of two separate tests

[3]C = comfrey

[4]T = tansy ragwort

[5]Rats were induced with Arochlor 1254 and S-9 fraction prepared as described by Ames et al. (1975).

[6]Ratio number = the average number of revertant colonies produced by test solution divided by the average number of normal revertants produced on control plates.

[7]Relative concentration was determined by the initial volume of extract rotary evaporated to dryness and then reconstituted to a new volume consisting of 0, 1, 2, 3 or 7 times the original concentration of extracted plant material.

[8]Control revertant number averaged 20 for TA98

Effect of *Senecio jacobaea* and Molybdenum on Copper Accumulation in Sheep[1,2]

R.D. White, R.A. Swick and P.R. Cheeke

Oregon State University
Corvallis, OR 97331

[1]Oregon Agricultural Experiment Station Technical Paper No. 6333.
[2]This project was supported by Project 156 of the Oregon Agricultural Experiment Station

Effects of Senecio jacobaea and Molybdenum on Copper Accumulation in Sheep

R.D. White, R.A. Swick and P.R. Cheeke

SUMMARY

Nine castrated male lambs were assigned to three groups as follows: 1) Control diet with 50 µg/g added Cu (Control+Cu); 2) Senecio jacobaea (SJ) diet with 50 µg/g added Cu (SJ+Cu); 3) SJ diet with 50 µg/g added Cu and 50 µg/g added Mo (SJ+Cu+Mo). Hematocrit values and liver Cu levels were determined prior to commencement of the treatments and then periodically determined during the course of the study. A steep initial rise (at least 13-fold) in liver Cu was observed in all treatments after about eight wks of diet consumption. This increase in liver Cu was maintained through 14 wks of diet consumption for the SJ+Cu and SJ+Cu+Mo groups with a slight decrease in liver Cu in the Control+Cu group. A decrease in liver Cu levels was observed in all groups after 18 wks of diet consumption but was still at least eightfold greater than pre-treatment levels in the Control+Cu and SJ+Cu groups and ninefold greater than pre-treatment levels in the SJ+Cu+Mo group. Treatment diets were then discontinued and all surviving lambs were placed on pasture and a control pellet without the added Cu. All lambs in the SJ+Cu and SJ+Cu+Mo groups had died by the end of 23 wks after commencement of the study, with all Control+Cu lambs surviving. The Control+Cu lambs had further decreases in liver Cu after 25 wks but liver Cu levels were still at least fivefold that of pre-treatment levels. No real changes in

hematocrit values were observed during the course of this study. Results from this study suggest that dietary molybdenum may actually increase intoxication by SJ rather than protect against hepatic copper accumulation associated with SJ.

(Key Words:) Copper toxicity, Senecio jacobaea, Lambs, Copper Molydenum

INTRODUCTION

Senecio jacobaea (SJ), commonly called tansy ragwort, is well known in the U.S. Pacific Northwest and other temperate regions of the world as a major toxic plant to livestock and is responsible for considerable livestock loss each year (Muth, 1968; Anon., 1971; Duby, 1975). SJ is one of several species of plants that contain a varying number of pyrrolizidine alkaloids (PA) for which the primary concern is hepatoxicity from the PA or their metabolites. Cattle and horses have been shown to be quite susceptible while sheep are partially resistant to SJ poisoning (Bull et al., 1968). Sheep have also been promoted as biological control agents to remove SJ plants from infested pastures (Mosher, 1979). It has been shown, however, that sheep are susceptible to copper (Cu) toxicity (Schaper and Luetje, 1931; Todd et al., 1962; Ishmael et al., 1972). SJ consumption causes liver accumulation of Cu in rats (Swick et al., 1980). Bull et al. (1956) reported that sheep consuming Heliotropium europaeum L., a PA containing plant, demonstrated increased hepatic Cu levels with an associated hemolytic crisis and increased mortality. Dietary molybdenum has been shown to decrease hepatic Cu stores in animals under certain conditions (Underwood, 1977). The objective of this experiment was to determine if dietary SJ predisposes sheep to Cu intoxication and to ascertain the effects of supplementary Mo in the diet.

MATERIALS AND METHODS

Nine Polypay wether lambs weighing between 13 and 17 kg were assigned to three groups as follows: 1) Control diet with 50 µg/g added Cu (Control+Cu); 2) SJ diet with 50 µg/g added Cu (SJ+Cu); 3) SJ diet with 50 µg/g added Cu and added Mo (SJ+Cu+Mo). The Mo in diet three was added at 50 µg/g for the first month and then changed to 10 µg/g thereafter. The adjustment in Mo content became necessary as the Mo-supplemented lambs refused to eat the diet containing 50 µg/g added Mo after the first month of diet consumption. These lambs readily accepted the diet again, however, after the Mo level was decreased to 10 µg/g. Diet intake was subsequently restricted for the other two groups until cumulative Cu intakes for all groups became approximately equal. Composition of diets is listed in Table 8. Animals were group fed; group feed intakes and periodic individual body weights were measured. Hematocrit values were determined by the micro-capillary method (Davidsohn and Bernard, 1969) prior to commencement of the treatments and then periodically at two to five week intervals during the course of the study. Liver Cu levels were determined prior to initiation of the treatments and after 8, 14, 18 and 25 wks of diet consumption. Liver biopsies were utilized for live animal tissue collection and were taken by aspiration using a 13Ga x 3 1/4 inch Becton Dickinson and Co. special needle, No. 468 LRT. Postmortem liver samples were collected at time of necropsy via tissue excision. Hepatic Cu levels were determined from samples of whole liver. Liver samples were subjected to wet ashing in 1:4 concentrated $HClO_4$:HNO_3 for six hours at 250 C after 24 h predigestion.

Appropriate dilutions were made with .1 N HCl and samples analyzed against Cu standards on a Perkin-Elmer model 303 atomic absorption (AA) spectrophotometer. Working standards were found to be within the acceptable range when verified against National Bureau of Standards bovine liver and oyster tissue reference standards. All glassware used in Cu analysis was soaked in 1N 1:1 $HCl:HNO_3$ for at least 24 h and rinsed four times with glass distilled water. Where applicable, treatment means were compared statistically by analysis of variance (Steel and Torrie, 1960).

RESULTS AND DISCUSSION

The consumption of supplemental Cu resulted in a steep initial rise in liver Cu levels in all treatment groups after eight weeks of diet consumption as shown in Figure 6. This 13-fold increase in liver Cu was maintained through 14 wks of diet consumption in the SJ+Cu and SJ+Cu+Mo groups but decreased slightly in the Control+Cu group. Decreases in liver Cu levels were observed in all groups after 18 wks of diet consumption but were still at least eightfold greater than pre-treatment levels. Also, two of the three animals in the SJ+Cu+Mo group had died by 18 wks. Treatment diets were discontinued after 18 wks diet consumption and surviving lambs placed on pasture and a control pellet without added Cu. All lambs in the SJ+Cu and SJ+Cu+Mo groups had died by the end of 23 wks with all Control+Cu lambs surviving. A further decrease in liver Cu level was observed in the Control+Cu group following the discontinuation of supplemental Cu. A similar initial increase followed by a decrease

in liver Cu levels over time was reported by Bull et al. (1956) for sheep given supplemental Cu following two months' grazing on Heliotropium europaeum infested and non-infested pastures.

Bull et al. (1956) also reported a hemolytic crisis associated with increased liver copper levels following consumption of H. europaeum without supplemental Cu. This was not evident, however, in our experiment as hematocrit values (Table 10) did not change during the course of the study.

Average daily gain (Table 10) was similar ($P>.05$) for all treatment groups. Feed, SJ, Cu and Mo intake was also similar between the appropriate groups as shown in Table 9.

It was interesting to note the voluntary anorexic reaction of the Mo-supplemented lambs when receiving 50 µg/g added Mo. Underwood (1977) described a similar voluntary rejection of high-Mo diets by rats and attributed this rejection to a learned or conditioned sensory, probably olfactory, recognition of Mo in the diet over a period of time.

The mortality exhibited in the present study may be reflective of toxicities associated with the consumption of SJ (Mortimer, 1970). Average survival times are shown in Table 10. Bull et al. (1956) believed that heliotrope hepatosis in sheep was due, in part, to altered Cu metabolism and the high concentration of Cu in liver cells in conjunction with an overall alteration of liver cell metabolism. The average survival time for the SJ+Cu+Mo group was about four wks less than that for the SJ+Cu group which may indicate that supplemental Mo at this level may increase SJ intoxication rather than protect against

hepatic Cu accumulation. Craig (1980) reported that sheep consuming twice their body weight in SJ demonstrated no noticeable adverse effects. Mortimer and White (1970), however, reported that mortalities occurred in sheep after consuming 30-40 percent of their body weight in SJ. The lambs in the present study died after consuming approximately 100 percent of their body weight in SJ. It is apparent from these conflicting results that more research is required to elucidate the toxic level for SJ and the interaction of SJ and hepatic Cu accumulation in sheep.

TABLE 8. PERCENT COMPOSITION OF LAMB DIET

Ingredient	Percent
Alfalfa or *Senecio jacobaea*[1]	52
Barley	30
SBM	10
Molasses	5
TM Salt	.5
Di Cal	.5
Mineral-Corn premix[2]	2

[1]Diets 2 and 3 contained 20% SJ from 17 JUN 81 to 30 SEP 81 and 40% SJ from 1 OCT 81 to 30 OCT 81. Diet 1 contained 52% Alfalfa; alfalfa was replaced by SJ in diets 2 and 3.

[2]50 µg/g Cu as $CuSO_4$ was added to each diet. Diet 3 contained 50 µg/g Mo as $(NH_4)_6Mo_7O_{24}.4H_2O$ from 17 JUN 81 to 16 JUL 81 and 10 µg/g Mo from 18 AUG 81 to 30 OCT 81.

TABLE 9 . FEED, SENECIO JACOBAEA, COPPER AND MOLYBDENUM INTAKES OF LAMBS[1]

	Average Feed intake kg	Average SJ intake kg	Average Cu intake g	Average Mo intake g
Control-Cu	172	--	8.6	--
SJ-Cu	172	42.6	8.6	--
SJ-Cu-Mo	166	40.7	8.3	2.1

[1]Includes only that amount of Cu and Mo added to the diet.

TABLE 10. AVERAGE DAILY GAIN, HEMATOCRIT, AND SURVIVAL TIME FOR LAMBS RECEIVING EITHER A CONTROL DIET WITH ADDED COPPER (Cu)[1], SENECIO JACOBAEA (SJ) WITH ADDED Cu OR SJ WITH ADDED Cu AND MOLYBDENUM (Mo)[1]

Diet	Daily Gain (g/d)	Hematocrit (%)	Survival Time (d)[3]
Control+Cu	195.3 ± 8.9[2]	37 ± 2	survived
SJ+Cu	168.8 ± 30.4	37 ± 2	161 ± 11
SJ+Cu+Mo	180.6 ± 55.1	37 ± 3	135 ± 16

[1]Cu and Mo added to diets at 50 g/g diet.

[2]Mean ± SD

[3]SJ+Cu and SJ+Cu+Mo group survival times significantly different (P .1).

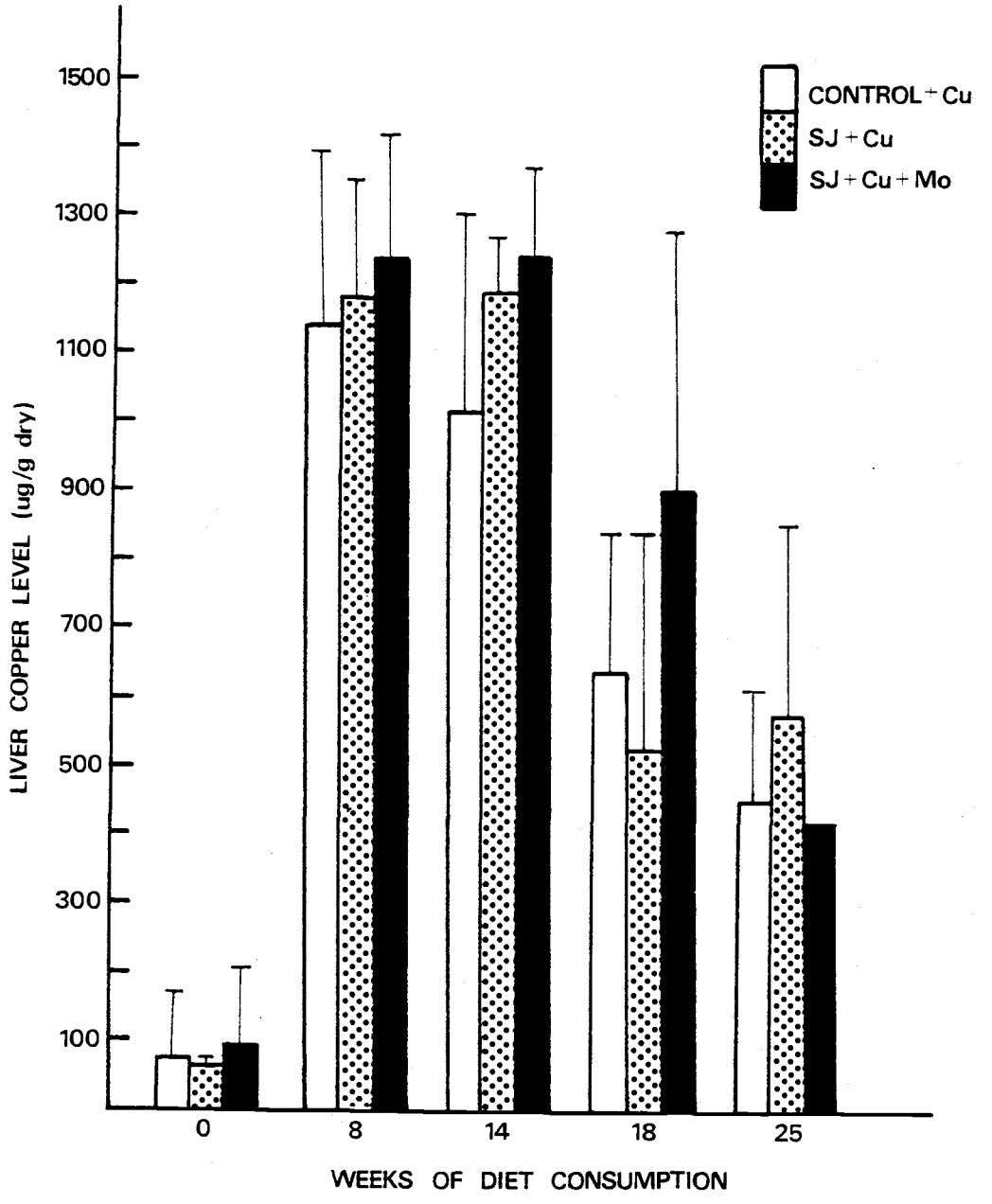


Figure 6. Liver copper levels in lambs consuming either a control diet with 50 µg/g added Cu (Control+Cu), a Senecio jacobaea diet with 50 µg/g added Cu (SJ+Cu) or a S. jacobaea diet with 50 µg/g added Cu and 50 µg/g added Mo (SJ+Cu+Mo). Values at 25 wks are comparative results for Control+Cu lambs after 25 wks and SJ+Cu and SJ+Cu+Mo lambs after 23 wks of consumption.

Effect of Esterase Inhibition on Pyrrolizidine (*Senecio*) Alkaloid Toxicity in Rats

Randy D. White and Peter R. Cheeke

Department of Animal Science
Oregon State University
Corvallis, OR 97331

Effect of Esterase Inhibition on Pyrrolizidine (Senecio) Alkaloid Toxicity in Rats

Randy D. White and Peter R. Cheeke

SUMMARY

The effects of 0.1% dietary tri-o-cresyl phosphate (TOCP) and 50 ppm 2-pyridine aldoxime methiodide (2-PAM) on Senecio jacobaea (SJ) toxicity in rats consuming 5% SJ diets and changes in the oral median lethal dose (LD_{50}) of a pyrrolizidine alkaloid (PA) mixture from Senecio longilobus (SL) caused by dietary exposure to organophosphates or carbamates were evaluated. Consumption of diets containing TOCP resulted in decreased 35 d weight gains in control animals and a slight decrease in feed intake with further decreased weight gains in SJ-fed animals. Decreased feed intakes and weight gains occurred in animals fed a control diet containing 2-PAM compared to animals fed the control diet without 2-PAM. Animals consuming a SJ+2-PAM diet had slight increases in feed intake and weight gain over animals receiving the SJ diet without 2-PAM. The average survival time of rats fed 5% SJ, 0.1% TOCP + 5% SJ and 50 ppm 2-PAM+5%SJ diets was approximately 60 d. The oral LD_{50} for a PA mixture extracted from SL at 72, 144 and 168 h post PA administration was 238,200 and 200 mg/kg, respectively. Concomitant dietary exposure to the organophosphate pesticide malathion caused a 16% increase in the 72 h LD_{50} (238 mg/kg) with a 16% decrease in the 144 and 168 h LD_{50} (168 and 168 mg/kg respectively) occurring compared to the control. Dietary exposure to the organophosphates coumaphos and TOCP resulted in consistent decreases in the 72, 144 and 168 h LD_{50} values (coumaphos: 126, 119 and

119 mg/kg; TOCP:168, 141 and 141 mg/kg) compared to the control. Dietary exposure to the carbamate sevin resulted in a similar 72 h LD_{50} (238 mg/kg) with decreased 144 h and 168 h LD_{50} values (168 and 168 mg/kg) compared to the controls. These results suggest increased PA toxicity associated with inhibition of esterase metabolism.

INTRODUCTION

Consumption of plants that contain pyrrolizidine alkaloids (PA) results in considerable livestock losses in the U.S. Pacific Northwest and other temperate regions of the world (Mattocks, 1968; Muth, 1968; Duby, 1975). The PA themselves are relatively non-toxic but hepatic enzyme dehydrogenation of the 1,2-dehydropyrrolizidine ring (Chesney et al., 1974) forming pyrrole (dihydropyrrolizidine) metabolites (Mattocks and White, 1971) causes a dramatic increase in toxicity (Mattocks, 1968; Culvenor et al., 1969). PA molecules contain one or more ester linkages. Alkaline hydrolysis (Mattocks, 1972b) in which the ester groups are removed (Bull et al., 1969) results in non-toxic (Schoental, 1968) acid and base moieties. Deesterification before pyrrole formation occurs may reduce the toxicity of PA. Culvenor et al. (1962) noted that benzyl mercaptan reacted with the PA heliotrine to replace the anion lost during alkyl-oxygen fission. Hayashi and Lalich (1968), in pursuit of this theory, reported improved survival time and growth rate in rats dosed with the PA monocrotaline followed by an injection of mercaptoethylamine or cysteine. It has been shown that pretreatment of rats with PA causes subsequent decreased pyrrole production (Mattocks and White, 1971). As initial pyrrolic metabolites may act as bifunctional alkylating agents (Mattocks, 1968; Culvenor et al., 1969), this decreased pyrrole production could have been due to a direct attack on the hepatic microsomal enzymes, including esterases, thereby preventing further deesterification. It has been indicated that a major mechanism of PA detoxification is that of de-esterification of PA (Mattocks, 1978). Organophosphates such as

coumaphos, tri-o-cresyl phosphate (TOCP) and malathion and carbamates such as carbaryl (sevin) are known as esterase inhibitors (Murphy, 1975) and TOCP has been shown to increase liver production of the highly toxic pyrrole metabolites from PA (Mattocks and White, 1971; Mattocks, 1978; Aldridge and Reiner, 1972). The compound 2-pyridine aldoxime methiodide (2-PAM) is effective in regenerating inhibited esterase and is used as an antidote for organophosphate pesticide intoxication (cholinesterase inhibition) (Murphy, 1975).

Inhibition of esterase metabolism and thus a probable PA detoxification mechanism could result in increased PA toxicity. The objectives of these studies were to: 1) delineate possible changes in the oral LD_{50} of a PA mixture (Senecio longilobus) that might result from dietary exposure to the pesticides coumaphos, TOCP, malathion and sevin; 2) determine if TOCP increases the toxicity of the PA-containing plant Senecio jacobaea (SJ); and 3) determine the effect of 2-PAM on SJ toxicity.

MATERIALS AND METHODS

Plant material. Senecio longilobus (SL) whole plant (excluding roots) was collected in the vicinity of Tucson, AZ and SJ flower tops and leaves were collected in the vicinity of Corvallis, OR. Plant materials were dried at 50°C, finely ground in a Wiley mill and stored at room temperature.

PA preparation. An ethanol extract was obtained from the dried, ground SL by the method of Campbell(1956). Repeated crystallization in methanol of the ethanol extract was then used to prepare the

composite mixture of PA. Resultant crystals were vacuum-desiccated for 24 h at room temperature and then stored at -30°C until used. The crystallized PA was prepared for oral administration by dissolving in 0.2 N HCl and neutralized to pH 5.6 with 1 N NaOH.

Control diet. The percent composition of the control diet utilized in each experiment was as follows: ground corn; 58.5% soybean meal, 30%, sucrose, 5%, corn oil, 3%; mineral mix (Jones and Foster, 1942), 3%; and vitamin mix (Cheeke, 1972), 0.5%.

Experiment 1

An oral LD_{50} in rats for a SL PA mixture and changes in the LD_{50} due to dietary exposure to the organophosphates coumaphos (97.1% active ingredient; Bayvet, Shawnee Mission, KS), TOCP (95.0% active ingredient; Eastman Kodak Co., Rochester, NY) and malathion (91.0% active ingredient; American Cyanimid Co., Wayne, NJ) and the carbamate sevin (99.8% active ingredient, Union Carbide Ag. Prod. Co., Inc., Research Triangle Park, NC) were determined. Eighty male Sprague-Dawley rats, initially weighing about 150 g, were assigned to five treatment groups. One group received control diet and the other four groups received a similar diet containing either 0.0075% coumaphos, 0.1% TOCP, 0.1% malathion, or 0.1% sevin, substituted for a portion of the corn in the control diet. The TOCP diet also contained 1 ppm Se (added as Na_2SeO_3) which has been shown to negate slight decreases in weight gain (Shull and Cheeke, 1973) previously demonstrated in rats fed diets containing 0.1% TOCP (Shull and Cheeke, 1975). Sixteen rats were assigned each diet group and received their respective diets ad libitum beginning seven days prior to PA administration.

A single oral dose (by gavage) PA administration utilizing four rats per dosage level of 50, 100, 200 and 400 mg per kg body weight per diet treatment was then performed. Rats continued ad lib. access to their respective diets and mortality at 72, 144 and 168 h post PA administration was determined. The method of LD_{50} calculation used was described by Thompson (1947) and Weil (1952). Weight gain and diet intake were measured for the seven days prior to PA administration and treatment means were compared statistically by one way analysis of variance and the Least Significant Difference procedures (Steel and Torrie, 1960).

Experiment 2

Dietary supplements of TOCP and 2-PAM were assessed for possible effects on SJ toxicity in rats. Five male Sprague-Dawley rats of about 95 g initial weight were assigned to each of six treatment groups as follows: 1) control diet only (Control); 2) control diet containing .1% TOCP and 1 ppm Se (TOCP-Control); 3) control diet containing 50 ppm 2-PAM (2-PAM-Control; 4) diet containing 5% SJ (5%SJ); 5) 5% SJ diet containing .1% TOCP and 1 ppm Se (TOCP+5%SJ); and 6) 5% SJ diet containing 50 ppm 2-PAM (2-PAM_5%SJ). The TOCP and SJ were substituted for a portion of the corn in the control diet and Se was extraneously added as Na_2SeO_3. The levels of 0.1% TOCP and 1 ppm Se were selected for use as the addition of 0.1% TOCP to rat diets has previously been shown to cause slight decreases in weight gain (Shull and Cheeke, 1973) but the addition of 1ppm Se has been shown to negate this effect (Shull and Cheeke, 1975). The level of 50 ppm 2-PAM was utilized as a 34% approximation of the human intravenous dosage

(1 g 2-PAM per 70 kg body weight) used in treating organophosphate insecticide poisoning (Murphy, 1975). Diets were fed ad libitum for 104 d or until mortality occurred. The animals were housed individually in meshed floor, stainless steel cages under controlled light, temperature and humidity. Feed intake and weight gain for the first 35 d of diet consumption and survival time were recorded for each animal. Treatment means were compared statistically by one way analysis of variance and compared by the Least Significant Difference procedure (Steel and Torrie, 1960).

RESULTS AND DISCUSSION

Experiment 1

The mortality profiles shown in Table 11 were used for LD_{50} determination by the method of Thompson (1947) and using tables generated by Weil (1952). The calculated oral LD_{50} for each treatment group is shown in Table 12. The decreases in the LD_{50} values over time conform with trends previously demonstrated (Bull et al., 1968) for the PA heliotrine having LD_{50}'s of 310 and 210 mg/kg at 72 and 168 h, respectively. A substantial decrease in LD_{50} by 144 h post PA administration, compared to the control, was demonstrated for each organophosphate and carbamate treatment. The degree of increased toxicity may coincide with the toxicities associated with those of the individual pesticides themselves as coumaphos, malathion and sevin have been shown to have oral LD_{50} values (Gains, 1969) of 16-41 mg/kg, 1.00-1.37 g/kg and 0.5-0.7 g/kg, respectively. The total pesticide

intake prior to PA administration (Table 13) for each treatment and considerably below these LD_{50} levels. Only marginal weight gain and feed intake differences were observed among treatment groups with the exception of the TOCP group (Table 13). Shull and Cheeke (1973) previously demonstrated that the addition of 0.1% TOCP to rat diets caused slight decreases in weight gains but the addition of 1 ppm Se tended to negate this effect (1975). As this group also had a decreased feed intake, the decreased weight gain may have been due to a combination of effects. The results suggest that esterase inhibition could have been responsible for the increased PA toxicities associated with the organophosphate and carbamate treatments as reflected by the individual toxicities of the respective pesticides.

Experiment 2

Feed intake and weight gain data for the first 35 d of diet consumption for each treatment group are shown in Table 14. Weight gain was reflective of feed intake as decreases in weight gain were observed in conjunction with decreases in feed intake. The exception to this was the TOCP-Control group which consumed a similar amount of feed to that of the Control group but still exhibited a significant ($P<0.05$) decrease in weight gain. The incorporation of 5% SJ into the diet caused further decreases in feed intake and weight gain. The inclusion of 2-PAM into the 5% SJ diet resulted in a slight increase in feed intake and weight gain over that of the 5% SJ group, whereas the TOCP+5%SJ group had a slight decrease in feed intake and a significant ($P<0.05$) decrease in weight gain compared to the 5% SJ group. The SJ

intake was similar ($P>1.0$) for all groups consuming SJ. The substantial decreases in weight gain observed for the TOCP groups may be associated with a direct toxicologic action of TOCP in the TOCP-Control group. Shull and Cheeke (1975) reported that TOCP-fed rats grew more slowly than pair-fed controls concluding that less efficient nutrient utilization had occurred. Mattocks (1978), using synthetic alkaloid analogues, demonstrated that rats predosed with the esterase inhibitor TOCP had increased *in vivo* liver pyrrole production following the administration of mono- and diester PA (retronecine) and Mattocks and White (1971) demonstrated increased liver pyrrole *in vitro* from a diester PA (retrorsine) after donor rats had been pretreated with an acute dose (approx. 350 mg) of TOCP 60 h before the rats were killed. As previously indicated (Mattocks, 1972b; Bull *et al.*, 1968; Schoental, 1968; Mattocks, 1978), ester hydolysis is a major detoxification mechanism for PA and this mechanism could be inhibited by TOCP via esterase inhibition. A coupling of the direct action of TOCP with that of increased pyrrole production associated with concomitant TOCP ingestion and PA exposure could have been the cause for further decreased weight gain observed in the TOCP+5%SJ group as this group consumed significantly ($P<0.05$) less TOCP than the TOCP-Control group.

All animals in the Control, 2-PAM-Control and TOCP-Control groups survived 104 d (Table 14). The addition of 2-PAM or TOCP had no effect on the average survival time of SJ consuming animals, although the mortality range for the TOCP+5%SJ group was considerably broader than that of the 5% SJ and 2-PAM+5% SJ groups.

The substantial decrease in weight gain observed in the TOCP+5% SJ group further indicates the need for avoidance of organophosphate insecticides when PA exposure occurs. As TOCP is not a potent esterase inhibitor (Murphy, 1975), it is still not certain if its effects were due to esterase inhibition, induction of microsomal enzymes with subsequent increased pyrrole production, nutritional inhibition or a combination of these effects. The use of 2-PAM as an antidote to PA toxicosis does not appear promising.

ACKNOWLEDGEMENTS

The coumaphos, malathion and sevin were gifts of these respective companies: Bayvet, Shawnee Mission, KA; American Cyanimid Agricultural Division, Wayne, NJ; and Union Carbide Ag. Prod. Co., Inc., Triangle Park, NC. This research was supported by Project 156 of the Oregon Agricultural Experiment Station and grants No. 113 and No. 123 from the Committee on Unsponsored Research Utilizing Laboratory Animals, Oregon State University, Corvallis, OR. The manuscript was issued as Oregon Agricultural Experiment Station Technical Paper No. _____.

TABLE 11.

EXPERIMENT 1. MORTALITY OF RATS FOLLOWING ORAL ADMINISTRATION OF PYRROLIZIDINE ALKALOIDS (SENECIO LONGILOBUS) AT LEVELS OF 50, 100, 200, AND 400 mg/kg

Treatment Group	Number of Dead Per Group of Four											
	72 h				144 h				168 h			
	50	100	200	400	50	100	200	400	50	100	200	400
Control	0	0	1	4	0	0	2	4	0	0	2	4
Coumaphos	0	1	4	3	0	1	4	4	0	1	4	4
TOCP	0	0	3	4	0	1	3	4	0	1	3	4
Malathion	0	0	0	4	0	0	3	4	0	0	3	4
Sevin	0	0	1	4	0	0	3	4	0	0	3	4

TABLE 12.

EXPERIMENT 1. ORAL MEDIAN LETHAL DOSE (LD_{50}) AND 95 PERCENT CONFIDENCE RANGE[a] FOR PYRROLIZIDINE ALKALOIDS (*SENECIO LONGILOBUS*)

Treatment Group	LD_{50} and 95% limits (mg/kg)		
	72 h	144 h	168 h
Control	238(121,467)	200(134,298)	200(134,298)
Coumaphos	126(77,205)	119(84,168)	119(84,168)
TOCP	168(119,238)	141(87,231)	141(87,231)
Malathion	283(200,400)	168(119,238)	168(119,238)
Sevin	238(121,467)	168(119,238)	168(119,238)

[a] LD_{50} and 95% limits calculated by the method of Thompson (1947) and with tables generated by Weil (1952).

TABLE 14.

EXPERIMENT 2. FEED INTAKE[a] AND WEIGHT GAIN[a] FOR THE FIRST 35 DAYS OF DIET CONSUMPTION AND SURVIVAL TIME[a] AND MORTALITY RANGE[b]

Treatment	Daily Weight Gain (g)	Total 35d Feed Intake (g)	35d TOCP Intake (g)	35d 2-PAM Intake (mg)	35d SJ Intake (g)	Survival Time (d)[c]	Mortality Range (d)
Control	6.1±0.8[d]	996± 90[d]	0	0	0	Survived	0
2-PAM-Control	5.1±0.1[e]	820± 24[e]	0	41±1	0	Survived	0
TOCP-Control	4.4±0.6[e]	939±196[de]	.939±.196[d]	0	0	Survived	0
5%SJ	1.9±0.6[f]	588± 51[f]	0	0	29±3	60±9	46-88
2-PAM+5%SJ	2.1±0.6[f]	679±120[ef]	0	34±6	34±6	59±6	50-61
TOCP+5%SJ	0.6±0.3[g]	557± 93[e]	.557±.093[e]	0	28±5	61±26	36-102

[a]Values given as mean ± SD

[b]Values represent the first and last days mortality occurred during the 104d feeding period.

[c]Survived indicates no mortality occurred during the 104d feeding period.

[d,e,f,g] Means in the same column with different superscripts differ ($P<0.05$).

Effects of Microsomal Enzyme Induction on the Toxicity of Pyrrolizidine (Senecio) Alkaloids Assessed by Changes in LD_{50}, Survival Time and Protective Activity of Cysteine

R.D. White, R.A. Swick and P.R. Cheeke

Department of Animal Science
Oregon State University
Corvallis, OR 97331

Effects of Microsomal Enzyme Induction on the Toxicity of Pyrrolizidine (Senecio) Alkaloids Assessed by Changes in LD_{50}, Survival Time and Protective Activity of Cysteine

R.D. White, R.A. Swick and P.R. Cheeke

SUMMARY

Effects of microsomal enzyme induction by phenobarbital (PB) injection (i.p.) and ingestion of Eucalyptus globulus foliage or phenothiazine (PT) on the toxicity of pyrrolizidine alkaloids (PA) of Senecio longilobus (SL) or Senecio jacobaea (SJ) were evaluated in rats. The oral median lethal dose (LD_{50}) for a PA mixture extracted from SL at 72, 144 and 168 h post PA administration was >320, 190 and 160 mg/kg, respectively. Induction of mixed function oxidases (MFO) activity by PB or dietary eucalyptus leaves resulted in only a marginal effect on the acute LD_{50} with no effective change at 168 h. The 72, 144 and 168 h LD_{50} values for PB treated rats were ca. 320, 190 and 160 mg/kg and for eucalyptus treated rats 127, 113 and 113 mg/kg, respectively. Dietary PT did not alter the toxicity of SJ but did cause reductions in feed intake and average daily gain. Supplemental cysteine increased ($P<0.05$) survival time in SJ-consuming rats with a concomitant increase ($P<0.05$) in SJ ingestion and caused slight increases in average daily gain. The simultaneous addition of cysteine and PT to a SJ containing diet resulted in an increased ($P<0.05$) survival time over SJ and SJ+PT diet groups and a slight decrease in survival time compared to the SJ+cysteine group.

INTRODUCTION

Considerable livestock losses in the United States and other regions of the world are directly related to consumption of plants that contain pyrrolizidine alkaloids (PA) (Duby, 1975; Muth, 1968). Senecio jacobaea (SJ) and Senecio longilobus (SL) are PA-containing plants found in the Unites States which are occasionally consumed by livestock (Bull et al., 1968; Roitman et al., 1979) and humans (Huxtable et al., 1979). Roitman et al. (1979) reported that the PA content of SL can be nearly 5% of its dry weight, making it a good source of alkaloid which can be extracted easily (Campbell, 1956). Ingested PA are metabolized to highly toxic pyrrole metabolites which cause acute hepatotoxicity (Bull et al., 1968; Mattocks, 1972a). Phenobarbital (PB) has been shown to be an effective inducer of the mixed function oxidases (MFO) and increases pyrrole (dehydropyrrolizidine) formation from PA in rats (Mattocks and White, 1971; Allen et al., (1972). Phenothiazine (PT), a commonly used livestock anthelmintic, has been identified as having a significant capacity to induce a detoxification enzyme in rat microsomal tissue (Wattenburg and Leong, 1965). Results from feeding studies conducted by Seawright et al. (1972) indicated that foliage of several species of trees when consumed by rats also induced MFO. It was also observed that foliage from one of these species of trees, Eucalyptus caleyi, was consumed by livestock on overgrazed pasture and increased their susceptibility to overdosing from carbon tetrachloride used as an anthelmintic. This type of MFO induction could have an effect on PA toxicity if concurrent

ingestion of eucalyptus foliage and PA-containing foliage occurred. Induction of pyrrole-producing enzymes by preadministration of PB or PT or by ingestion of eucalyptus foliage could increase the toxicity of PA that might be reflected by decreases in survival time or median lethal dose (LD_{50}). Buckmaster et al. (1976) demonstrated an increase in survival time for SJ-fed rats when they were supplemented with the amino acid cysteine. It is not known what effect MFO induction may have on the protective activity of cysteine. The objectives of these studies were to: 1) determine the oral LD_{50} in rats for a PA mixture extracted from SL; 2) delineate possible changes in the LD_{50} following the induction of hepatic microsomal enzymes by determining the LD_{50} for oral PA in rats previously exposed to PB or dietary eucalyptus leaves; 3) assess toxicologic implications of concomitant ingestion of PT and diets containing SJ; and 4) assess possible effects PT ingestion may have on the protective activity of cysteine against SJ intoxification.

MATERIALS AND METHODS

Plant material. SJ flower tops were collected in the vicinity of Corvallis, OR, and SL whole plant excluding roots was collected in the vicinity of Tucson, AZ. The plant materials were dried at 50°C, finely ground in a Wiley mill and stored at room temperature. Eucalyptus globulus leaves (EL) were collected in the vicinity of Corvallis, OR, lyophilized, finely ground in a Wiley mill and stored at 4°C until used.

PA preparation. An ethanol extract was obtained from the dried, ground SL plant material by the method of Campbell (1956). A composite mixture of PA was then prepared by repeated crystallization in methanol of the ethanol extract. Resultant crystals were vacuum-desiccated for 24 h at room temperature and then stored at -30°C until used. The crystallized PA was prepared for oral administration by dissolving in 0.2 N HCL and neutralized to pH 6.1 with 1N NaOH.

Experiment 1

An oral LD_{50} in rats of a SL PA mixture and changes in the LD_{50} due to microsomal enzyme induction were determined. Forty-eight male Sprague-Dawley rats, initially weighing about 160 g, were assigned to three treatment groups. Two groups received a control diet and one group (EL+PA) received a similar diet containing 10% dried, ground EL. Diet compositions are shown in Table 15. Sixteen rats were assigned to each diet group and received their respective diets ad libitum beginning eight days prior to PA administration. One group (PB+PA), receiving the control diet, also was given daily intraperitoneal (i.p.) injections with a solution of sodium phenobarbital (30.0 mg per ml sterile water) at a rate of 50 mg per kg body weight beginning four days prior to PA administration. The other control diet group (C+PA) received no extraneous treatment aside from PA administration. Eight days after the commencement of diet consumption, a single oral (by gavage) PA administration utilizing four rats per dosage level of 40, 80, 160 and 320 mg per kg body weight per diet treatment was performed. Mortality at 72, 144 and 168 h post administration of PA was

determined. The method of LD_{50} calculation described by Thompson (1947) and Weil (1952) was used.

Experiment 2

Phenothiazine, a commonly used livestock anthelmintic with microsomal enzyme inducing properties was assessed for possible effects on SJ toxicity with and without supplemental cysteine for protective activity. Sixty male rats of approximately 105 g initial body weight were randomly distributed into six dietary treatments of ten rats each. Diet compositions are shown in Table 16. Diets were fed ad libitum and protective activity of the additions to the SJ containing diets was assessed by comparing survival time means of these treatments to the controls. In addition, average daily gain, feed intake and SJ intake were compared for the treatments. Treatment means were compared statistically by analysis of variance and protected Least Significant Difference procedure (Snedecor and Cochran, 1967).

RESULTS AND DISCUSSION

Experiment 1

The mortality observed within each dosage level and treatment groups is shown in Table 17. Based on these mortality profiles, the LD_{50} calculations were determined by the method of Thompson (1947) and with tables generated by Weil (1952). The calculated LD_{50} are shown in Table 18. Bull et al. (1968) reported 72 h LD_{50} values of 80 and 85 mg/kg for rats (200 g weight) which were i.p. injected with the

pure Senecio alkaloids seneciphylline and senecionine, respectively, which are found in SL. The 7-day LD_{50} values of 80 and 38 mg/kg in rats i.v. injected with the Senecio alkaloids seneciphylline and retrorsine (also found in SL), respectively, have been reported (Anon., 1949). The 7-day i.p. LD_{50} for PA preparations obtained from SJ has been reported as being approximately 130-140 mg/kg for rats (Shull et al., 1976a; Shull et al., 1976b).

It was not known if the decreased LD_{50}'s for the EL+PA group was due to increased pyrrole production associated with induction of MFO from eucalyptus leaf ingestion or due to a decrease in body condition. The EL+PA group had an average decrease in body weight of about 30 g per rat during the eight days prior to PA administration as opposed to about a 40 g per rat weight increase for the C+PA and PB+PA groups during this same period. The average daily diet intake during this eight day period was about 8g/rat/day for the eucalyptus diet group compared to about 15g/rat/day for the control diet groups. There did appear to be, however, at least some acute affect due to PB induction of MFO as no mortality occurred at 72 h in the C+PA group whereas a 25% and 50% mortality had occurred at 160 and 320 mg/kg dosage levels in the PB+PA group (Table 17).

The decreases in LD_{50} over time for the SL PA (Table 18) conform with trends previously demonstrated by Bull et al. (1968) for the PA heliotrine which had 72 and 168 h LD_{50}'s of 310 and 210 mg/kg respectively. Caution should be taken in selecting chronic dose rates based on LD_{50} determinations for PA. Bull et al. (1968) reported that 4 LD_{50} of heliotrine could be administered to rats at a rate of 0.1 LD_{50}

three time per week for over 98 days whereas a larger dose of 0.4 LD_{50} resulted in one rat dying after a single injection and 6 others after 3, 4 or 5 injections.

It appears that the 168 h LD_{50} of 160 mg/kg for SL PA conforms with previously reported LD_{50} values for Senecio and other PA (Bull et al., 1968; Anon., 1949; Shull et al., 1976a; Shull et al., 1976b). Induction of MFO by i.p. injection of PB or dietary eucalyptus leaves resulted in only a marginal effect on the acute LD_{50} but had no effect on the 168 h LD_{50}.

Experiment 2

Dietary PT appeared to have no major effect in altering the toxicity of SJ with survival time being decreased by only 4 days in rats fed SJ with added PT (Table 19). The addition of PT, however, reduced feed intake and average daily gain. Supplemental cysteine showed protective activity against SJ toxicity by significantly increasing ($P<0.05$) survival time from 50 to 62 days even though SJ intake was greater ($P<0.05$) in the cysteine+SJ treatment and, in all cases, the addition of cysteine increased average daily gain slightly. When cysteine and PT were both added to the SJ diet, survival time was increased over the SJ and SJ+PT treatment groups but was still slightly less than that for the SJ+cysteine group.

Phenothiazine has been identified as an inducer of benz-α-pyrene hydroxylase, a detoxification enzyme present in rat microsomal tissue (Wattenburg and Leong, 1965). Induction of microsomal enzymes by PT

has also been shown to be effective in reducing the incidence of carcinomas in animals ingesting bracken fern (Pteridium aquilinum), a poisonous plant with an undetermined toxin (Pamukcu et al., 1971). Mattocks and White (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 of this experiment that PT was mildly active in inducing pyrrole formation and that induction of N-oxidation or conjugation pathways by PT was unlikely since toxicity of SJ was not reduced.

Since the protective effect of cysteine was not enhanced by PT, increased rate of pyrrole formation does not appear to cause an increase in non-toxic thiol conjugates even when sulfhydryls are readily available. Deesterification of the PA before pyrrole formation occurs, however, may reduce the toxicity of PA. Culvenor et al. (1962) noted that benzyl mercaptan reacted with the PA heliotrine to replace the anion lost during alkyl-oxygen fission. Hayashi and Lalich (1968), in pursuit of this theory, reported improved survival time and growth rate in rats dosed with the PA monocrotaline followed by an injection of mercaptoethylamine or cysteine. It could therefore 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.

ACKNOWLEDGEMENTS

This research was supported by Project 156 of the Oregon Agricultural Experiment Station and grant No. 116 from the Committee on Unsponsored Research Utilizing Laboratory Animals, Oregon State University, Corvallis. The manuscript was issued as Oregon Agricultural Experiment Station Technical Paper No. _____.

TABLE 15.

EXPERIMENT I. PERCENT COMPOSITION OF THE CONTROL RAT DIET

Ingredient	%
Ground corn	58.5
Soybean meal	30.0
Sucrose	5.0
Corn oil	3.0
Mineral mix[a]	3.0
Vitamin mix[b]	0.5
Eucalyptus leaf[c]	0

[a]Jones and Foster Mineral Mix, Nutritional Biochemicals Corp., Cleveland, OH.

[b]Cheeke (1972).

[c]In the eucalyptus-containing diet, 10% eucalyptus leaf meal replaced 10% corn in the control diet.

TABLE 16.

EXPERIMENT 2. PERCENT COMPOSITION OF THE CONTROL RAT DIET

Ingredient	%
Ground corn	53.5
Soybean meal	30.0
Alfalfa meal	5.0
Senecio jacobaea (SJ)[a]	0
Sucrose	5.0
Corn oil	3.0
Mineral mix[b]	3.0
Vitamin mix[c]	0.5
Cysteine[d]	0
Phenothiazine[d,e]	0

[a] In the SJ-containing diet, 5% SJ replaces the 5% alfalfa meal in the control diet.

[b] Jones and Foster Mineral Mix, Nutritional Biochemicals Corp., Cleveland, OH.

[c] Cheeke (1972)

[d] Addition of 1.0% cysteine and 0.15% phenothiazine were made in place of corn in the control diet.

[e] 94% Phenothiazine (Stocklin Supply, Portland, OR).

TABLE 17.

EXPERIMENT 1. MORTALITY OF RATS FOLLOWING ORAL ADMINISTRATION OF PYRROLIZIDINE ALKALOIDS (SENECIO LONGILOBUS) AT LEVELS OF 40, 80, 160 AND 320 mg/kg

Treatment Group	Number of Dead per Group of Four											
	72 h				144h				166h			
	40	80	160	320	40	80	160	320	40	80	160	320
PA[a]	0	0	0	0	0	0	1	4	0	0	2	4
PB+PA[b]	0	0	1	2	0	0	1	4	0	0	2	4
EL+PA[c]	0	0	4	3	0	0	4	4	0	0	4	4

[a]PA group began control diet 8 d prior to PA administration and received no other extraneous treatment.

[b]PB+PA group began control diet 8 d prior to PA administration and began daily i.p. administration of sodium phenobarbital (50 mg/kg) 4 d prior to PA administration.

[c]EL+PA group began 10% eucalyptus diet 8 d prior to PA administration.

TABLE 18.

EXPERIMENT 1. ORAL MEDIAN LETHAL DOSE (LD_{50}) AND 95 PERCENT CONFIDENCE RANGE[a] FOR PYRROLIZIDINE ALKALOIDS (SENECIO LONGILOBUS)

Treatment Group	LD_{50} and 95% limits (mg/kg)		
	72 h	144 h	168 h
PA[b]	>320[e]	190(160,226)	160(135,190)
PB+PA[c]	ca. 320[f]	190(160,226)	160(135,190)
EL+PA[d]	127(93,173)	113(113,113)	113(113,113)

[a] LD_{50} and 95% limits calculated by the method of Thompson (1947) and with tables generated by Weil (1952).

[b] PA group began control diet 8 d prior to PA administration and received no other extraneous treatment.

[c] PB+PA group began control diet 8 d prior to PA administration and began daily i.p. administration of sodium phenobarbital (50 mg/kg) 4 d prior to PA administration.

[d] EL+PA group began 10% eucalyptus diet 8 d prior to PA administration.

[e] >320 mg/kg indicated as no mortality was observed at 72 h. post PA administration (refer to Table 17).

[f] ca.320 mg/kg indicated as 1 rat had died at 160 mg/kg and 2 at 320 mg/kg (refer to Table 17) but these were insufficient results for a more accurate determination.

TABLE 19.

EXPERIMENT 2. EFFECT OF PHENOTHIAZINE (PT) AND CYSTEINE (cys) ON THE TOXICITY OF SENECIO JACOBAEA (SJ) (MEAN ± SEM)

Treatment	Survival Time (days)	SJ Intake (g)	Average Daily Gain (g) (first 14 days)
Control	survived	0	7.17 ± 0.17^{d}
PT	survived	0	5.95 ± 0.26^{c}
SJ	50 ± 3^{a}	27.0 ± 2.0^{a}	2.10 ± 0.14^{ab}
SJ+PT	46 ± 3^{a}	22.8 ± 3.1^{a}	1.89 ± 0.18^{a}
SJ+cys	62 ± 3^{b}	34.1 ± 2.4^{b}	2.79 ± 0.19^{b}
SJ+PT+cys	55 ± 4^{ab}	28.7 ± 2.1^{ab}	2.85 ± 0.18^{b}

a,b,c,d Means in same column followed by different superscripts are significantly different ($P<0.05$).

CONCLUSION AND SUGGESTIONS FOR FUTURE RESEARCH

Mutagenic responses of Senecio jacobaea (SJ) plant, pyrrolizidine alkaloids (PA) and metabolites in goat milk were evaluated by the use of the Salmonella/mammalian-microsome mutagenicity test and the response from an SJ plant extract was compared to those from alfalfa, lettuce, thread-leaf groundsel, comfrey and bracken. Bioassays were conducted to evaluate changes in PA toxicity due to concomitant copper (Cu) and molybdenum (Mo) ingestion, esterase inhibition, microsomal enzyme induction and dietary cysteine supplementation.

An SJ plant acetone extract produced toxic (without liver microsomes present) and positive (with microsomal activation) responses in the mutagenicity test. Milk from SJ-fed goats produced positive mutagenic responses following microsomal activation. The PA extracted from SJ and Senecio longilobus and jacobine and monocrotaline, however, produced only negative mutagenic responses. Acetone extracts from Senecio longilobus, lettuce and alfalfa produced only negative responses while a bracken acetone extract caused a positive response following bioactivation with liver microsomes. Comfrey acetone extract produced a toxic response in the absence of liver microsomes which was negated in the presence of the microsomal system. These results suggested that an unknown mutagenic/toxic component may exist in SJ plant material other than PA. It appears that this mutagenicity test system could be utilized as a tool for the isolation of the actual fraction in the SJ acetone extract that contains the mutagenic agent. This could possibly be accomplished by subsequent mutagenicity testing of resultant

separatory fractions from the acetone extract such as water, ether, chloroform, etc. Once the mutagenic component was isolated in a less complex fraction, techniques such as high pressure liquid chromatography, gas chromatography, nuclear magnetic resonance and mass spectrometry could be employed for specific identification of this agent.

The dietary addition of Cu or Cu and Mo to SJ diets fed to lambs caused only marginal effects on liver Cu levels and no apparent hemolytic effects and mortality in the SJ diet groups did not appear to be correlated to liver Cu accumulation. The lambs in this study died after consuming approximately 100 percent of their body weight in SJ with lambs fed the SJ+Cu+Mo diet dying about four wks before and consuming about five percent less SJ than lambs fed the SJ+Cu diet. The conflicting results of Mortimer and White (1970), where mortality in sheep occurred after consuming 30-40 percent of their body weight in SJ, and those of Craig (1980), where sheep consuming twice their body weight in SJ demonstrated no noticeable adverse effects, suggest that more research is required to elucidate the toxic level for SJ and the interaction of SJ, Cu and Mo in sheep.

Deesterification has been indicated as a major mechanism of PA detoxification (Mattocks, 1978). Esterase inhibition may, therefore, increase PA toxicity which was indicated when dietary exposure to the esterase inhibiting organophosphates coumaphos, tri-o-cresyl phosphate (TOCP) and malathion and the carbamate carbaryl resulted in decreased PA LD_{50} values in rats. The inclusion of TOCP in SJ diets fed to rats only caused decreased weight gains with similar survival times to rats

fed just an SJ diet. TOCP, however, is considered to not be a potent esterase inhibitor but is considered to more effectively cause delayed neurotoxic effects (Murphy, 1975). As coumaphos caused a greater increase in PA toxicity and is considered a more potent esterase inhibitor than TOCP, it is suggested that coumaphos be incorporated into SJ diets to evaluate the effects of esterase inhibition on PA toxicity associated with entact SJ plant. Experiments with rats fed SJ+ coumaphos should be conducted to measure both survival time and liver pyrrole production compared to rats fed SJ without coumaphos.

Microsomal enzyme induction by phenobarbital (PB) or phenothiazine (PT) appeared to have no major effect in altering PA toxicity in rats. Rats pretreated with PB had similar PA LD_{50} values to those for rats not receiving PB and rats fed PT in an SJ diet had similar survival times to rats fed just the SJ diet while rats fed the SJ diet with supplemental cysteine had increased survival times. It was not known, however, if decreased PA LD_{50} values for rats pretreated with dietary eucalyptus leaves were a result of microsomal enzyme induction from eucalyptus leaf-ingestion or due to decreased body condition as this group averaged approximately 4 g per day weight decrease compared to an average of approximately 5 g per day weight increase for control animals during the eight day period prior to PA administration. Mattocks and White (1971) reported that both pyrrole and N-oxide metabolites were significantly increased during in vitro liver metabolism of retrorsine when rats were pretreated with PB. These results may indicate then that PB induces detoxification enzymes to a slightly greater extent than those enzymes responsible for pyrrole production.

Miranda et al. (1981c) showed that dietary cysteine and butylated hydroxyanisole caused increased glutathione S-transferase activity as well as decreased PA toxicity in mice while butylated hydroxyanisole also caused a decrease in hepatic aminopyrine demethylase activity. Experiments to evaluate the profile of induced hepatic microsomal enzymes such as epoxide hydrase, animopyrine demethylase and cytosolic glutathione S-transferase could be of value.

Continued experimentation with other compounds previously shown to have protective effects against PA toxicity and the development of an economic dietary supplement for livestock could result in a substantial contribution to agriculture. Allen et al. (1972) showed that rats pretreated with chloramphenicol had an absence of mortaltity and unaffected growth rate when given the PA monocrotaline. Chloramphenicol, however, has not been approved for dietary use in livestock where chlortetracycline has (Feed Additive Compendium 1982, The Miller Publishing Company, Minneapolis, Minn.). Chloramphenicol and chlortetracycline have similar antibiotic effects in that they inhibit the 50S and 30S subunits of 70S ribosomes, respectively (Stryer, 1981). This may or may not be the protective mechanism of chloramphenicol but comparative studies with chlortetracycline on PA toxicity could have implications toward livestock protection. Other dietary agents such as sulfur amino acids (Retief, 1962; McLean, 1970; Cheeke and Garman, 1974; Buckmaster et al., 1976) vitamins B_6 and B_{12} (McLean, 1970) and antioxidants ethoxyquin (Miranda et al., 1981a; Kim and Jones, 1982) and butylated hydroxyanisole (Miranda et al., 1981c; Kim and Jones, 1982) have been shown to have protective activity against PA toxicity

in laboratory animals. Further research is required to determine which agents (alone or in combination) and their appropriate dosage provide the greatest protection in not only laboratory animals but livestock as well.

BIBLIOGRAPHY

Aeschbacher, H. U. and H. P. Wurzner. 1980. An evaluation of instant and regular coffee in the Ames mutagenicity test. Toxicol. Letters 4:139.

Alderson, T. and A. M. Clark. 1966. Interlocus specificity for chemical mutagens in Aspergillus nidulans. Nature 210:593.

Aldridge, W. N. and E. Reiner. 1972. Enzyme Inhibitors as Substrates. North Holland Publishing Co., Amsterdam.

Allen, J. R., C. F. Chesney and W. J. Frazee. 1972. Modifications of pyrrolizidine alkaloid intoxication resulting from altered hepatic microsomal enzymes. Toxicol. Appl. Pharmacol. 23:470.

Allen, J. R., I. C. Hsu and L. A. Carstens. 1975. Dehydroretronecine induced rhabdomyosarcomas in rats. Cancer Res. 35:997.

Ames, B. N., W. E. Durston, E. Yamasaki and F. D. Lee. 1973. Carcinogens are mutagens: a simple test system combining liver homogenates for activation and bacteria for detection. Proc. Natl. Acad. Sci. U.S.A. 70:2281.

Ames, B. N., J. McCann and E. Yamasaki. 1975. Methods for detecting carcinogens and mutagens with the Salmonella/mammalian microsome mutagenicity test. Mutation Res. 31:347.

Anon. 1949. Senecio and Related Alkaloids. Eli Lilly and Co. Indiana, U.S.A., Res. Today 5:55.

Anon. 1971. Tansy ragwort, an increasing problem in western Oregon. Oregon State Exten. Circ. No. 796. Oregon State University, Corvallis.

AOAC. 1980. Official Methods of Analysis (13th Ed.). Association of Official Analytical Chemists, Washington, D.C.

Brink, N. G. 1966. The mutagenic activity of heliotrine in Drosophila I. Complete and mosaic sex linked lethals. Mutation Res. 3:66.

Brink, N. G. 1969. The mutagenic activity of the pyrrolizidine alkaloid heliotrine in Drosophilia melanogaster, II. Chromosome rearrangements. Mutation Res. 8:139.

Buckmaster, G. W., P. R. Cheeke and L. R. Shull. 1976. Pyrrolizidine alkaloid poisoning in rats: protective effects of dietary cysteine. J. Anim. Sci. 43:464.

Bull, L. B., C. C. J Culvenor and A. T. Dick. 1968. The Pyrrolizidine Alkaloids. John Wiley and Sons, Inc., New York.

Bull, L. B., A. T. Dick, J. C. Keast and G. Edgar. 1956. An experimental investigation of the hepatotoxic and other effects on sheep of consumption of Heliotropium europaeum L.: Heliotrope poisoning of sheep. Aust. J. Agric. Res. 9:281.

Campbell, J. G. 1956. An investigation of the hepatotoxic effects in the fowl of ragwort, with special reference to the induction of liver tumors with seneciphylline. Proc. Roy. Soc. Edinburgh Sect. B Biol. 66:111.

Cheeke, P. R. 1972. Antioxidant activity of torula yeast. Nutr. Rpts. Internat. 5:159.

Cheeke, P. R. 1977. Progress in development of alfalfa as a feedstuff for non-ruminants. Feedstuffs 49:48.

Cheeke, P. R. and G. R. Garman. 1974. Influence of dietary protein and sulfur amino acid levels on the toxicity of Senecio jacobaea (tansy ragwort) to rats. Nutr. Rpts. Internat. 9:197.

Chesney, C. F., I. C. Hsu and J. R. Allen. 1974. Modifications of the in vitro metabolism of the hepatotoxic pyrrolizidine alkaloid monocrotaline. Res. Comm. Chem. Path. and Pharmacol. 8:567.

Clark, A. M. 1959. Mutagenic activity of the alkaloid heliotrine in Drosophila. Nature 183:731.

Clark, A. M. 1960. The mutagenic activity of some pyrrolizidine alkaloids in Drosophila. Z Vererbungsl. 91:74.

Commoner, B., A. J. Vithayathil, P. Dolara, S. Nair, P. Madyastha and G. C. Cuca. 1978. Formation of mutagens in beef and beef extract during cooking. Science 201:913.

Craig, A. M. 1980. Special report 578, Agric. Exp. Sta., Oregon State University, Corvallis, p. 58.

Culvenor, C. C. J., A. T. Dann and A. T. Dick. 1962. Alkylation as the mechanism by which the hepatoxic pyrrolizidine alkaloids act on cell nuclei. Nature (London) 195:570.

Culvenor, C. C. J., D. T. Downing, J. A. Edgar and M. V. Jago. 1969. Pyrrolizidine alkaloids as alkylating and antimitotic agents. Ann. N.Y. Acad. Sci. 168:837

Davidsohn, I. and J. B. Bernard. 1969. Clinical Diagnosis (14th Ed.). W. B. Saunders Co., Philadelphia.

Deinzer, M. L., B. Arbogast, D. R. Buhler and P. R. Cheeke. 1982. Analysis of pyrrolizidine alkaloids in milk. Anal. Chem., submitted.

Dickinson, J. O. 1974. Pyrrolizidine alkaloids and milk transfer. West. Vet. 12:26.

Dickinson, J. O. 1980. Release of pyrrolizidine alkaloids into milk. Proc. West. Pharmacol. Soc. 23:377.

Dickinson, J. O. and R. R. King. 1978. The transfer of pyrrolizidine alkaloids from Senecio jacobaea into the milk of lactating cows and goats. In: Effects of Poisonous Plants on Livestock (R. F. Keeler, K. R. Van Kampen and L. F. James, eds.). p201. Acacemic Press, New York.

Duby, G. D. 1975. Tansy ragwort: a toxic threat to livestock. Mod. Vet. Pract. 56:185.

Evans, I. A. 1979. Bracken carcinogenicity. Res. Vet. Sci. 26:339.

Evans, W. C. 1976. Bracken thiaminase-mediated neurotoxic syndromes. Bot. J. Linnean Soc. 73:113.

Fox, D. W., M. C. Hart, P. S. Bergeson, P. B. Jarrett, A. E. Stillman and R. J. Huxtable. 1978. Pyrrolizidine (Senecio) intoxication mimicking Reye syndrome. J. Pediatrics 93:980.

Furuya, T. and K. Araki. 1968. Studies on constitutents of crude drugs, I. Alkaloids of Symphytum officinale. Chem Pharm. Bull. 16:2512.

Gains, T. B. 1969. Acute toxicity of pesticides. Toxicol. Appl. Pharmacol. 14:515.

Goeger, D. E., P. R. Cheeke, D. R. Buhler and J. A. Schmitz. 1979. Effect of tansy ragwort on dairy goats and goat milk transfer of pyrrolizidine alkaloids. In: Symposium on Pyrrolizidine (Senecio) Alkaloids: Toxicity, Metabolism and Poisonous Plant Control Measures. (P. R. Cheeke, ed.). p. 77. Nutrition Research Institute, Oregon State University, Corvallis.

Goeger, D. E., P. R. Cheeke, J. A. Schmitz and D. R. Buhler. 1982. Toxicity of tansy ragwort (Senecio jacobaea) to goats. Am. J. Vet. Res. 43:252.

Green, M. E. Ben-Hur and E. Ridlis. 1980. Application of mutagenicity test for milk. J. Dairy Sci. 63:358.

Hayashi, Y. and J. J. Lalich. 1968. Protective effect of mercaptoethylamine and cysteine against monocrotaline intoxication in rats. Toxicol. Appl. Pharmacol. 12:36.

Hirono, I., H. Mori and M. Haga. 1978. Carcinogenicity of Symphytum officinale. J. Natl. Cancer Inst. 61:865.

Hooper, P. T. 1972. A study of the pathology of pyrrolizidine alkaloid poisoning with particular reference to lesions in the central nervous system. Ph.D. Thesis. University of Melbourne, Victoria, Australia.

Hoover, R. and J. F. Fraumeni, Jr. 1975. Cancer mortality in U.S. countries with chemical industries. Environ. Res. 9:196.

Hopkirk, C. S. M. and I. J. Cunningham. 1936. Biological experiment with jacobine extracted from ragwort by J. R. Hosking and C. W. Brandt. New Zealand J. Sci. Technol. 17:645.

Huxtable, R. J. 1979. Herbal teas and pyrrolizidine alkaloids. In: Symposium on Pyrrolizidine (Senecio) Alkaloids: Toxicity, Metabolism and Poisonous Plant Control Measures. (P. R. Cheeke, ed.) p. 87. Nutrition Research Institute, Oregon State University, Corvallis.

Ishmael, J., C. Gopinath and J. McC. Howell. 1972. Experimental chronic copper toxicity in sheep: Biochemical and hematological studies during the development of lesions in the liver. Res. Vet. Sci. 13:22.

Jago, M. V., J. A. Edgar, L. W. Smith and C. Culvenor. 1970. Metabolic conversion of heliotridine-based pyrrolizidine alkaloids to dehydroheliotridine. Mol. Pharmacol. 6:402.

Johnson, A. E. 1976. Changes in calves and rats consuming milk from cows fed chronic lethal doses of Senecio jacobaea (tansy ragwort). Am. J. Vet. Res. 37:107.

Jones, J. H. and C. Foster, 1942. A salt mixture for use with basal diets either high or low in phosphorus. J. Nutr. 24:245.

Kier, L. D., E. Yamasaki and B. N. Ames. 1974. Detection of mutagenic activity in cigarette smoke condensates. Proc. Natl. Acad. Sci. U.S.A. 71:4159.

Kim, H. L. and L. P. Jones. 1982. Protective effects of butylated hydroxyanisole, ethoxyquin and disulfiram on acute pyrrolizidine alkaloids poisoning in mice. Res. Comm. Chem. Path. Pharmacol. 36:341.

Koletsky, A., R. Oyasu and J. K. Reddy. 1978. Mutagenicity of the pyrrolizidine (Senecio) alkaloid lasiocarpine in the Salmonella/microsome test. Lab. Invest. 38:352.

Mattocks, A. R. 1968. Toxicity of pyrrolizidine alkaloids. Nature (London) 217:723.

Mattocks, A. R. 1969. Dihydropyrrolizine derivatives from unsaturated pyrrolizidine alkaloids. J. Chem. Soc. (London) Sect. C. 1155.

Mattocks, A. R. 1972a. Acute hepatoxicity and pyrrolic metabolites in rats dosed with pyrrolizidine alkaloids. Chem-Biol. Interact. 5:227.

Mattocks, A. R. 1972b. Toxicity and metabolism of Senecio alkaloids. In: Phytochemical Ecology. (J. B. Harbourne, ed.). p. 179. Academic Press, London.

Mattocks, A. R. 1978. Recent studies on mechanisms of cytotoxic action of pyrrolizidine alkaloids. In: Effects of Poisonous Plants on Livestock. (R. F. Keeler, K. R. VanKamper and L. F. James, eds.). p. 177. Academic Press, New York.

Mattocks, A. R. and I. N. H. White. 1970. Estimation of metabolites of pyrrolizidine alkaloids in animal tissues. Anal. Biochem. 38: 529.

Mattocks, A. R. and I. N. H. White. 1971. The conversion of pyrrolizidine alkaloids to N-oxides and to dihydropyrrolizidine derivatives by rat-liver microsomes in vitro. Chem.-Biol. Interact. 3:383.

McCann, C. P., R. J. McLaren, R. L. DeLay and J. K. Matsushima. 1973. Digestive tract cannulas for ruminants. Proc. West. Sect. Amer. Soc. An. Sci. 24:377.

McCann, J., E. Choi, E. Yamasaki and B. N. Ames. 1975. Detection of carcinogens as mutagens in the Salmonella/microsome test: assay of 300 chemicals. Proc. Natl. Acad. Sci. U.S.A. 72:5135.

McCann, J. and B. N. Ames. 1976. Detection of carcinogens as mutagens in the Salmonella/microsome test: assay of 300 chemicals: discussion. Proc. Natl. Acad. Sci. U.S.A. 73:950.

McLean, E. K. 1970. The toxic actions of pyrrolizidine (Senecio) alkaloids. Pharmacol. Rev. 22:429.

Miranda, C. L., H. M. Carpenter, P. R. Cheeke and D. R. Buhler. 1981a. Effects of ethoxyquin on the toxicity of the pyrrolizidine alkaloid monocrotaline and on hepatic drug metabolism in mice. Chem.-Biol. Interact. 37:95.

Miranda, C. L., M. C. Henderson and D. R. Buhler. 1981b. Dietary copper enhances the hepatotoxicity of Senecio jacobaea in rats. Toxicol. Appl. Pharmacol. 60:418.

Miranda, C. L., R. L. Reed, P. R. Cheeke and D. R. Buhler. 1981c. Protective effect of butylated hydroxyanisole against the acute toxicity of monocrotaline in mice. Toxicol Appl. Pharmacol. 59: 424.

Mohabbat, O., M. S. Youono, A. A. Merzad, R. N. Srivastava, G. G. Sedig and G. N. Aram. 1976. An outbreak of hepatic veno-occlusive disease in Northwestern Afghanistan. Lancet 2:269.

Mortimer, P. H. and E. P. White. 1970. Toxicity of some Composite (Senecio) weeds. Proc. 28th N.Z. Weed and Pest Control Conf., N.Z. Agri. Dept., p.88.

Mosher, W. 1979. Use of sheep in control of tansy ragwort. In: Symposium on Pyrrolizidine (Senecio) Alkaloids: Toxicity, Metabolism and Poisonous Plant Control Measures. (P. R. Cheeke, ed.). p. 157. Nutrition Research Institute, Oregon State University, Corvallis.

Muller, D., J. Neeles, E. Deparade and P. Arni. 1980. The activity of S9-liver fractions from seven species in the Salmonella/mammalian-microsome mutagenicity test. Mutation Res. 70:279.

Murphy, S. D. 1975. Pesticides. In: Toxicology: The Basic Science of Poisons. (L. J. Casarett and J. Doull, eds.). p. 408. Macmillan, New York.

Muth, O. H. 1968. Tansy ragwort (Senecio jacobaea), a potential menace to livestock. JAVMA 153:310.

Newberne, P. M. and A. E. Rogers. 1973. Nutrition, monocrotaline and aflatoxin B_1 in liver carcinogenesis. Plant Food Man. 1:23.

Pamukcu, A. M., L. W. Wattenburg, J. M. Price and G. T. Bryan. 1971. Phenothiazine inhibition of intestinal and urinary bladder tumors induced in rats by bracken fern. J. Natl. Cancer Inst. 47:155.

Pierson, M. L., P. R. Cheeke and E. O. Dickinson. 1977. Resistance of the rabbit to dietary pyrrolizidine (Senecio) alkaloid. Res. Comm. Chem. Path. Pharmacol. 16:561.

Ramsdell, H. S. and D. R. Buhler. 1981. High-performance liquid chromatographic analysis of pyrrolizidine (Senecio) alkaloids using reversed-phase styrene-divinylbenzene resin column. J. Chromatogr. 210:154.

Retief, G. P. 1962. The use of crytalline methionine as a treatment for liver damage in race horses. J.S. Afr. Vet. Med. Assoc. 33: 405.

Rogers, Q. R., H. D. Knight and B. A. Gulick. 1979. Proposed method of diagnosis and treatment of pyrrolizidine alkaloid toxicity in horses. In: Symposium on Pyrrolizidine (Senecio) Alkaloids: Toxicity, Metabolism and Poisonous Plant Control Measures. (P.R. Cheeke, ed.). p. 145. Nutrition Research Institute, Oregon State University, Corvallis.

Roitman, J. N., R. J. Molyneux and A. E. Johnson. 1979. Pyrrolizidine alkaloid content of Senecio species. In: Symposium On Pyrrolizidine (Senecio) Alkaloids: Toxicity, Metabolism and Poisonous Plant Control Measures. (P.R. Cheeke, ed.). p. 23. Nutrition Research Institute, Oregon State University, Corvallis.

Shaper, D. F. and R. W. Luetje. 1931. Copper poisoning in sheep and cattle after the control of fruit tree pests by copper sulfate solutions. Vet. Bull. 1:172.

Schoental, R. 1959. Liver lesions in young rats suckled by mothers treated with the pyrrolizidine (Senecio) alkaloids lasiocarpine and retrorsine. J. Bact. Pathol. 77:485.

Schoental, R. 1968. Toxicology and carcinogenic action of pyrrolizidine alkaloids. Cancer Res. 28:2237.

Schoental, R., M. E. Fowler and A. Coady. 1970. Islet cell tumors of the pancreas found in rats given pyrrolizidine alkaloids from Amsinckia intermidia fisch and mey and from Heliotropium supinum L. Cancer Res. 30:2127.

Seawright, A. A., D. P. Steele and R. E. Menrath. 1972. Seasonal variation in hepatic microsomal oxidative metabolism in vitro and susceptibility to carbon tetrachloride in a flock of sheep. Austral. Vet. J. 48:488.

Sher, S. 1971. Drug enzyme induction and drug interactions: literature tabulation. Toxicol. Appl. Pharmacol. 18:780.

Shull, L. R. 1976. Selenium effects and species differences in hepatic metabolism of pyrrolizidine alkaloids and other xenobiotics. Ph.D. Dissertation. Oregon State University, Corvallis.

Shull, L. R., G. W. Buckmaster and P. R. Cheeke. 1976a. Effect of pyrrolizidine alkaloids on liver microsome mixed-function oxidase activity in rats. J. Anim. Sci. 43:1024.

Shull, L. R., G. W. Buckmaster, and P. R. Cheeke. 1976b. Factors influencing pyrrolizidine (Senecio) alkaloid metabolism: species, liver sulfhydryls and rumen fermentation. J. Anim. Sci. 43: 1247.

Shull, L. R. and P. R. Cheeke. 1973. Antiselenium activity of tri-o cresyl phospate in rats and Japanese quail. J. Nutr. 103:560.

Shull, L. R. and P. R. Cheeke. 1975. Tri-o-cresyl phosphate inter-relationships with selenium, vitamin E and sulfur amino acids in the rat: growth, hemolysis and glutathione peroxidase. Nutr. Repts. Intl. 11:39.

Snedecor, G. W. and W. G. Cochran. 1967. Statistical Methods (6th Ed). The Iowa State University Press, Ames, IA.

Steel, R. G. D. and J. H. Torrie. 1960. Principles and Procedures of Statistics, A Biometrical Approach (2nd Ed.). McGraw-Hill Book Co., New York.

Stillman, A. E., R. Huxtable, P. Consroe, P. Kohner and S. Smith. 1977. Hepatic veno-occlusive disease due to the pyrrolizidine (Senecio) poisoning in Arizona. Gastroenterology 73:349.

Stryer, L. 1981. Biochemistry (2nd Ed.). W. H. Freeman and Co., San Francisco.

Swick, R. A. 1980. Dietary factors influencing the toxicity of tansy ragwort (Senecio jacobaea) in animals. M.S. Thesis. Oregon State University, Corvallis.

Swick, R. A. 1981. Senecio jacobaea: toxicity and effects on mineral metabolism in animals. Ph.D. Thesis. Oregon State University, Corvallis.

Swick, R. A., P. R. Cheeke, C. L. Miranda and D. R. Buhler. 1982. The effect of consumption of the pyrrolizidine alkaloid-containing plant Senecio jacobaea on iron and copper metabolism in the rat. J. Toxicol. Environ. Health (in press).

Tandon, B. N., H. D. Tandon, R. K. Tandon, M. Narndranathan and Y. K. Joshi. 1976. An epidemic of veno-occlusive disease of liver in Central India. Lancet. 2:271.

Taylor, A. and N. C. Taylor. 1963. Protective effect of Symphytum officinale on mice bearing spontaneous and transplant tumors. Proc. Soc. Exp. Biol. Med. 114:772.

Thompson, W. R. 1947. Use of moving averages and interpolation to estimate median-effective dose. Bact. Rev. 11:115

Thorpe, E. and E. J. H. Ford. 1968. Development of hepatic lesions in calves fed with ragwort (Senecio jacobaea). J. Comp. Path. 78:195.

Todd, J. R. and R. H. Thompson. 1963. Studies on chronic copper poisoning: II. Biochemical studies on the blood of sheep during hemolytic crisis. Brit. Vet. J. 119:161.

Underwood, E. J. 1977. Trace Elements in Human and Animal Nutrition (4th Ed.). Academic Press, New York.

Warren, F. L. 1970. In: The Alkaloids (R.H.F. Manske, ed.). Academic Press, New York. 12:245.

Watt, J. M. and M. G. Breyer-Brandwijk. 1962. Medicinal and Poisonous Plants of Southern and Eastern Africa. Second Edition. E. S. Livingstone Ltd., Edinburgh.

Wattenburg, L. W. and J. L. Leong. 1965. Effects of phenothiazine on protective systems against polycyclic hydrocarbons. Cancer Res. 25:365.

Wehner, F. C., P. G. Thiel and S. J. vanRensburg. 1979. Mutagenicity of Alkaloids in the Salmonella/microsome system. Mutation Res. 66:187.

Weil, C. S. 1952. Tables for convenient calculations of median effective dose (LD_{50} or ED_{50}) and instructions in their use. Biometrics 8:249.

White, I.N.H., A. R. Mattocks and W. H. Butler. 1973. The conversion of the pyrrolizidine alkaloid retrorsine to pyrrolic derivative in vivo and in vitro and its acute toxicity to various animal species. Chem.-Biol. Interact. 6:207.

Wynder, E. L. 1975. Introductory remarks. Cancer Res. 35:3238.

Yamanaka, H., M. Nago and T. Sugimura. 1979. Mutagenicity of pyrrolizidine alkaloids in the Salmonella/mammalian-microsome test. Mutation Res. 68:211.

APPENDIX

Effect of Microsomal Enzyme Induction on Toxic Responses from Dietary *Senecio jacobaea* and Supplemental Copper in Rats[1,2]

R.D. White and P.R. Cheeke

Oregon State University
Corvallis, OR 97331

[1]Oregon Agricultural Experiment Station Technical Paper No. _____.

[2]This research was supported by Project 156 of the Oregon Agricultural Experiment Station and grant No. 109 from the Committee on Unsponsored Research Utilizing Laboratory Animals, Oregon State University, Corvallis.

Effect of Microsomal Enzyme Induction on Toxic Responses from Dietary *Senecio jacobaea* and Supplemental Copper in Rats

R.D. White and P.R. Cheeke

SUMMARY

The effect of microsomal enzyme induction with phenobarbital (PB) on toxic responses from diets containing *Senecio jacobaea* (SJ) and supplemental copper (Cu) was evaluated in rats. Young male rats were fed diets containing 50 ppm Cu and 0, 5, 7.5 or 10% SJ. Two additional groups receiving 50 ppm added dietary Cu and 0 or 5% SJ were also injected (i.p.) with phenobarbital (PB) three times per week. After 28 days of diet consumption, body weights and average daily gains were decreased for rats fed SJ; the administration of PB had no effect on body weight or average daily gain. Spleen and kidney weights were increased in rats consuming dietary SJ. The administration of PB had no added effect on kidney weights but caused an increase in spleen weights for rats fed 5% SJ. All treatment groups with the exception of one (0%SJ+PB) had similar liver weights with this group having increased liver weights over those for the 5%SJ and 5%SJ+PB groups. Liver pyrrole content was increased for all groups receiving SJ diets. PB administration caused only a slight increase in pyrrole content for SJ-fed rats. It appeared that pyrrole production was only indirectly associated with Cu accumulation in this experiment. Only marginal increases in liver Cu content were observed in the SJ-fed groups with an apparent trend toward Cu accumulation. The results, however, suggested a possible decrease in toxic potency for the SJ used in this study.

INTRODUCTION

Senecio jacobaea (SJ), commonly called tansy ragwort, is a poisonous plant containing pyrrolizidine alkaloids (PA) and is responsible for considerable livestock loss in the U.S. Pacific Northwest and other temperate regions of the world. The primary toxicologic concern is hepatotoxicity resulting from irreversible tissue damage following PA metabolism by the mixed function oxidase (MFO) enzyme system to highly toxic pyrrole (dehydropyrrolizidine) metabolites (Mattocks, 1972a; Mattocks, 1968; Bull et al., 1968). Cattle (Johnson, 1979; Thorpe and Ford, 1968) and horses (Rogers et al., 1979; Bull et al., 1968) are quite susceptible to SJ toxicity while sheep are resistant and have been promoted as biological control agents to remove the plant from infested pastures (Mosher, 1979). It has been shown, however, that sheep are quite susceptible to copper (Cu) toxicity (Todd et al., 1962, Ishmael et al., 1972). Swick et al. (1982) demonstrated liver accumulation of Cu in rats consuming SJ. The relationship between pyrrole production and Cu accumulation in animals has not been determined. Administration of compounds that are known MFO inducers to animals consuming diets containing SJ and moderate levels of Cu should increase the Cu status of the liver if pyrroles are responsible for this effect. Phenobarbital (PB) has been shown to be an effective inducer of the MFO causing increased pyrrole formation from PA (Allen et al., 1972; Mattocks and White, 1971). Some pesticides (Sher, 1971; Wattenburg and Leong, 1965) and dietary constituents (Seawright et al., 1972) that sheep may be exposed to are also known to induce MFO.

The objective of this study was to determine if pyrrole metabolites are responsible for hepatic Cu accumulation in SJ intoxicated rats.

MATERIALS AND METHODS

Five male Sprague-Dawley rats of about 85 g initial weight were assigned to each of 6 treatments: control, control+PB, 5%SJ, 5%SJ+PB, 7.5%SJ and 10%SJ. Copper as $CuSO_4$ was added to each diet at a level of 50 ppm Cu and diets were fed ad libitum for 28 days. Diet compositions are shown in Table 20. In addition to the dietary treatment, the control+PB and 5% SJ+PB groups also received intraperitoneal (i.p.) injections with a solution of sodium phenobarbital (30.0 mg per ml sterile water) at a rate of 50 mg PB per kg body weight administered three times per week. At the end of the 28-day feeding period, rats were sacrificed via cranial fracture and necropsies were performed. Blotted weights of liver, spleen and kidney were measured from each rat. Livers were then stored at -30°C until analyses for Cu and bound pyrrole content could be performed.

Hepatic Cu levels were determined from samples of whole liver. Liver samples were subjected to wet ashing in 1:4 concentrated $HClO_4$: HNO_3 for 6 h at 250°C after 24 h predigestion. Appropriate dilutions were made with .1N HCl and samples analyzed against Cu standards by atomic absorption spectrophotometry. Working standards were found to be within the acceptable range when verified against National Bureau of Standards bovine liver and oyster tissue reference standards. All glassware used in Cu analysis was soaked in 1N 1:1 HCl:HNO_3 for at

least 24 h and rinsed four times with glass distilled water. Feed Cu levels were determined in a similar manner.

Liver tissue from the rats was analyzed for pyrrole content by the method of Mattocks and White (1970). Liver tissue was minced, then homogenized in ethanolic 5% mercuric chloride via a Potter Elvehjem tissue grinder. Color development was with Ehrlich's reagent (3% dimethylaminobenzaldehyde in ethanolic BF_3) and the absorbances at 565 and 625 nm were measured against an ethanol blank. Corrected pyrrole content, expressed as O.D. units/g liver, was determined.

Weekly body weights and feed intakes were also measured. Means between treatments for parameters measured were analyzed by analysis of variance and when significantly different, compared by the Least Significant Difference (LSD) procedure (Steel and Torrie, 1960).

RESULTS AND DISCUSSION

The data in Table 21 show that rats fed SJ had lower body weights and average daily gains than those fed the control diet. These reductions were probably attributed to the toxic effects of the plant as total feed consumption in general was similar among groups and no trends existed that might implicate Cu consumption as a causitive factor. Miranda et al. (1981b) reported similar results concluding that supplementary copper at this level had no significant effect on body weights and feed intake of rats given 5% SJ. A trend toward decreased body weight and average daily gain did appear, however, as the level of dietary SJ was increased. The administration of PB did not compound the effect on body weight or daily gain beyond that imposed

by the incorporation of 5% SJ into the diets.

Organ weights are shown in Table 22. Spleen and kidney weights were significantly increased ($P<.05$) in rats consuming dietary SJ. Swick et al. (1982) demonstrated similar spleen and kidney weight increases for rats consuming 5%SJ and 50 ppm added Cu. The administration of PB had no added effect on kidney weights but caused a significant increase ($P<.05$) in spleen weights for rats consuming 5%SJ. All treatment groups with the exception of the Control+PB group had similar liver weights. The Control+PB group had similar liver weights to the Control, 5%SJ and 5%SJ+PB groups but had significantly increased ($P<.05$) weights to those from the 7.5% and 10% SJ groups. Swick et al. (1982) and Miranda et al. (1981b), however, demonstrated significant increases ($P<.05$) in liver weights for rats receiving 5% SJ compared to controls.

Liver pyrrole content (Table 22) was significantly increased for all groups receiving SJ diets compared to groups receiving the control diet. PB administration caused only a slight increase in pyrrole content for rats receiving the 5% SJ diet. This is consistent, however, with previously reported profiles for pyrrole production and disappearance following preadministration of PB prior to injections of PA. Allen et al. (1972) reported that rats preadministered with PB had significant increases ($P<.001$) in hepatic pyrrole content occurring within one hour after PA injection with a subsequent sharp decline thereafter to a level only slightly higher than that of PA injected rats not receiving PB which was maintained through 48 h post PA administration. Shull (1976) reported a similar trend. Liver in the present

study was collected about 36 h after the final PB administration.

Slight increases in liver Cu content were observed in the SJ-fed groups (Table 22) with an apparent trend toward accumulation as the 5% SJ group consumed 19% less Cu than the Control group and the 5%SJ+PB group consumed 24% less Cu than the Control+PB group. This trend toward Cu accumulation, however, was only marginal compared to the 19-fold increase in liver Cu content for rats fed diets containing 5%SJ and 50 ppm Cu reported by Swick et al. (1982). Bull et al. (1956) reported that sheep consuming Heliotropium europaeum, PA containing plant, demonstrated increased hepatic Cu levels and concluded that this hepatogenous chronic copper poisoning in sheep was directly associated with the liver damage produced by the PA causing predisposition to high liver Cu values. Based on the results with rats it appears that pyrrole production is indirectly associated with Cu accumulation via increased hepatotoxic effects associated with increased pyrrole production. The insignificant increases in hepatic Cu levels in this study, as opposed to the significant increases reported by Swick et al (1982), may have been indicative of decreased SJ toxic potency due to prolonged SJ storage. Dickinson and King (1978) reported a reduction in PA content from 0.16% to 0.012% (dry wt.) after six months SJ storage. Swick (1980) reported survival times of 39, 53 and 57 days for rats fed diets containing SJ that had been stored 1, 14 and 26 months, respectively, again indicating decreased toxicity associated with prolonged SJ storage. The SJ used in the present study had been stored from 7-18 months. As only marginal increases in hepatic Cu levels were observed due to PA

exposure, it was not possible to determine if microsomal enzyme induction has an effect on PA induced Cu accumulation.

TABLE 20. PERCENT COMPOSITION OF RAT DIETS

Ingredient	Dietary Treatments			
	Control	5%SJ	7.5%SJ	10%SJ
Ground Corn	58.5	53.5	51.0	48.5
Soybean Meal	30.0	30.0	30.0	30.0
Sucrose	5.0	5.0	5.0	5.0
Corn Oil	3.0	3.0	3.0	3.0
SJ	0.0	5.0	7.5	10.0
Mineral Mix[a]	3.0	3.0	3.0	3.0
Vitamin Mix[b]	.5	.5	.5	.5
Cu[c]	50 ppm	50 ppm	50 ppm	50 ppm

[a] Jones and Foster Mineral Mix, Nutritional Biochemicals Corp., Cleveland, OH.

[b] Cheeke (1972).

[c] 50 µg/g Cu as $CuSO_4$ was added to each diet.

TABLE 21. FEED CONSUMPTION AND PERFORMANCE[a] FOR RATS FED CONTROL OR SENECIO JACOBAEA (SJ) DIETS CONTAINING 50 PPM ADDED COPPER (Cu) FOR 28 DAYS

Treatment	Total Feed Consumed (g)	Total SJ Consumed (g)	Feed Cu Content (ppm)[b]	Total Cu Consumed (mg)[c]	Average Daily Gain
Control	552±59^{g}	0	63.8±1.4^{e}	35.3±3.9^{fg}	5.8±1.0^{h}
Control+PB[d]	524±70^{fg}	0	63.8±1.4^{e}	33.5±4.4^{efg}	4.7±1.6^{h}
5%SJ	424±118^{ef}	21.4±5.9^{e}	66.7±3.5^{e}	28.5±7.9^{ef}	1.8±1.0^{i}
5%SJ+PB[d]	401±79^{e}	23.0±4.0^{e}	66.7±3.5^{e}	26.7±5.3^{e}	1.9±0.5^{i}
7.5%SJ	469±74^{efg}	35.2±5.5^{f}	82.3±5.2^{f}	38.6±6.1^{g}	1.7±0.5^{i}
10%SJ	450±58^{efg}	45.0±5.8^{g}	73.4±5.4^{ef}	33.0±4.3^{efg}	1.1±0.6^{i}

[a] Values are mean ± SD.

[b] Feed Cu content determined on feed samples atomic absorption.

[c] Consumed Cu values based on atomic absorption determinations of feed sample Cu levels.

[d] Phenobarbital (PB) was administered by injection (i.p.) at a rate of 50 mg/kg, three times per week.

[e,f,g] Means in same column with different superscripts differ ($P<.05$).

[h,i] Means in same column with different superscripts differ ($P<.01$).

TABLE 22. SPLEEN, KIDNEY AND LIVER WEIGHTS[a] AND LIVER COPPER (Cu) AND PYRROLE CONTENT[a] FROM RATS FED CONTROL OR SENECIO JACOBAEA (SJ) DIETS CONTAINING 50 PPM COPPER (Cu) FOR 28 DAYS

Treatment	Organ Weights (% of body weight)			Liver Cu (ppm/dry wt.)	Liver Pyrrole (O.D. Units/g liver)
	Spleen	Kidney	Liver		
Control	.26±.04^{c}	.78±.03^{c}	4.64 ± .43^{cd}	9.4 ± 4.2^{c}	.05±.02^{f}
Control+PB[b]	.23±.02^{c}	.88±.08^{c}	4.97 ± .63^{c}	11.0 ± 4.1^{c}	.05±.03^{f}
5%SJ	.50±.12^{d}	1.14±.22de	4.47 ± .61^{cd}	18.8±11.0^{c}	.33±.07^{g}
5%SJ+PB[b]	.61±.10^{e}	1.09±.04^{d}	4.22±1.07^{cde}	50.4±47.5^{cd}	.38±.10^{g}
7.5%SJ	.62±.12^{e}	1.12±.80de	3.98 ± .80de	31.1±19.0^{cd}	.29±.06^{g}
10%SJ	.70±.03^{e}	1.26±.18^{e}	3.39 ± .39de	68.1±64.4^{d}	.36±.13^{g}

[a] Values are mean ± SD

[b] Phenobarbital (PB) was administered by injection (i.p.) at a rate of 50 mg/kg, three times per week.

[c,d,e] Means in same column with different superscripts differ ($P<.05$).

[f,g] Means in same column with different superscripts differ ($P<.01$).