PYRROLIZIDINE ALKALOIDS IN FOODS

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I. INTRODUCTION

In addition to the many well-known major nutrients (protein, fat, carbohydrate and fiber) and minor nutrients (vitamins, minerals and non-essential compounds), foods contain thousands of naturally present toxic plant compounds. Some are carcinogenic in animals, and thus may be potentially carcinogenic in people. Many of these compounds are commonly termed "nature's pesticides" because they are often toxic to predators, such as insects and animals, thereby conferring a competitive advantage to the plant that produces them. Although these chemicals are in every meal we eat, they have received little attention compared to that given to the relatively minor residues of synthetic chemicals such as polychlorinated biphenyls (PCBs) and pesticides. Our food contains greater than 10 000-fold more natural toxins than the synthetic kind, and

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in terms of metabolic reactions, our bodies are not able to distinguish between the two. Despite the popular notion equating "natural" and "healthy," it is clear that natural toxins pose a far greater health risk than that posed by synthetic chemicals in our foods.

One important and well-known class of naturally occurring chemicals in foods is the pyrrolizidine alkaloids. Pyrrolizidine alkaloids are a large group of compounds; more than 350 pyrrolizidine alkaloids have been isolated from over 6000 plant species. The majority of pyrrolizidine alkaloids are toxic. Many of these have been shown to cause cancer in animals, and are therefore potentially carcinogenic in people.

People become intoxicated by pyrrolizidine alkaloids in a variety of ways. Epidemics of pyrrolizidine alkaloid poisoning have typically occurred when large numbers of people eat foods made from small grains contaminated with seeds from pyrrolizidine alkaloid-containing plants. Other less spectacular poisoning incidents occur from the use of certain dietary supplements and traditional "remedies," direct consumption of pyrrolizidine alkaloid-containing plants, or through residues in food products, such as eggs, meat, milk and honey.

The real extent of human poisoning by pyrrolizidine alkaloids has probably gone woefully underreported. Pyrrolizidine alkaloid poisonings in people have been referred to as an "iceberg disease" because reported cases of pyrrolizidine alkaloid poisoning are probably only a small percentage of the true incidence (Huxtable *et al.*, 1977). This is due to several factors, not the least of which is the difficulty inherent in determining the etiology of a chronic disease that often has a long latency period. In addition, the toxic effects have characteristics similar to those of other diseases, such as chronic alcoholism; pyrrolizidine alkaloids are seldom analyzed in dietary supplements and traditional remedies; and there has been a lack of medical inquiry stemming from the misconception that natural compounds are healthful rather than potentially harmful.

This is not intended to be an exhaustive review of all aspects of pyrrolizidine alkaloids. For that, I recommend Robin Mattock's book *Chemistry and Toxicology of Pyrrolizidine Alkaloids* (Mattocks, 1986). Although it was published more than 15 years ago, it remains the definitive source for in-depth descriptions of nearly all aspects of pyrrolizidine alkaloids.

II. PLANT SOURCES

Pyrrolizidine alkaloids are produced by thousands of species of flowering plants in several higher plant families, but the genera responsible for most of the outbreaks of human poisonings are in Fabaceae (aka Leguminosae),

TABLE I

SOME PLANTS CONTAINING (OR SUSPECTED OF CONTAINING) PAs WHICH HAVE BEEN USED AS EITHER HERBAL MEDICINES OR FOODS

Plant	Medicine (M) or food (F)	Country or region	Reference
Apocynaceae			
Holarrhena antidysenterica	M	Sri Lanka	Arseculeratne *et al.* (1981)
Asteraceae (Compositae)			
Adenostyles alliariae	M	Italy	Sperl *et al.* (1995)
Ageratum conyzoides	M	China	Roeder (2000)
Cacalia decomposita (matarique)	M	United States	Sullivan (1981)
Cacalia hastate, hupensis	M	China	Roeder (2000)
C. yatabei	F	Japan	Hikichi *et al.* (1978)
Chromolaena odoranta	M	China	Roeder (2000)
Eupatorium cannabinum, chinense, fortunei, japonicum	M	China	Roeder (2000)
Farfugium japonicum	M	Japan	Furuya *et al.* (1971)
Liatris punctata	M	Southwestern US	Mead *et al.* (1992)
Ligularia dentata	F	Japan	Asada and Furuya (1984)
Packera candidissma	M	Mexico, Southwestern US	Bah *et al.* (1994)
Petasites japonicus	F, M	Japan	Hirono *et al.* (1973)
Senecio abyssinicus	M	Nigeria	Williams and Schoental (1970)
S. aureus	M	United States	Wade (1977)
S. bupleuroides	M	Africa	Watt and Breyer-Brandwijk (1962)
S. burchelli	F, M	South Africa	Rose (1972)
S. coronatus	M	South Africa	Rose (1972)
S. discolor	M	Jamaica	Asprey and Thornton (1955)
S. doronicum	M	Germany	Roder *et al.* (1980)
S. inaequidens	F	South Africa	Rose (1972)
S. jacobaea (ragwort)	M	Europe	Schoental and Pullinger (1972); Wade (1977)
S. longilobus (*S. douglassi*)	M	United States	Stillman *et al.* (1977); Huxtable (1979)
S. monoensis	M	United States	Huxtable (1980)
S. nemorensis ssp. *fuchsii*	M	Germany	Habs *et al.* (1982)
S. pierotii	F	Japan	Asada and Furuya (1982)
S. retrorsus (*S. latifolius*)	M	South Africa	Rose (1972)
S. vulgaris (common groundsel)	F	Japan	Hikichi and Furuya (1976)
Trichodesma africana	M	Asia	Omar *et al.* (1983)

TABLE I *(continued)*

SOME PLANTS CONTAINING (OR SUSPECTED OF CONTAINING) PAs WHICH HAVE BEEN USED AS EITHER HERBAL MEDICINES OR FOODS

Plant	Medicine (M) or food (F)	Country or region	Reference
Tussilago farfara (coltsfoot)	M	Japan; China?	Culvenor *et al.* (1976)
	M	Norway	Borka and Onshuus (1979)
Boraginaceae			
Anchusa officinalis	M	Europe	Broch-due *et al.* (1980)
Arnebia euchroma	M	China	Roeder (2000)
Cordia myxa	M	China	Roeder (2000)
Cynoglossum amabile, lanceolatum, zeylanicum	M	China	Roeder (2000)
C. geometricum	M	East Africa	Schoental and Coady (1968)
C. officinale	M	Iran	Coady (1973)
Hackelia floribunda (western false forget-me-not)	M	United States	Hagglund *et al.* (1985)
Heliotropium eichwaldii	M	India	Datta *et al.* (1978a, b); Gandhi *et al.* (1966)
H. europaeum	M	India; Greece	IARC (1976)
H. indicum	M	India, Africa, South American, China	Schoental and Coady (1968); Hoque *et al* (1976) Roeder (2000)
H. ramossissimum (ramram)	M	Arabia	Macksad *et al.* (1970); Coady (1973)
H. supinum	M	Tanzania	Schoental and Coady (1968)
Symphytum officinale (comfrey)	F, M	Japan (and elsewhere)	Hirono *et al.* (1978)
S. x uplandicum	F, M	General	Hills (1976)
S. x uplandicum	M	Europe	Roeder *et al.* (1992)
Fabaceae (Leguminosae)			
Cassia auriculata	M, F	Sri Lanka; India	Arseculeratne *et al.* (1981)
Crotalaria albina, assamica, mucronata, sessiliflora, tetragona	M	China	Roeder (2000)
C. brevidens	F	East Africa	Coady (1973)
C. fulva	M	Jamaica	Barnes *et al.* (1964); McLean (1970, 1974)
C. incana	M	East Africa	Schoental and Coady (1968)
C. juncea	M, F	India	Chopra (1933); Watt and Breyer-Brandwijk (1962)
C. laburnifolia	M	Tanzania	Schoental (1968) Coady (1973)
	F	Asia	
C. mucronata	M	Tanzania	Coady (1973)

TABLE I *(continued)*
SOME PLANTS CONTAINING (OR SUSPECTED OF CONTAINING) PAs WHICH HAVE BEEN USED AS EITHER HERBAL MEDICINES OR FOODS

Plant	Medicine (M) or food (F)	Country or region	Reference
C. recta	M, F	Tanzania	Schoental and Coady (1968); Coady (1973)
C. retusa	M, F	Africa; India	IARC (1976); Watt and Breyer-Brandwijk (1962)
C. verrucosa	M	Sri Lanka	Arseculeratne *et al.* (1981)
Orchidaceae			
Liparis nervosa	M	China	Roeder (2000)
Scrophulariaceae			
Castilleja integra	M	Southwestern US	Mead *et al.* (1992)
C. rhexifolia	M	Southwestern US	Stermitz and Suess (1978)
Pedicularis sp.	M	Southwestern US	Schneider and Stermitz (1990)

Updated from Mattocks (1986).

Asteraceae (Compositae) and Boraginaceae (Table I). Pyrrolizidine alkaloid-producing plants are common worldwide, and are considered noxious weeds in many geographical areas. So abundant are pyrrolizidine alkaloids that they are estimated to be in 3% of the world's flowering plants (Winter and Segall, 1989). While alkaloids are found in many parts of the plant, they are typically associated with seeds or fruit, but other parts may also contain pyrrolizidine alkaloids. In fact, the highest recorded pyrrolizidine alkaloid content of any plant, 18% dry weight, was found in the leaves of *Senecio riddellii* (Molyneux and Johnson, 1984).

III. CHEMICAL STRUCTURES OF PYRROLIZIDINE ALKALOIDS

The majority of pyrrolizidine alkaloids, and all of the ones discussed in this review, contain an eight-membered necine base, which can either be saturated or contain a double bond in the 1,2-position (Figure 1). The presence of this double bond is an important determinant in the hepatoxicity of pyrrolizidine alkaloids in that only those with 1,2-unsaturation are hepatotoxic. Other variants of the necine base are also found, such as the

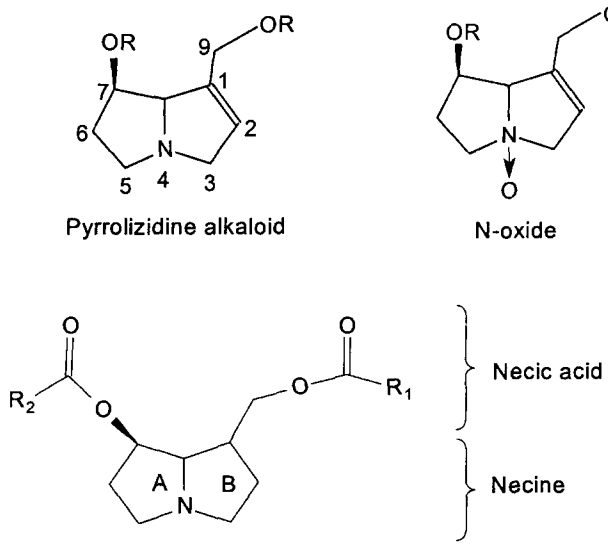


FIG. 1. Chemical structures of the various component features of pyrrolizidine alkaloids and their *N*-oxides.

discontinuous ring structure, otonecine, in petasitenine and senkirkine (Figure 2). The necine moiety is often esterifed to constituents called necic acids, which vary significantly in structure.

Necic acids may be absent (as in retronecine; Figure 2) or they may be present as open esters (heliosupine) or as macrocyclic esters (senecionine, monocrotaline, petasitenine and senkirkine). Other features of necic acids include an epoxide (petasitenine), saturation (monocrotaline) or unsaturated carbon nuclei (senecionine). Pyrrolizidine alkaloids with a cyclic diester moiety are generally more potently toxic, and are more likely to form more cellular DNA cross-links than the other forms (Kim *et al.*, 1993, 1995). Usually coexisting in the plant with pyrrolizidine alkaloids, often in greater quantities, are *N*-oxides, such as indicine *N*-oxide (Figure 2), which often may represent the majority of the total pyrrolizidine alkaloid content. *N*-Oxides usually become reduced to the basic alkaloids during the process of extraction.

IV. PYRROLIZIDINE ALKALOIDS IN FOODS AND HERBAL MEDICINES

Since pyrrolizidine alkaloid-containing plants are distributed worldwide, poisoning incidences are, predictably, seen in many geographical locations.

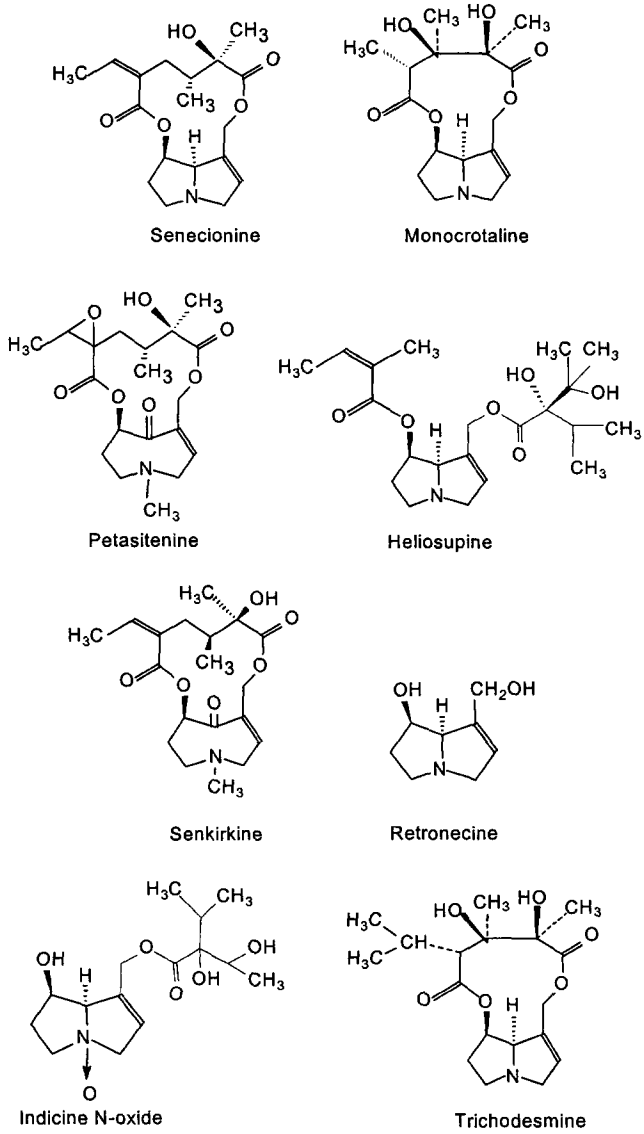


FIG. 2. Chemical structures of several representative pyrrolizidine alkaloids. Some pyrrolizidine alkaloids have necic acids esterified to the necine base. Necic acids may be absent (retronecine), or may be present as open esters (heliosupine) or as macrocyclic esters (senecionine, monocrotaline, petasitenine and senkirkine). Other possible features of necic acids may include an epoxide (petasitenine), saturation (monocrotaline) or unsaturated carbon nuclei (senecionine). *N*-Oxides, represented here by indicine *N*-oxide, usually coexist with the reduced pyrrolizidine alkaloid in the plant.

The majority of reports of outbreaks of pyrrolizidine alkaloid poisoning have largely been limited to third world countries. Generally, these have been outbreaks where hundreds, and sometimes thousands, have been poisoned from eating staple foods made from cereal crops contaminated with seeds from pyrrolizidine alkaloid-containing weeds. More recently, however, along with an increasing reliance on unconventional medicine and the use of herbal supplements and traditional medicines, there has been a sharp rise in the number of poisonings in industrialized countries. A survey of some of the plants reported to be responsible for incidences of human poisonings, whether from foods or medicines, is presented in Table II.

A. PYRROLIZIDINE ALKALOIDS IN FOODS

Numerous sporadic outbreaks of pyrrolizidine alkaloid intoxications have been recorded (Table II). These are largely attributed to consumption of staple small grains contaminated with seeds from *Senecio*, *Heliotropium*,

TABLE II

SOME LARGE-SCALE POISONING OUTBREAKS CAUSED BY PA-CONTAMINATED FOODS OR BY HERBAL MEDICINES

Plant	PA (if identified)	Country or region	Number of cases	Reference
Senecio ilicifolius; *S. burchelli*	Senecionine?	South Africa	80	Willmont and Robertson (1920)
Senecio spp.	–		12	Selzer and Parker (1951)
Heliotropium lasiocarpum	Heliotrine; lasiocarpine	Central Asia	28	McLean (1970)
Crotalaria fulva	Fulvine	West Indies	Endemic	Bras *et al.* (1954); Bras and Hill (1956)
Heliotropium lasiocarpum		Central Asia	61	McLean (1970)
Heliotropium popovii	Heliotrine	Afghanistan	7800	Mohabbat *et al.* (1976); Tandon *et al.* (1978)
Crotalaria nana	Crotananine; cronaburmine	India	67	Tandon *et al.* (1976), Siddiqi *et al.* (1978)
Heliotropium popovii	Heliotrine	Tajikistan	3906	Chauvin *et al.* (1993); Mayer and Luthy (1993)

Adapted from Mattocks (1986).

Crotalaria and other genera of pyrrolizidine alkaloid-containing plants. As with many episodes of food intoxication caused by naturally occurring toxins from plants and fungi, they are often associated with consumption of a contaminated staple food supply that is locally grown.

Pyrrolizidine alkaloids are one of the few classes of natural toxins to be regulated in foods or herbal supplements. In 1992, the German Federal Health Agency established a maximum allowable daily intake of 0.1 μg of pyrrolizidine alkaloids and *N*-oxides in herbal supplements. One μg per day is allowed if the intake is limited to 6 weeks per year (German Federal Health Bureau, 1992; Edgar and Smith, 2000). The United States has no such regulation. Currently, there is no limit on intake of pyrrolizidine alkaloid-containing foods, herbal supplements or other products, or the amount of pyrrolizidine alkaloids that a herbal supplement can contain. Pyrrolizidine alkaloids enter the food chain in a variety of ways – through grain, milk, honey, eggs and herbal medicines. Because these items can at times be contaminated at concentrations that exceed the German regulations, a real risk is posed to human health by pyrrolizidine alkaloids in foods.

1. Pyrrolizidine alkaloids in staple foods

The first report of human poisonings due to pyrrolizidine alkaloids recorded in scientific literature occurred in 1918 in the George District of Cape Province, South Africa (Willmont and Robertson, 1920). Dubbed "*Senecio* disease," it was apparently caused by contamination of cereal grains with seeds of *Senecio ilicifolius* and *S. burchelli*, which were made into bread. The problem appeared to be exacerbated by "old-fashioned" mills that did not efficiently winnow the grain, and the fact that bread was the staple diet of the largely poor populace. The outbreak was responsible for some 80 cases over a 10-year period, most of whom were children. The symptoms, which were often fatal, included nausea, vomiting, acute gastric pain, ascites and hepatic distension, and had an onset of 2 weeks to 2 years or longer. The investigators noted similarities in symptoms and post-mortem signs between this outbreak and Moltendo disease in South Africa, Winton's disease in New Zealand, and Picton disease in Nova Scotia, all diseases in livestock known to be caused by *Senecio*.

Seltzer and Parker (1951) published details of clinical and post-mortem findings of *Senecio* poisoning in Cape Town, South Africa, similarly attributed to contamination of wheat with seeds from *Senecio* sp. Of the 12 cases described, six were fatal. The most common and most constant early symptoms in all patients were abdominal pain and swelling, followed by rapidly developing ascites and hepatomegaly. Surviving victims recalled

that the tainted bread had an abnormal taste, some describing it as "musty," others as "bitter." These investigators noted the similarity of these signs to those of Budd–Chiari syndrome, a hepatic disease characterized by obstruction of the trunk and large branches of the hepatic vein.

An outbreak of veno-occlusive disease (VOD) that was probably caused by consumption of cereals contaminated with seeds of *Crotalaria* sp. containing pyrrolizidine alkaloids occurred in the Sarguja district of India in November–December 1975 (Tandon *et al.*, 1976). Forty-two per cent of the 67 recorded cases died. A follow-up study reported post-mortem signs characteristic of pyrrolizidine alkaloid poisoning, which included changes ranging from acute hemorrhagic centrilobular necrosis, progressive sclerosis to nonportal cirrhosis, and in the terminal phases, occlusion of the terminal veins (Tandon *et al.*, 1977).

A much larger outbreak of VOD occurred in northwestern Afghanistan. This was caused by consumption of bread made from wheat contaminated with seeds of *Heliotropium*, which contained heliotrine (Figure 3) (Mohabbat *et al.*, 1976; Tandon *et al.*, 1978). Approximately 7800 were affected. In a small number of cases, clinical improvement was observed after several months of hospitalization.

Another extensive outbreak of pyrrolizidine alkaloid poisoning occurred in 1992 in southern Tajikistan, caused by consumption of bread contaminated with *Heliotropium popvoii* (Chauvin *et al.*, 1993; Mayer and Luthy, 1993). The outbreak was exacerbated by political instability that led to a blockade of the Farkhar region, causing a 2-month delay of the wheat harvest and subsequent famine. Because of this delay, *Heliotropium* had time to go to seed, and then contaminate the wheat at harvest. In the area affected by the outbreak, a poisoning rate of 4% occurred, and a total of 3906 cases of VOD was recorded. The overall fatality rate was approximately 1.3%. Attack rates favored younger children and adolescents, while the case–fatality rate increased with age. The most dominant alkaloid in samples of contaminated wheat was heliotrine (Mayer and Luthy, 1993).

2. *Pyrrolizidine alkaloids in milk*

Another potential source of pyrrolizidine alkaloids in the human food chain is from the milk of animals that have ingested pyrrolizidine alkaloid-producing plants. The pyrrolizidine alkaloid, jacoline, was detected in the milk of four lactating cows given *Senecio jacobaea* (tansy ragwort) via rumen canula at the dose rate of 10 g kg^{-1} day^{-1} for 2 weeks. The plants, which contained five pyrrolizidine alkaloids, had an average pyrrolizidine alkaloid content of 0.16% dry weight. The dosed cows exhibited marked changes in blood leukocyte count, sorbitol dehydrogenase values, and mild

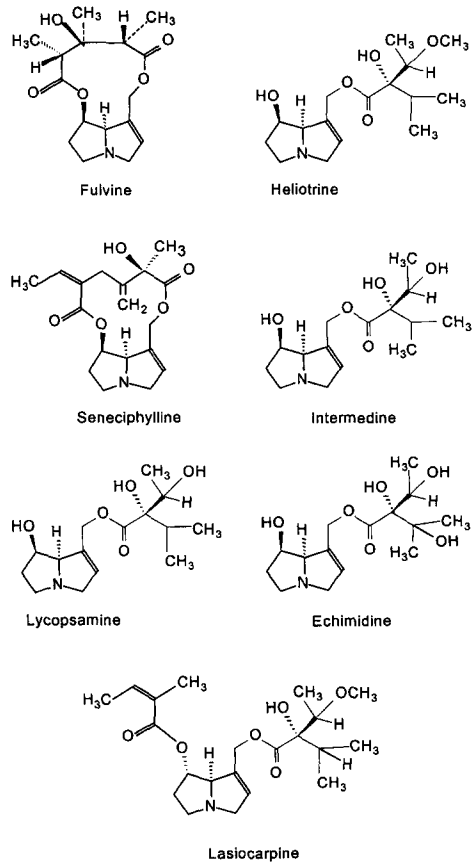


FIG. 3. Selected pyrrolizidine alkaloids found in folk medicines and foods that have been involved in human poisoning incidents. Fulvine, from *Crotalaria fulva*, an ingredient in some "bush teas," is responsible for endemic veno-occlusive disease (VOD) in the West Indies. Heliotrine, from *Heliotropium popvoii* and spp., is the etiological agent of large endemics of VOD in Afganistan and Tajikistan. Seneciphylline, a potent hepatotoxin in *Senecio*-based teas, is extensively used in southwestern United States and Northern Mexico, where it is known under various names. It has also been found in honey. Intermedine is one of the major hepatotoxic alkaloids found in the widely used traditional herb comfrey *Symphytum officinale* and Siberian comfrey *S. uplandicum*. Lycopsamine is another major alkaloid in comfrey. Echimidine was found in commercial Australian honey made from bees pollinating *Echium plantagineurm*. Australian researchers recently found lasiocarpine, among other alkaloids, in commercial eggs at concentrations ranging from 1.2 to 9.7 μg of total alkaloid per egg. Chickens were given a wheat-based feed grain contaminated with *Heliotropium europaeum*. Intermedine, lycopsamine and senecionine were found in *Liatris punctata*, also known as "cachana," "grey feather" and "blazing star." This herb is traditionally used by some Native Americans and Hispanics in the southwestern United States as a diuretic, for throat inflammation, laryngitis, cough and nosebleeds.

liver pathology, consistent with hepatotoxicity. However, no such changes were noted in calves nursing from these cows. Jacoline was found in concentrations ranging from 9.4 to 16.7 μg per 100 mL of milk (Dickinson *et al.*, 1976). Milk from goats fed tansy ragwort (1% body weight per day) caused either a negative or a mildly positive mutagenic response in the *Salmonella*/mammalian microsome (Ames) assay (White *et al.*, 1984). Another study confirmed that transfer of pyrrolizidine alkaloids in milk can cause hepatic toxicity. Rats fed a diet of milk from goats fed tansy ragwort (7.5 ng of pyrrolizidine alkaloid per g dry weight) for 180 days with a calculated total pyrrolizidine alkaloid intake of 0.96 mg per rat had swollen hepatocytes of centrilobular distribution and biliary hyperplasia, indicating hepatic toxicity due to pyrrolizidine alkaloids (Goeger *et al.*, 1982). Importantly, these hepatic signs were similar to those found in rats fed tansy directly at concentrations of 1%, 0.1%, 0.01% and 0.001% (corresponding to pyrrolizidine alkaloid intakes of 39.77, 5.04, 0.52, and 0.05 mg per rat). In this study, no changes were noted in two calves fed the pyrrolizidine alkaloid-containing goats' milk.

3. Pyrrolizidine alkaloids in honey

Honey is another food source found to contain pyrrolizidine alkaloids. Initial investigations found pyrrolizidine alkaloids at relatively low concentrations of 0.3–3.9 ppm in honey produced from bees foraging fields of *S. jacobea* (Deinzer *et al.*, 1977). The type of alkaloids found in the honey – senecionine, seneciphylline, jacoline, jaconine, jacobine and jacozine – reflected those found in the local plant. In one study, *Senecio* pollen counts in honey samples were correlated with pyrrolizidine alkaloid concentrations. Honey that contained *S. jacobea* pollen (15–21 grains g^{-1}) with total alkaloid concentrations of 0.011–0.056 mg kg^{-1} contained jacobine, jacozine, seneciophylline and senecionine. Honey samples containing two grains or less did not contain detectable alkaloids (Crews *et al.*, 1997). These authors did not detect pyrrolizidine alkaloids in honey purchased in retail outlets.

Australian researchers discovered that honey produced from stands of the purple flower *Echium plantagineurm*, known as "Patterson's Curse" or "Salvation Jane," contained a pyrrolizidine alkaloid content ranging between 0.27 and 0.95 ppm (Culvenor *et al.*, 1981). The main alkaloid found in honey obtained from four suppliers in Victoria and New South Wales contained echimidine, with smaller amounts of 7-acetyllycopsamine, 7-acetylintermedine, echiumine, uplandicine, lycopsamine, intermedine (Figure 3) and a novel alkaloid. Given usual patterns of honey consumption and the relatively low concentrations of alkaloids found, there is probably little, if any, human health risk posed by pyrrolizidine alkaloids in honey.

4. Pyrrolizidine alkaloids in eggs

While there have been many instances of poultry being poisoned by pyrrolizidine alkaloids in their feed, one report has indicated that pyrrolizidine alkaloids can enter the human food chain via eggs (Edgar and Smith, 2000). Three flocks of chickens from a small-scale egg producer were given wheat-based feed grain contaminated with *Heliotropium europaeum*, at a concentration estimated to be 0.6% by weight. Analysis of the wheat uncovered the presence of the pyrrolizidine alkaloids supinine, heleurine, heliotrine, europine and lasiocarpine. These pyrrolizidine alkaloids (as well as others, probably as a result of metabolism) were found at concentrations ranging from 1.2 to 9.7 μg per egg.

5. Pyrrolizidine alkaloids in meat

Residues of pyrrolizidine alkaloids have not been found in meat from animals ingesting alkaloid-containing plants. There are several possible reasons for this. These compounds are metabolized into reactive pyrrolic intermediates with relatively short half-lives, which rapidly bind to cellular macromolecules, such as GSH, proteins and DNA. Thus, it is unlikely that sufficiently large amounts of residues of pyrrolizidine alkaloids *per se* would exist in meat products to be of any risk to the consumer. One possible exception to this would be if an animal is slaughtered only hours after grazing on contaminated pasture. In any event, it is not likely that pyrrolizidine alkaloids in meat pose a significant health threat.

B. PYRROLIZIDINE ALKALOIDS IN TRADITIONAL REMEDIES AND MEDICINES

In North America, where crop weeds are controlled by herbicides and foods regularly inspected, the biggest risk of exposure to pyrrolizidine alkaloids comes primarily from so-called herbal "remedies," herbal teas and folk medicines (Huxtable, 1989). In contrast to the sporadic outbreaks of poisonings in many third world countries, poisonings from consumption of pyrrolizidine alkaloid-contaminated botanical folk medicines account for a consistent, regular occurrence of cases.

Taken as a whole, the majority of herbal drinks are harmless and in some cases may even be beneficial. However, some herbal teas that are widely sold in health food stores contain pyrrolizidine alkaloids that have been implicated in many cases of human poisonings.

The serious health risk posed by pyrrolizidine alkaloid-containing plant-based supplements compelled the German Federal Health Bureau to

establish regulations limiting intake. Unfortunately, in the United States, recent legislation has moved in the opposite direction, away from consumer protection. The ironically misnamed "Dietary Supplement Health and Education Act" of 1994, now exempts "natural" food products such as folk medicines and teas from safety regulations that normally apply to prescription drugs. In fact, this law placed dietary supplements into a new category, distinct from food or drugs, that is now exempt from the rules the US Food and Drug Administration used against questionable products. Protected by this legislation, manufacturers and dispensers of herbal supplements are not required to provide information on content, effectiveness (or lack thereof), or possible adverse effects. Furthermore, in the case of botanical supplements, there is usually no reliable information available about how and where the plant was collected, or what part of the plant went into the product. Such factors have a bearing on the content of potentially toxic compounds. Compounded by the commonly held belief that equates "natural" with health and well-being, the popularity of botanicals used for dietary supplements has dramatically increased in industrialized countries.

The occurrence of liver disease resulting from ingestion of herbal teas containing pyrrolizidine alkaloids have been known for some time. There is a longstanding tradition of medicinal herb use in Jamaica and the West Indies (Asprey and Thornton, 1955). In the 1950s, Bras and co-workers reported on endemic VOD in Jamaica (Bras *et al.*, 1954). Liver biopsies, taken mostly from children, showed nonportal liver pathology typical of pyrrolizidine alkaloid ingestion with the key features of inflammation and veno-occlusion with intact hepatic portal triads, termed "nonportal" cirrhosis. More than 50% of the patients affected in Jamaican outbreaks fully recovered after sodium and fluid restriction. In the West Indies, acute VOD, which largely affects children, is associated with consumption of folk medicines made from *Crotalaria fulva*, which contains the pyrrolizidine alkaloid fulvine (Figure 3) (McLean, 1970). These medicines, or "bush teas," which are hot-water infusions prepared from various plants, are generally bitter tasting, and are traditionally given to children as a tonic for ailments as diverse as a cold, teething pain and gastric problems.

A number of reports described clinically evident liver diseases resulting from either short- or long-term use of pyrrolizidine alkaloid-containing herbs. Probably the most popular and widely used pyrrolizidine alkaloid-containing medicinal herb is comfrey (*Symphytum*). Since Greek and Roman antiquity, this herb has been part of the offical pharmacopeia of many cultures, and has been used as a "cure-all" for a variety of ailments and complaints. In fact, the species name *officinale* indicates its place in medieval herbs (Huxtable, 1989). Comfrey and Russian comfrey (*S. uplandicum*) are cultivated throughout Europe, North America and Australia.

The name comfrey may have been derived from the Latin *confirmare*, "to strengthen," owing to its reputed ability to promote overall health and well-being, as well as heal fractured bones more quickly. Other uses for comfrey have included external use for wounds, and internal use for joint inflammation, gout, haematomas, gastritis, diarrhea, rheumatoid arthritis, bronchitis, backache and various allergies (Stickel and Seitz, 2000). Comfrey can also be found in a range of cosmetics and personal-care products, such as shampoo, skin creams, bath oils and various ointments.

Another popular form is a comfrey-pepsin capsule marketed as a digestive aid. According to Huxtable (1989), one preparation claiming to contain comfrey leaves contained 40 mg kg^{-1} pyrrolizidines and 230 mg kg^{-1} pyrrolizidine *N*-oxides, while another claiming to contain comfrey root had a total content of 2900 mg kg^{-1}, consisting of 400 mg kg^{-1} pyrrolizidines and 2500 mg kg^{-1} of the *N*-oxides. As people can take several of these capsules per day, these products represent a major source of exposure to pyrrolizidines.

Comfrey contains a mixture of pyrrolizidine alkaloids, including intermedine, acetylintermedine, lycopsamine, acetyllycopsamine, symphytine, echimidine and symviridine, all of which are hepatotoxic (Huxtable, 1989). The overall alkaloid content of the plant varies from 0.003 to 0.2% for dry leaves and 0.2 to 0.4% for root (Roitman, 1981). These variations are mostly due to species, age of plant, location and season collected. Roitman found 8.5 mg of total alkaloids in a cup of comfrey root tea. Mattocks observed that because the leaf contains a relatively low concentration of alkaloids (which are largely *N*-oxides), and because the acute toxicity in rats of comfrey alkaloids is approximately half that of other pyrrolizidine alkaloids such as heliotrine (Culvenor *et al.*, 1980), it is unlikely that people can be *acutely* poisoned by drinking comfrey tea (Mattocks, 1986). He predicted that a person weighing 60 kg would require 700 cups of comfrey tea (with 8 mg total alkaloids) to be so poisoned. However, poisoning by pyrrolizidine alkaloids is accumulative and generally chronic. Predictably, chronic comfrey use has been involved in a number of poisonings in people.

Ridker *et al.* (1985) reported hepatic VOD and centrilobular necrosis in a 49-year-old woman who was a "heavy consumer" of herbs, vitamins, and natural food supplements and a regular drinker of commercial comfrey tea. Additionally, she took six comfrey-pepsin capsules per day. From these sources, and from the regularity of use, the authors calculated that she had consumed 15 μg kg^{-1} day^{-1} or a minimum of 85 mg of pyrrolizidine alkaloids in the 4-months previous to her admission to hospital.

In another case, a 13-year-old boy was diagnosed with classic VOD who had for 2–3 years regularly consumed comfrey tea as a home treatment prescribed by a homeopath (Weston *et al.*, 1987). The authors were con-

fident that comfrey ingestion was the only plausible explanation for this condition.

That regular use of comfrey products has serious health consequences was confirmed in a later clinical report of hepatic VOD diagnosed in a 47-year-old woman who was given a prescription of comfrey from a homeopathic doctor to cure abdominal pain, fatigue and allergies (Bach *et al.*, 1989). The patient consumed as many as 10 cups of comfrey tea per day, as well as comfrey-pepsin capsules "by the handful."

A fatal case of hepatic VOD associated with regular comfrey use was reported in a 23-year-old man who ate 4–5 steamed comfrey leaves 1–2 weeks before the onset of symptoms (Yeong *et al.*, 1990). Other possible causes of VOD were excluded by these investigators. In another report, McDermott and Ridker described classic VOD associated with heavy comfrey use (McDermott and Ridker, 1990).

Other, less common, medicinal plants that contain pyrrolizidine alkaloids have been reported to cause poisonings. Daily consumption of the pyrrolizidine alkaloid-containing herbal medicines by a pregnant woman resulted in transmission of fatal VOD to her newborn infant (Roulet *et al.*, 1988). The responsible pyrrolizidine alkaloid was identified as senecionine from the herb *Tussilago farfara*, which the mother purchased from a pharmacy. This herb, which is also known as *coltsfoot*, or the Old English designation *coughwort*, has been used since antiquity as a cough suppressant. Assuming a pyrrolizidine alkaloid content in the tea of 0.6 mg per kg dry weight, the authors calculated a cumulative transplacental exposure to the baby of approximately 0.125 mg total pyrrolizidine alkaloids per kg body weight. This was the first report of transplacental transmission of a pyrrolizidine alkaloid in people. It was later reported that the tea involved in this fatal case of poisoning also contained roots of *Petasites officinalis* (Spang, 1989).

Veno-occlusive disease was diagnosed in an 18-month-old boy who had regularly consumed a herbal tea mixture since the 3rd month of life (Sperl *et al.*, 1995). The tea, given to the boy to promote "healthy development," was gathered by the boy's mother in her own garden. It consisted of peppermint leaves and *Adenostyles alliariae* or "*Alpendost*," which the mother misidentified as coltsfoot (also likely to be toxic!). The two plants can easily be confused, especially after the flowering period. Analysis of the tea mixture revealed high amounts of seneciphylline (Figure 3) and its *N*-oxide. The authors calculated that the child had consumed at least 60 μg per kg body weight per day of pyrrolizidine alkaloids over 15 months. Fortunately, the child recovered completely within 2 months following conservative treatment.

Several wildflowers native to the southwestern United States and northern Mexico in common use as folk medicines have been shown to

contain pyrrolizidine alkaloids. There have been several poisoning incidents in which these plants are implicated. A 6-month-old girl regularly given home-brewed tea made from *Senecio longilobus* developed VOD which progressed over 2 months to extensive hepatic fibrosis (Stillman *et al.*, 1977). Tea made from this plant had 3 mg g^{-1} of pyrrolizidine alkaloids, and 10.5 mg g^{-1} of *N*-oxides. By preparing a tea made according to the mother's recipe, the authors calculated that during the 2 weeks before admission, the child received between 70 and 140 mg of pyrrolizidine alkaloids, clearly a toxic dose. Indigenous to the southwestern United States and northern Mexico, and known by Hispanics as "gordolobo yerba," hot-water infusions of *Senecio longilobus* are used as a gargle and cough suppressant.

Another medicinal plant native to the southwestern United States, *Pedicularis*, also known as "betony," "lousewort," "Indian Warrior," and "Elephant Head," among other appellations, contains pyrrolizidine as well as toxic quinolizidine alkaloids through transfer by root parasitism from alkaloid-containing host plants, such as *Senecio triangularis* (Schneider and Stermitz, 1990). The major alkaloid found in this plant was senecionine. This is in stark contrast with the commonly held view that this plant is safe for use by children. For example, this plant was described in the "Bible" of herbal remedies entitled *Medicinal Plants of the Mountain West* (Moore, 1979) in this way:

> Betony is an effective sedative for **children** [my emphasis]. It acts as a mild relaxant ... quieting anxieties and tension ... Large quantities may cause a befuddled lethargy and some interference of motor control ... It wouldn't hurt to test a particular collection before administering freely, since the potency of various species is variable.

Castellija sp., or Indian paint brush, a native southwestern plant that has some traditional medicinal use, may contain pyrrolizidine alkaloids through root parasitism from another medicinal pyrrolizidine alkaloid-containing plant, *Liatris punctata* (Mead *et al.*, 1992). Also known as "cachana," "grey feather" and "blazing star," *Liatris* is used as a diuretic, to treat throat inflammation and laryngitis, and as a cough suppressant. Burning *Liatris* root is inhaled as a cure for nosebleeds and tonsillitis (Moore, 1979). *Liatris punctata* was found to contain senecionine, intermedine and lycopsamine, in addition to a novel open-diester pyrrolizidine alkaloid, punctanecine (Mead *et al.*, 1992). Another medicinal Indian paint brush, *C. rhexifolia*, contains pyrrolizidine alkaloids (Stermitz and Suess, 1978).

Senecionine and other hepatotoxic pyrrolizidine alkaloids and *N*-oxides (0.36% in aerial parts, 0.76% in roots) were found in *Packeria candidissima*, a Mexican medicinal herb commonly used to treat kidney ailments, ulcers,

and for its antiseptic properties (Bah *et al.*, 1994). Among Mexicans in rural and urban areas of the state of Chihuahua and by Hispanics in southwestern United States, *Packeria* is commonly known as "chucaca," "lechuguilla de la sierra," "té de milagros" and "hierba de milagro."

There are several other reports of fatal and non-fatal liver disease resulting from use of various pyrrolizidine alkaloid-containing herbal products (Lyford *et al.*, 1976; McGee *et al.*, 1976; Margalith *et al.*, 1985; Jones and Taylor, 1989).

V. TOXICITY OF PYRROLIZIDINE ALKALOIDS

Many pyrrolizidine alkaloids are carcinogenic in a number of animal models. (Schoental, 1968; Hirono, *et al.* 1973, 1978), although there are not sufficient data to conclude that they are carcinogenic in people. Much of the quantitative data on the toxicity of pyrrolizidine alkaloids are from shorter-term lethality studies. These data can give some indication about the relative toxicity of various pyrrolizidine alkaloids. There is a considerable difference in the toxicity among pyrrolizidine alkaloids (Table III). For example, the more potent alkaloids to which people are exposed, such as trichodesmine, senecionine and seneciphylline, have acute lethal toxicity (i.e. LD_{50}) values of 25, 50 and 77 mg kg^{-1}, respectively, while for heliotrine, lycopsamine and heliotrine *N*-oxide the LD_{50} values are 296, >1000 and *c.* 5000, respectively (Table III). Monocrotaline is in the middle range of these toxins.

Similarly, there is a considerable range of susceptibility among animal species to pyrrolizidine alkaloids. Acute lethal toxicity of retrorsine, a

TABLE III

COMPARATIVE ACUTE TOXICITY OF SOME PYRROLIZIDINE ALKALOIDS

Alkaloid	LD_{50} (mg kg^{-1}, i.p.)[a]
Trichodesmine	25
Senecionine	50
Seneciphylline	77
Lasiocarpine	77
Monocrotaline	109
Heliotrine	296
Lycopsamine	>1000
Heliotrine *N*-oxide	*c.* 5000

[a]Male rats, intraperitoneal (i.p.) administration.

Adapted from Mattocks (1986) and references therein.

12-membered α,β-unsaturated pyrrolizidine alkaloid similar to senecionine, varies from 34 mg kg^{-1} in rats to 279 mg kg^{-1} in quail and over 800 mg kg^{-1} in guinea pigs (White *et al.*, 1973). Like most other toxins, a major factor in species susceptibility to pyrrolizidine alkaloids is the ability of that animal to metabolize the parent compound into the active, electrophilic intermediate. Generally, susceptible animals form more of the reactive pyrrole than do resistant animals. Some evidence indicates that pyrrolizidine alkaloids may be degraded and hence detoxified by rumen microflora in resistant species.

Postulated as a possible etiology of Indian childhood cirrhosis, which is characterized by excessive hepatic copper accumulation (Morris *et al.*, 1994; Aston *et al.*, 1996), copper and pyrrolizidine alkaloids have been shown to exhibit true hepatotoxic synergism in a number of animal models. In rats, retrorsine and copper given concurrently result in significantly greater mortality, liver pathology and hepatic copper accumulation compared to that seen when either of these agents was administered alone (Morris *et al.*, 1994). Likewise, heliotrope hepatotoxicity in sheep was markedly enhanced by concurrent or subsequent administration of copper (Howell *et al.*, 1991). Those authors noted that hepatic copper concentrations were higher in sheep given heliotrope + copper compared to those given the same amount of copper alone.

Serum copper concentrations have been used as a noninvasive indicator for monocrotaline-induced cardiopulmonary toxicity (Molteni *et al.*, 1988). Retrorsine passing to rat neonates via breast milk results in the accumulation of hepatic copper, an impairment of the rise in serum ceruloplasmin, and a decrease in hepatic metallothionein and serum albumin levels (Aston *et al.*, 1996). The authors suggested that accumulation of liver copper and reduction of copper-binding proteins could result in an increase of free copper that might enter into pro-oxidant Fenton-type reactions, resulting in the generation of oxygen free radicals. The mechanism of the synergy between copper and pyrrolizidine alkaloids probably centers around the fact that both compete for the same pool of cellular glutathione (GSH). Copper uptake and incorporation into metallothionein is intimately associated with reduced GSH, and GSH is generally thought to act as a protectant against oxidant damage (Freedman *et al.*, 1989). In fact, depletion of GSH potentiates metal toxicity in a variety of animals and cell systems (Freedman *et al.*, 1989). Similarly, GSH depletion resulted in an increase of the release of active pyrroles from rat liver, which are then available for macromolecular alkylation and toxicity (Yan and Huxtable, 1995).

As a group, pyrrolizidine alkaloids are hepatotoxic, but depending on the alkaloid, other extra-hepatic effects may result from ingestion. One important distinction of pyrrolizidine alkaloid poisoning is that it is

progressive, once it has been initiated by exposure to even a single moderate dose. The most prevalent pyrrolizidine alkaloid-caused diseases in people are hepatic VOD, pulmonary hypertension and cor pulmonale, or congestive right heart failure (Huxtable, 1990). Hepatotoxicity commonly results from pyrrolizidine alkaloids found in *Senecio* species, such as senecionine, retrorsine, seneciphylline and riddelliine, while cardiopulmonary toxicity is more likely to result from *Crotalaria* alkaloids, such as monocrotaline. Trichodesmine, an alkaloid very similar to monocrotaline (Figure 2), is a neurotoxin (Yan *et al.*, 1995).

In people, the most common clinical sequela is VOD caused by an occlusion of the smaller branches of the hepatic vein due to endothelial proliferation and medial hypertrophy (Huxtable, 1989). This occlusion leads to centrilobular congestion and a pooling of blood. Clinically, VOD can be divided into an acute, a subacute and a chronic phase.

The acute phase is characterized by a rapid onset of nausea, emesis, abdominal pain, distension, portal hypertension, reduced urinary output, hepatomegaly and ascites (Stillman et al., 1977; Sperl et al., 1995). In this phase, poisoning victims may either expire, recover completely (especially if pyrrolizidine alkaloid ingestion is discontinued), or progress to the subchronic and chronic phases (Stillman *et al.*, 1977). The subchronic phase involves persistent hepatomegaly and recurrent ascites. The chronic phase consists of cirrhosis and liver failure, and may be delayed for months or years following exposure. There is no specific antidote for pyrrolizidine alkaloid poisoning, except supportive treatment.

Aside from being occasionally seen as a result of chemotherapy and bone marrow transplantation, VOD is diagnostic for pyrrolizidine alkaloid poisoning. It has a poor long-term prognosis, and death may occur anywhere from 2 weeks to over 2 years following exposure. Children are more vulnerable than adults to VOD.

A small number of pyrrolizidine alkaloids, of which monocrotaline is the best example, result in cardiopulmonary toxicity. The pathology and pulmonary toxicology of monocrotaline is well studied. As with other pyrrolizidine alkaloids, the toxicity depends upon hepatic metabolic activation, which, in this case, results in the formation of monocrotaline pyrrole, or dehydromonocrotaline. Unlike hepatotoxic pyrrolizidine alkaloids such as senecionine, monocrotaline exerts its primary toxicity in an organ distant to the location of metabolic activation. The lung appears not to activate monocrotaline to any appreciable extent. Monocrotaline had no effect when perfused through isolated rat lungs, but induced pulmonary toxicity when first perfused through isolated liver (Lafranconi and Huxtable, 1984). The selectivity of monocrotaline for the lung is dependent upon the binding of the monocrotaline pyrrole by red blood

cells, where it is stabilized during transport to the lung (Wilson *et al.*, 1992). Toxicity to the lung proceeds through an inflammatory response, for which platelet activation plays a role. In animals, this results in pulmonary hypertension in which an abrupt elevation in pulmonary arterial pressure occurs, and acute cor pulmonale. This pathology, while not known to occur in people exposed to monocrotaline, closely follows acute respiratory distress syndrome (ARDS), a syndrome known to be caused by a variety of environmental agents.

As mentioned earlier, diagnosis pyrrolizidine alkaloid poisoning is often difficult, especially when the disease takes a chronic course. There are several factors that confound a correct diagnosis. These include the varying interval between ingestion and development of symptoms, and that health care providers may not query the patient about herbal supplement use. Unfortunately, there is little general appreciation that natural "organic" supplements and tonics may indeed be harmful. Furthermore, pyrrolizidine alkaloid-related diseases can easily be confused with other pathologies. For example, chronic hepatic cirrhosis induced by pyrrolizidine alkaloids is clinically indistinguishable from that caused by alcohol, infection, or any number of other etiologies. Veno-occlusive disease has been confused with viral hepatitis (Datta *et al.*, 1978a). Pyrrolizidine intoxication may also have a few symptoms in common with Reye's syndrome, as was documented in the fatal poisoning case of a 2-year-old child (Fox *et al.*, 1978).

Despite the fact that pyrrolizidine alkaloids are known to be carcinogenic in animals, and are genotoxic in a variety of short-term *in vitro* and *in vivo* systems, there is insufficient evidence to conclude that long-term exposure to pyrrolizidine alkaloids causes cancer in people.

VI. METABOLISM OF PYRROLIZIDINE ALKALOIDS

Like thousands of other "pro-toxicants" such as aflatoxin B_1, benzo[a]pyrene, and dimethylnitrosamine, pyrrolizidine alkaloids are not toxic *per se*, but must first be metabolized by endogenous enzymes following ingestion of the plant material. Several classes of enzymes act on pyrrolizidine alkaloids. In mammals, the most important examples are the cytochromes P450 (CYP). Cytochromes P450 are a large group of hemoprotein enzymes that are present in greatest quantities in the liver, but are also found in the lung, kidney, brain and other organs. CYPs utilize reduced NADPH + H^+ as a cofactor together with NADPH cytochrome P450 reductase to reduce active site iron to the ferrous form to catalyze insertion of oxygen into the substrate. Hundreds of CYP isoforms have been isolated, each having various substrate specificities.

Metabolic conversion of pyrrolizidine alkaloids results in several possible products with varying degrees of toxicity. The three main phase I enzymatic reactions acting on pyrrolizidine alkaloids in the liver are: hydrolysis, resulting in the formation of a free necic acid and necic base; *N*-oxidation, to form the *N*-oxides, which are of modest toxicity (this pathway is therefore considered a detoxification); and dehydrogenation to form reactive and toxic pyrroles, also called dehydroalkaloids (Figure 4). As with many other proximal, reactive intermediates (such as the aflatoxin 8,9-epoxide, and benzo[*a*]pyrene diol-epoxide), pyrroles are electrophilic and inherently unstable, and react quickly with endogenous nucleophilic macromolecules.

The current hypothesis for the mechanism for production of the pyrrolic intermediate involves an initial CYP-mediated hydroxylation of the pyrrolizidine alkaloid at C-8, producing a chemically unstable carbinolamine, which spontaneously loses the hydroxyl group, then a proton, giving the dehydropyrrolizidine (Mattocks, 1986; Rajski and Williams, 1998) (Figure 5). There are several fates for these reactive pyrroles, which have a half-life of a few seconds.

In liver tissue from people and rodents, CYP 3A4 is an important isoform catalyzing dehydrogenation. This isoform is the most prevalent in human liver (approximately 60% of total), and is inducible by compounds such as barbiturates, dexamethazone and erythromycin (Guengerich, 1989). This enzyme has broad substrate specificity; indeed, approximately half of the drugs currently on the market are substrates for CYP 3A4 (Ueng *et al.*, 1997). The prototype activity of CYP 3A4, which is the basis of diagnosing 3A4 activity of various tissues, is the oxidative conversion of the calcium channel blocker nifedipine (NF) to its main metabolite, dehydronifedipine. Good evidence of the critical role of CYP 3A4 in pyrrolizidine alkaloid activation was provided by the observation that two mechanism-based inhibitors of human liver CYP 3A4, gestodene and triacetyloleandomycin, inhibited the conversion of senecionine to dehydrosenecionine and senecionine *N*-oxide in human liver microsomes (Miranda *et al.*, 1991).

N-Oxidation of pyrrolizidine alkaloids appears to be catalyzed by CYP 2C11 and to a lesser extent by CYP 3A. In hepatic microsomal preparations from guinea pig, *N*-oxide formation is also catalyzed by flavin-containing monooxygenases (FMO) (Williams *et al.*, 1989). The formation of the toxic pyrroles and detoxified *N*-oxides occurs by different pathways. This conclusion has been reached largely from the observation that plant *N*-oxides are not activated to pyrroles in microsomal preparations *in vitro*. The actual mechanism of *N*-oxide formation has not been elucidated.

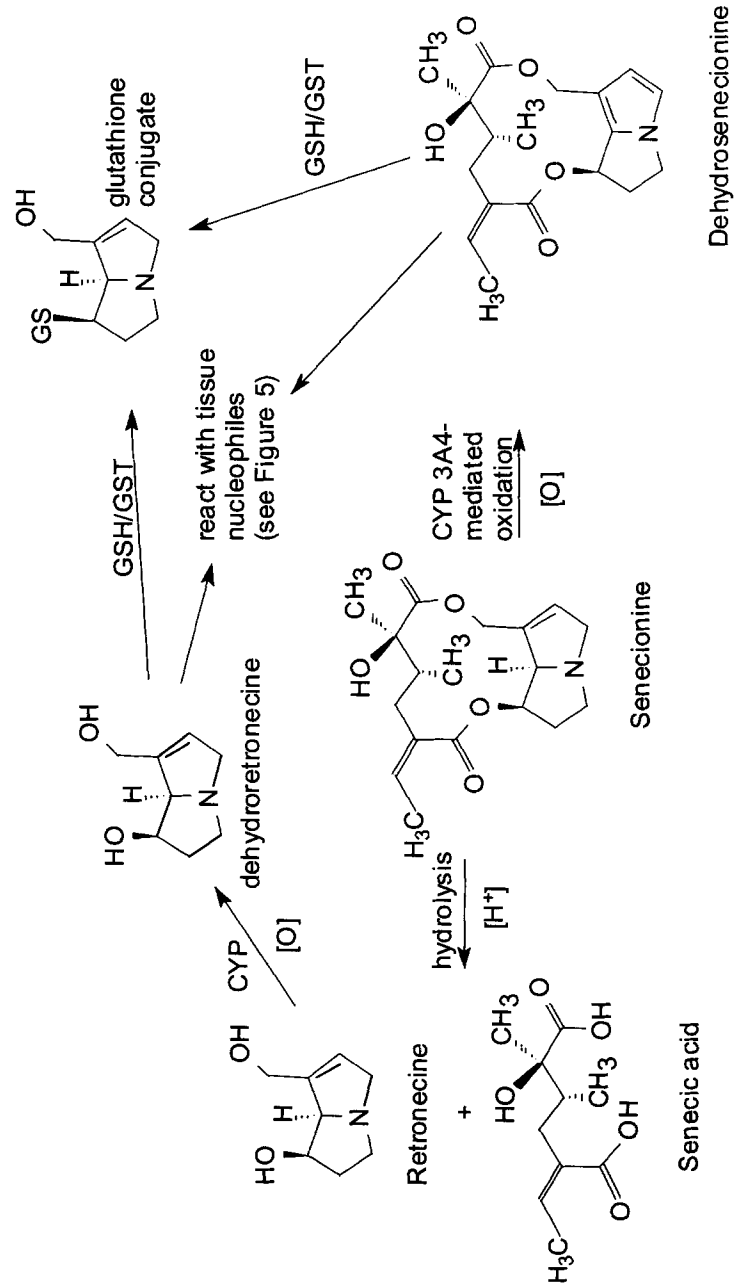
An important route of detoxification is mediated by the universal detoxifying enzyme, glutathione *S*-transferase (GST), to produce various glutathione (GSH) conjugates that are water soluble and hence more easily excretable than the parent compound (Figure 4). Glutathione *S*-transferases are a group of cytosolic homo- and heterodimeric proteins that catalyze the detoxification of a large number of compounds which, like pyrrolizidine alkaloids, are metabolized to electrophilic intermediates (Boyer, 1989). Like the CYPs, the GSTs are a multienzyme family with varying substrate specificities. Which GST isoforms are involved in catalysis of pyrrolic detoxification has not yet been elucidated. Various GSH conjugates were frequently the major detoxified products of pyrrolizidine alkaloids in isolated, perfused rat liver (Yan *et al.*, 1995). Activated pyrroles may polymerize, resulting in detoxified products.

A variety of cellular nucleophiles are known to react with pyrrolic pyrrolizidine alkaloids and hence may be involved in detoxifying Sn1-type reactions. The thiols GSH and cysteine (Robertson *et al.*, 1977), and thiol resins (Glowaz *et al.*, 1992) efficiently react with pyrroles. Dietary cysteine resulted in protection against pyrrolizidine alkaloid-induced hepatoxicity compared to control rats without cysteine (Miranda *et al.*, 1982). Glutathione and cysteine, but not methionine, competed with λ-phage DNA for pyrrolic cross-linking, indicating that thiols with a free sulfhydryl group are sufficiently reactive to compete with DNA for reaction with pyrroles (Coulombe *et al.*, 1999).

VII. MECHANISM OF TOXIC ACTION

Enzymatic oxidation of pyrrolizidine alkaloids to the pyrrole results in the production of a bifunctional intermediate with reactive, electrophilic centers at C-7 and C-9 by conjugation of the pyrrole nitrogen lone pair. These electrophilic centers react with a variety of nucleophilic cellular macromolecules, possibly the most critical of which are various nucleoside bases in DNA, resulting in the formation of cross-links between two strands of DNA (DNA–DNA cross-links) or between one strand of DNA and some cellular protein (DNA–protein cross-links; DPCs) (Figure 5). It has been postulated that nucleophilic substitution at C-7 is favored over C-9 owing to greater stablilization of the secondary carbonium ion at C-7 (Huxtable and Cooper, 2000). In cultured cells, various pyrrolic pyrrolizidine alkaloids form an approximately equal proportion of these cross-links, and are inherently DNA repair-resistant (Kim *et al.*, 1993, 1995).

A number of reports strongly support the hypothesis that the formation of DNA cross-links is an important molecular event in the toxicity of


glutathione conjugate
GSH/GST
GSH/GST
react with tissue nucleophiles (see Figure 5)
dehydroretronecine
CYP
[O]
CYP 3A4-mediated oxidation
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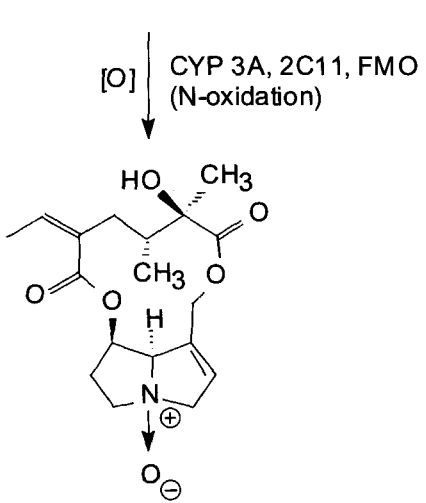
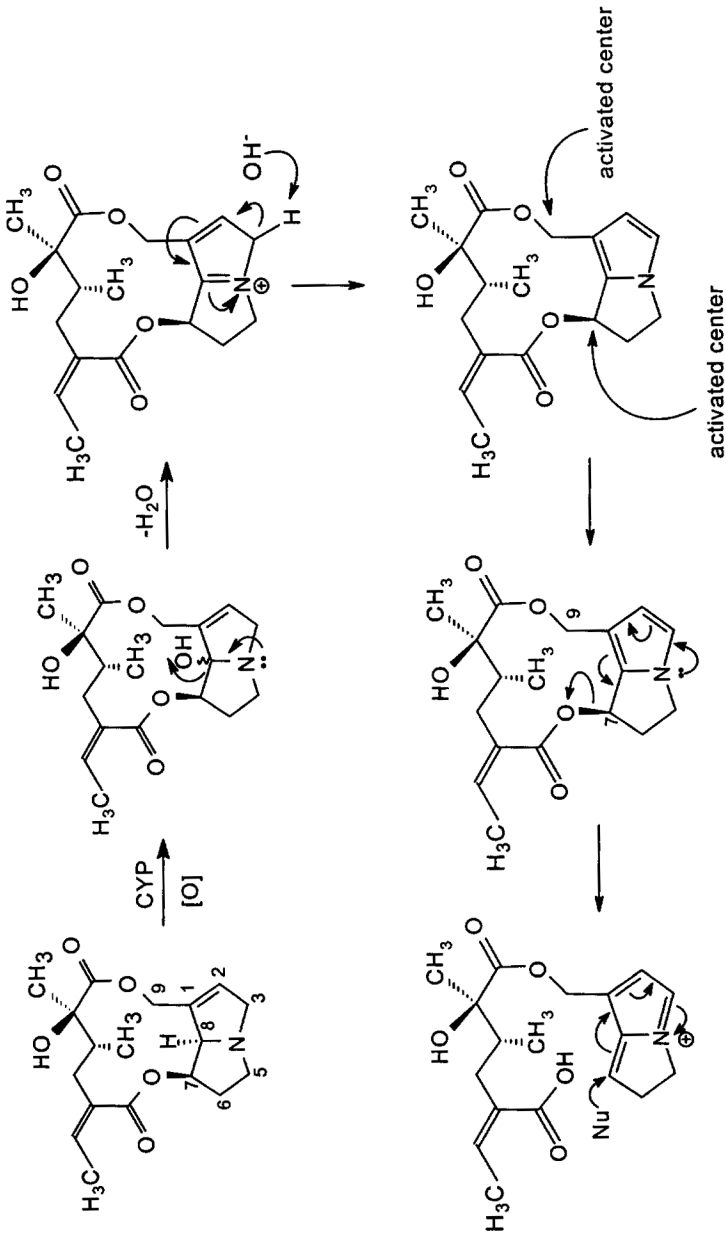
Senecionine
Dehydrosenecionine
hydrolysis
[H+]
Retronecine
+
Senecic acid



Senecionine N-oxide

FIG. 4. Metabolic fate of a representative pyrrolizidine alkaloid senecionine showing the various phase I and phase II reactions. Alkaloids are metabolized by a variety of enzymes, the most important of which are the cytochromes P450 (CYP). Activated pyrrolic intermediates may react with glutathione (GSH) under catalysis of glutathione *S*-transferase (GST), resulting in a water-soluble conjugate that is easily excreted. Pyrrolic intermediates may also form cross-links with tissue nucleophiles, such as DNA and proteins, resulting in toxicity. Alternatively, the pyrrole may be detoxified by CYP or flavin monooxygenases (FMO) to *N*-oxides, or hydrolyzed to retronecine and senecic acid.


CYP
[O]
-H2O
OH-
activated center
activated center
Nu

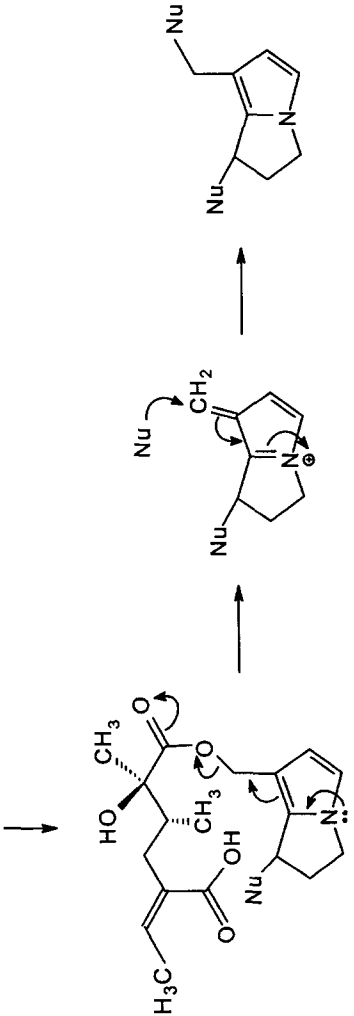


FIG. 5. Postulated sequential mechanism of cytochrome P450-(CYP)-mediated oxidative activation of senecionine, and formation of a cross-link. Generation of the pyrrolic intermediate results in activation to electrophilic centers at the C-7 and C-9 by conjugation of the pyrrole nitrogen lone pair. Once formed, the activated, bifunctional pyrrolic intermediate can form cross-links with cellular nucleophiles (Nu), such as DNA, and protein, such as actin. It has been postulated that nucleophilic substitution at C-7 is favored over C-9 owing to greater stablilization of the secondary carbonium ion at C-7. Adapted from Huxtable and Cooper (2000) and Rajski and Williams (1998).

pyrrolizidine alkaloids. For example, the degree to which various pyrrolic pyrrolizidine alkaloids cause cytotoxic end points such as megalocyte formation and inhibition of colony formation correlates with the formation of DNA cross-links in cultured mammalian cells (Kim *et al.*, 1993). Megalocytosis is seen in the liver of animals exposed to pyrrolizidine alkaloids. It is a condition where cells have an unusually large cellular and nuclear volume, due to the antimitotic effect of the pyrrolizidine alkaloids where biosynthesis of cellular precursors occur, but without formation of mitotic spindles and mitosis (Figure 6). Another report demonstrated that DNA cross-link formation by pyrrolizidine alkaloids interferes with DNA replication. Dehydrosenecionine (DHSN) and dehydromonocrotaline (DHMO) interrupt the polymerase chain reaction amplification of a segment of pBR322 (Kim *et al.*, 1999). This implies that cross-linking by activated pyrrolizidine alkaloids is functionally significant in the cell.

There is a significant difference in cross-link potency among pyrrolizidine alkaloids. In a series of structure–activity studies, it was found that structural features, most notably the presence of α,β-unsaturation and a macrocyclic diester, confer potent cross-link and megalocytic activity to pyrrolizidine alkaloids in cultured bovine kidney (MDBK) cells (Kim *et al.*, 1993, 1999). In this system, macrocyclic pyrrolizidine alkaloids with α,β-unsaturation (seneciphylline, senecionine, riddelliine and retrorsine) were significantly more potent DNA cross-linkers and inducers of megalocytosis than was the α,β-saturated monocrotaline. The open diester pyrrolizidine alkaloids (latifoline and heliosupine) were less potent than monocrotaline, and the simple necine base retronecine was the least potent of any of these. Ironically, indicine *N*-oxide, which had demonstrated activity against acute leukemia in clinical trials, but was discontinued because of myelosupression and severe hepatotoxicity in human subjects (King *et al.*, 1987), did not induce any measurable DNA cross-links (Kim *et al.*, 1993).

The rank order of relative DNA cross-link potencies is often reflected in the animal toxicity of these pyrrolizidine alkaloids. For example, the acute toxicities in rats of senecionine, seneciphylline and retrorsine are approximately equal, but significantly higher than that of monocrotaline (Huxtable and Cooper, 2000).

Chemically reactive pyrrolic pyrrolizidine alkaloids share a common pyrrolic substructure with reductively activated bifunctional mitomycins, such as mitomycin C, which preferentially cross-link 5′-CG sequences within DNA (Woo *et al.*, 1993). There is conflicting evidence as to whether activated pyrrolizidine alkaloids share similar cross-linking base or DNA sequence specificity. Several DNA bases have been shown to be involved in covalent interactions and/or cross-links by pyrrolic pyrrolizidine alkaloids. For example, dehydroretronecine reacts with purine and pyrimidine

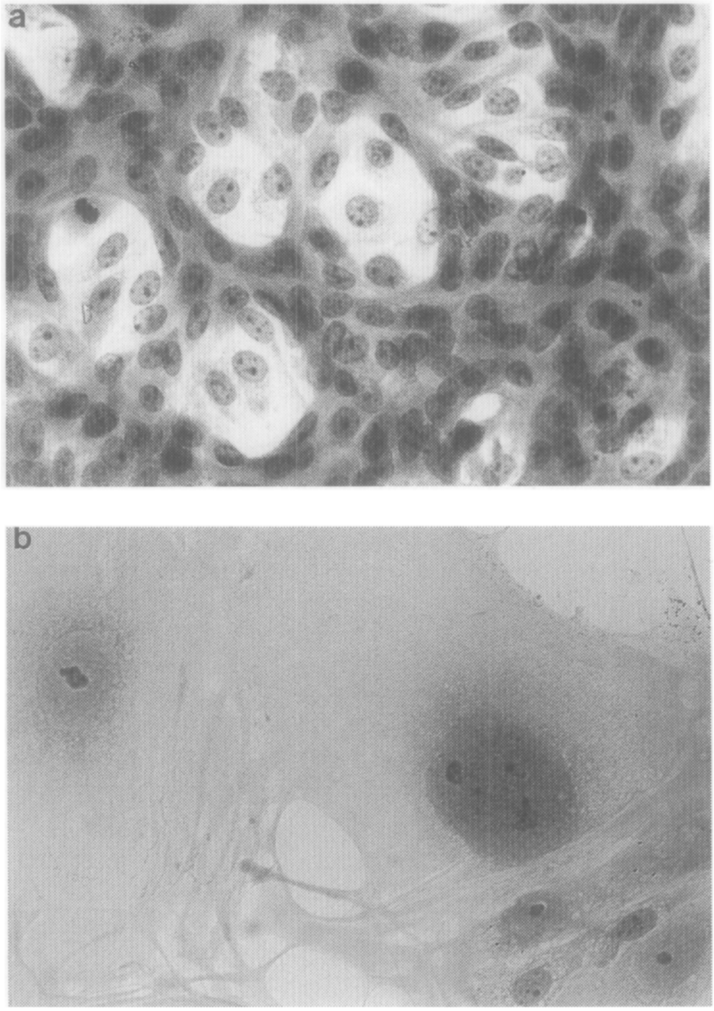


FIG. 6. Phase-contrast photomicrographs of pyrrolizidine alkaloid-induced megalocytosis in Madin–Darby bovine kidney (MDBK) cells. Cells were cultured with either (a) vehicle (dimethylsulfoxide, DMSO) or (b) seneciphylline (500 μM) for 2 h, washed, supplemented with fresh medium, then cultured for 6 weeks (×360). Reproduced from Kim *et al.* (1993), by permission.

nucleosides such as the N^2 of deoxyguanosine (dG) and N^6 of deoxyadenosine (dA). The O^2 sites of uridine and deoxythymidine or thymidine (dT) have also been identified as targets (Robertson, 1982; Wickramanayake *et al.*, 1985). Dehydromonocrotaline and dehydroretrorsine preferentially cross-

linked dG-to-dG at a 5′-CG sequence in synthetic duplex DNA (Weidner *et al.*, 1990). Dehydromonocrotaline was shown to cross-link at the N-7 position of guanine in a 35 bp fragment of pBR322 with a preference for 5′-GG and 5′-GA sequences. However, because these authors examined only alkylation at guanyl residues, no light was shed on whether DHMO alkylated at other sites (Pereira *et al.*, 1998).

Our laboratory has recently shown that the pyrrolic pyrrolizidine alkaloids dehydrosenecionine, dehydroretrorsine and dehydromonocrotaline had little, if any, discernible sequence specificity when cross-linked to a series of synthetic ^{32}P end-labeled oligonucleotides of varying DNA sequence (Coulombe and Rieben, 2002). These oligonucleotide targets were 14- or 24-base poly dA-T that had different central base sequences such as 5′-d(CG), 5′-d(GC), 5′-d(TA); 5′-d(CGA), 5′-d(GCA) or 5′-d(TAA). Similar model oligonucleotides have been used to determine possible sequence recognition of other bifunctional electrophiles, such as mitomycin C (Weidner *et al.*, 1990).

Less is known about the characteristics of the pyrrolizidine alkaloid-induced DNA–protein cross-link. Initial studies from our laboratory showed that a major protein released by DNAse I treatment of DNA–protein complexes purified from pyrrolizidine alkaloid-treated bovine kidney cell (MDBK) nuclei had a molecular weight of *c.* 43 kD, was acidic (pI ≈ 4.5) and had a two-dimensional electrophoretic pattern similar to that of cells treated with cisplatinum, a benchmark bifunctional cross-linker known to cross-link DNA with actin (Kim *et al.*, 1995). A follow-up study using an anti-actin multiple antigen peptide (MAP) antibody confirmed that actin is the major protein involved in pyrrolizidine alkaloid-induced DNA–protein cross-links in human breast carcinoma (MCF-7) and in Madin–Darby bovine kidney (MDBK) cells (Coulombe *et al.*, 1999). The cross-link pattern by two pyrrolic pyrrolizidine alkaloids dehydrosenecionine and dehydromonocrotaline was similar to that of cisplatinum as well as mitomycin C, a pyrrolic anti-cancer drug, although these benchmark compounds were more potent cross-linkers than the pyrrolizidine alkaloids (Figure 7).

The involvement of actin in pyrrolizidine alkaloid-induced DPCs is a reasonable expectation due to the abundance of this protein in the nuclear matrix. Actin is also a target for cross-linkers such as cisplatinum and trivalent chromium (Miller *et al.*, 1991), and mitomycin C (Coulombe *et al.*, 1999). Inasmuch as the megalocytic and antimitotic effects of pyrrolizidine alkaloids coincide with cross-linking potency (Kim *et al.*, 1993), it is plausible that the antimitotic action of pyrrolic pyrrolizidine alkaloids *in vitro* and *in vivo* may be explained, at least in part, by their ability to cross-link DNA with actin. In addition to actin, other protein

targets that were later identified in dehyrdomonocrotaline-treated cultured pulmonary artery endothelial cells included galectin-1 and protein-disulfide isomerase (Lame *et al.*, 2000). The functional significance of a monocrotaline cross-link to these other proteins is not known.

VIII. CONTROL OF PYRROLIZIDINE ALKALOIDS AND FUTURE PROSPECTS

Usual practices of treating cereal crops with herbicides to reduce weed infestation and subsequent quality assurance steps such as grain inspection can help prevent large-scale outbreaks of poisoning due to contamination of pyrrolizidine alkaloid-containing seeds or plants in staple food. Modern winnowing techniques readily separate toxic *Heliotropium*, *Senecio* and *Crotalaria* seeds from cereal grain, at least to acceptable tolerances. However, it is likely that drought, famine and political instability will again conspire to produce another large-scale outbreak of human poisonings.

Periodic exposure to small amounts of pyrrolizidine alkaloids in foods such as milk, honey and eggs is also likely to occur. University-based outreach and information programs may be one mechanism by which producers can become better educated on ways to minimize potential pyrrolizidine alkaloid contamination in their products. For example, honey producers may benefit from learning if plants their bees are likely to pollenate contain pyrrolizidine alkaloids. They can then move their hives to other fields, thereby preventing or reducing contamination.

The many poisoning cases resulting from consumption of "safe" and "natural" supplements emphasizes the urgent need for greater awareness of the potential adverse health effects of herbal products. Years of traditional use of a botanical supplement is no guarantee of safety. This caveat is especially true in the case of pyrrolizidine alkaloids and other chemicals, whose effects can be delayed months or even years. Such chronic toxicoses are notoriously difficult to diagnose.

Public health campaigns against the use of *Crotalaria fulva* have been very effective in reducing VOD in Jamaica (Mattocks, 1986). In the United States, unfortunately, much of the popular literature on herbal supplements, as well as nearly all of the product "fact sheets" provided by health food stores (produced by the companies that produce the herb products), are misleading. In the case of pyrrolizidine alkaloid-containing plants like comfrey and coltsfoot, new legislation is needed to protect consumers.

Clearly, existing laws, such as the Dietary Supplement Health and Education Act Congress, need to be significantly modified in the interest of consumer protection. In an article entitled "Herbal Roulette," Consumer's

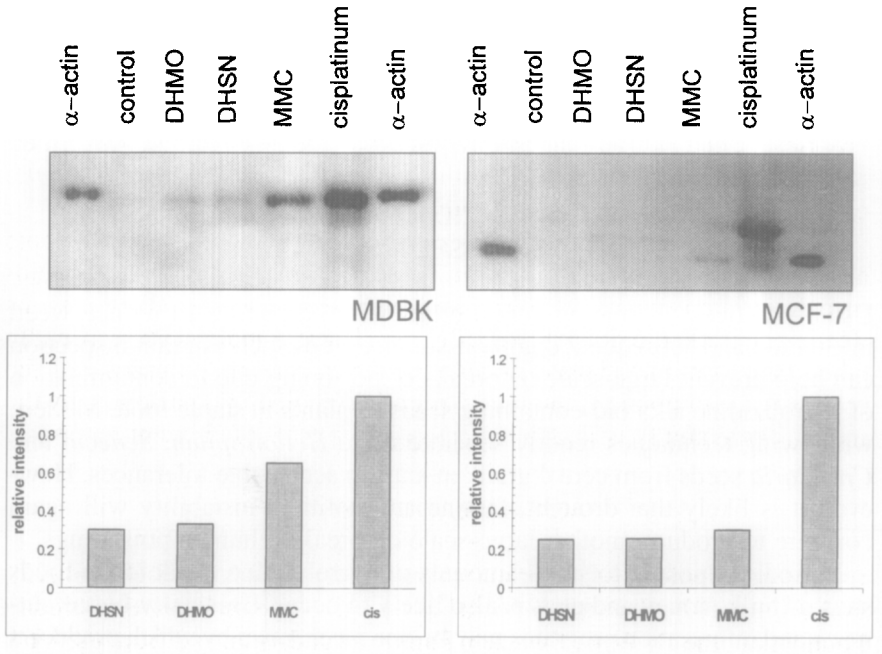


FIG. 7. Western immunoblots showing the presence of actin isolated from proteins released from purified DNA–protein cross-links in Madin–Darby bovine kidney (MDBK; left) and human breast carcinoma MCF-7 (right) nuclei treated with DMSO vehicle (control), and the pyrrolic pyrrolizidine alkaloids dehydrosenecionine (DHSN), or dehydromonocrotaline (DHMO). Anti-cancer cross-linking agents mitomycin C (MMC), or cisplatinum are included for reference. Released proteins that were originally cross-linked to 40 μg nuclear DNA were loaded onto SDS-PAGE (11% running, 4.5% stacking) gels, electorphoresed, transferred to membranes, then probed with a monoclonal anti-actin multiple antigen peptide (MAP) antibody that recognizes an epitope conserved in all actin isoforms. α-Skeletal actin was used as a standard (α-actin). Relative densitometric intensities of actin signals were normalized to that observed from cisplatinum-induced DNA–protein cross-links. Reproduced from Coulombe *et al.* (1999) by permission.

Union recommended that changes to the law should include: clearer disclaimers, in large type, stating that any claims of safety and efficacy are strictly the claims of the manufacturer, and have not been confirmed by the FDA; consistent manufacturing and content standards; and banning clearly dangerous supplements (Consumer's Union, 1995). Another worthwhile step would be to adapt the approach of the German Commission E, which was established to evaluate the safety and efficacy of herbal supplements on the basis of clinical trials and comprehensive risk assessment analyses. The Commission has published more than 320 monographs on herbs

(Klepser and Klepser, 1999). In the absence of these steps, people in the United States will continue to receive misinformation about the usefulness and safety of their "health" foods. In any event, an industry and government-sponsored campaign to increase awareness among herbalists, natural food proprietors, and Native American shamans on the deadly potential of their products will help. A few high-profile product liability cases may be the only impetus to motivate the industry to self-regulate.

Medical providers and users of herbs should be aware that young people are especially susceptible to the toxic effects of pyrrolizidine alkaloid-containing supplements. Medical providers should also query their patients on their possible use of home remedies and herbal supplements, especially when there are unexplained symptoms. An increased recognition of the relatively uncommon symptoms of pyrrolizidine alkaloid poisoning by the medical community will also increase the accuracy of incident reporting.

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