Toxic and Teratogenic Alkaloids of Western Range Plants

Richard F. Keeler

Livestock that graze in the range areas of the western United States are in a hazardous toxicologic environment. Many plants, which may be part of the routine diet of these livestock, readily produce acute toxicosis or teratogenic effects. Nitrogen-containing compounds, especially alkaloids, are the active principles in many of these botanically-unrelated plants. These alkaloids represent a variety of unrelated structural types common only in the classic sense that alkaloids are nitrogen-containing and biologically highly active. Recent work in the author's laboratory has established the teratogenic potential of three range genera—*Veratrum*, *Lupinus*, and *Astragalus*. Three teratogens, all steroidal alkaloids, have been isolated and structurally elucidated from *Veratrum* and shown to be responsible for the natural cyclopic effects. Experimental work suggests a similarity between the *Lupinus* and *Astragalus*-induced malformations and malformations induced by the highly active nitrogen-containing lathyrogens.

Studies of poi onous plant effects on livestock have historically centered around determining what plants were responsible for deaths among animals ingesting the plants (Kingsbury, 1964; Muenscher, 1951). On the basis of plant identity and clinical signs of mildly poisoned animals, efforts were made to alter grazing patterns or to acquire a suitable antidote to cope with the problem. Fundamental studies on isolating active compounds and investigating the physiologic and pharmacologic effects were often not performed, or were done on related plants of the genus by organic chemists and pharmacologists interested in potential drugs. Correlation of the two sets of data has been slow.

While most knowledge deals with effects on the ingesting animal (Kingsbury, 1964; Muenscher, 1951; Watt and Breyer-Brandwijk, 1962), recent studies in the author's laboratory have been directed at the teratogenic effect of range plants—that is, malformations in offspring from animals ingesting the plants. The historical information described deals primarily with the former, while the current research described deals with the chemistry of teratogenic effects being studied in this laboratory.

HISTORICAL SURVEY OF TOXIC EFFECTS OF ALKALOIDS FROM RANGE PLANTS

Hundreds of species of poisonous plants populate the ranges upon which livestock graze in the western United States (Kingsbury, 1964; Muenscher, 1951). Hundreds of other plants serve as useful livestock feed. Toxicity and teratogenicity problems center around the uneven distribution of these two kinds in grazing areas. Poisonous plants often predominate and, because of limited availability of useful forage, become a significant portion of the diet of range animals. Two closeup examples of predominant distribution of certain poisonous plants are shown in Figure 1. Livestock are reasonably selective in their eating habits and usually selectively graze plants that have little or no toxicity. However, many exceptions exist.

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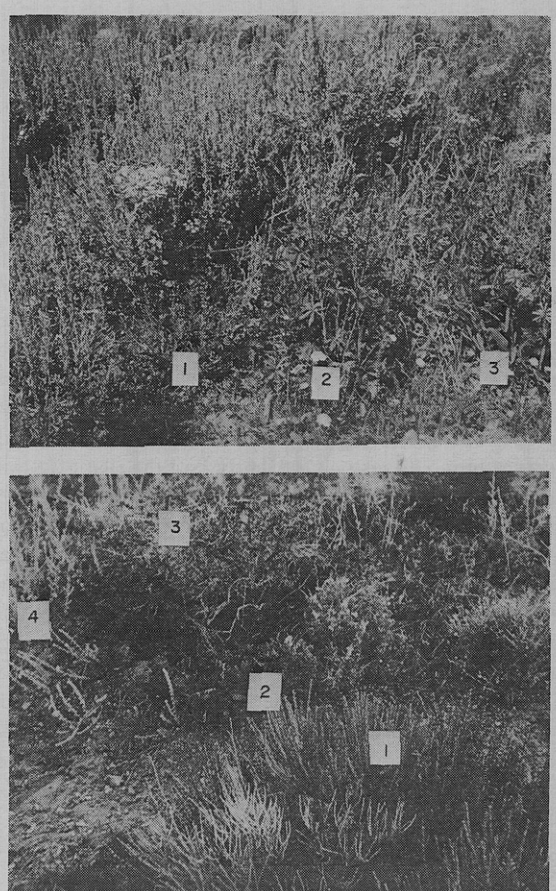

Figure 1. Closeups of heavy distribution of poison plants

Top: 1. *Lupinus*, 2. *Delphinium*, 3. *Helenium*
Bottom: 1. *Tetradymia*, 2. *Astragalus*, 3. *Sarcobatus*, 4. *Halogeton*

Deaths from poisonous plant ingestion in range animals vary from a few to as many as a few thousand (Kingsbury, 1964; Muenscher, 1951). Deaths in the thousands are not uncommon when animals are grazing in areas heavily populated by *Lupinus* sp. (lupine), *Astragalus* and *Oxytropis* sp. (loco), *Delphinium* (larkspur), *Halogeton*, and other genera.

Figure 2. *D. barbeyi*, **a plant containing polycyclic diterpene alkaloids**

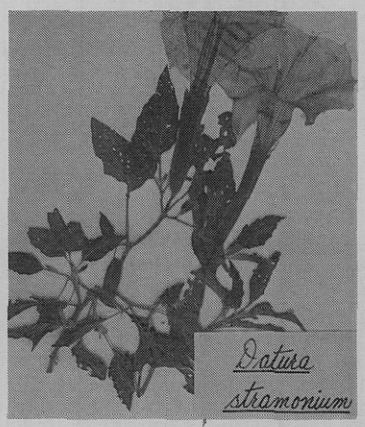

Figure 4. *D. stramonium*, **a plant containing tropane alkaloids**

The deleterious compounds of poisonous plants, where known, represent several chemical classes. Extensive reviews on poisonous plants are available that cite original literature investigating chemical classes (Kingsbury, 1964; Muenscher, 1951; Watt and Breyer-Brandwijk, 1962). Alkaloids are among the most potent of the classes of compounds found in poisonous plants, and are, perhaps, the most important because of widespread distribution.

POLYCYCLIC DITERPENE CLASS

Alkaloids of the polycyclic diterpene class are found in the *Aconitum* and *Delphinium* genera (monkshood and larkspur) (Cook, 1950; Cook and Beath, 1952; Kingsbury, 1964; Muenscher, 1951). Larkspur accounts for more cattle deaths than any other poisonous plant (Kingsbury, 1964). Sheep and horses are much less susceptible. *D. barbeyi* (Figure 2), one of the most dangerous species, is succulent and grows at high elevations in moist areas. Other members of the genus are found even in dry sagebrush ranges. Livestock evidently find the plant desirable since they readily eat it even when other forage is available. Among the complex alkaloids found in *Aconitum* and *Delphinium* is lycoctonine, whose structure (Przybyeska and Marion, 1956) is shown in Figure 3. *D. barbeyi* contains this and other diterpenoid alkaloids (Cook, 1950; Cook and Beath, 1952). The pure alkaloids induce toxicity signs in laboratory animals (Cook, 1950) similar to those in plant-poisoned domestic livestock. Signs include (Kingsbury, 1954; Muenscher, 1951; *U.S. Dept. Agr.*, 1963) loss of muscular control, salivation, trembling, rapid and weak respiration and heart action, bloating, and ultimate respiratory paralysis.

TROPANE CLASS

Alkaloids of the tropane class are found in members of the *Atropa*, *Datura*, and *Hyoscyamus* genera (Fodor, 1967; Kingsbury, 1964; Muenscher, 1951). The latter two are responsible for significant numbers of plant poisonings in livestock even though the plants are evidently distasteful (Kingsbury, 1964; Muenscher, 1951). *D. stramonium*, or jimsonweed (Figure 4), is representative of this group. A large, coarse annual herb with large, tubular flowers, it is widely distributed throughout the United States, especially the southwest. Atropine, scopolamine, and hyoscyamine are the common tropane alkaloids from *Datura* and other members of this group (Fodor, 1967; Kingsbury, 1964; Muenscher, 1951). Hyoscyamine structure is shown in Figure 5. The toxicity of plant parts is high, since alkaloid concentration is high. While the signs of the plant poisoning vary somewhat with the relative concentration of the various alkaloids, they include (Kingsbury, 1964; Muenscher, 1951) all signs characteristic of the individual tropane alkaloids such as thirst, vision disturbance, flushed skin, hyperirritability of the central nervous system, delirium, elevated temperature, rapid and weak heartbeat, convulsions, coma, and death.

PYRIDINE CLASS

Alkaloids of the pyridine class are found in members of *Conium*, *Lobelia*, and *Nicotiana* genera (Kim, 1965; Kingsbury, 1964; Muenscher, 1951). *C. maculatum* is shown in Figure 6. These genera are widely distributed throughout the United States and are commonly available to grazing livestock. Hemlock, Indian tobacco, and wild and cultivated tobaccos are common names of these plants which are all distasteful to animals, but cause animal poisonings under adverse circumstances. The pyridine alkaloids they contain are typified by coniine, lobeline, and nicotine, whose structures (Kim, 1965; Kingsbury, 1964) are shown in Figure 7. Knowledge of the toxicity of these plants dates to antiquity and, on the basis of symptoms, perhaps Socrates was poisoned by an extract of *Conium* (Kingsbury, 1964). Signs of *Conium* poisoning (Kingsbury, 1964; Muenscher, 1951) include nervousness,

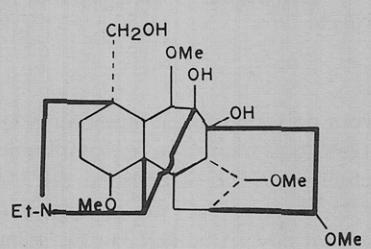

Figure 3. Structure of the diterpenoid alkaloid, lycoctonine

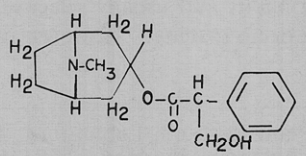

Figure 5. Structure of the tropane alkaloid, hyoscyamine

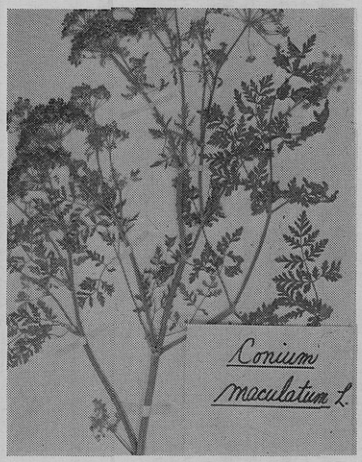

Figure 6. *C. maculatum*, **a plant containing pyridine alkaloids**

Figure 8. *S. ambrosioides*, **a plant containing pyrrolizidine alkaloids**

trembling, ataxia, dilation of pupils, weak heart beat, coldness of extremities, coma, and death from respiratory failure. Signs of *Lobelia* intoxication (Kingsbury, 1964; Muenscher, 1951) are sluggishness, salivation, diarrhea, anorexia, coma, and death. Signs of intoxication of *Nicotiana* (Kingsbury, 1964; Muenscher, 1951) include shaking and twitching of muscles, weakness, rapid weak pulse, cold extremities, paralysis, and death.

PYRROLIZIDINE CLASS

Alkaloids of the pyrrolizidine class are found in members of the *Amsinkia*, *Crotalaria*, *Senecio*, and other genera (Kingsbury, 1964; Muenscher, 1951). These common plants are distributed throughout range areas, are hepatotoxic to livestock, and the alkaloids they contain are established as the responsible compounds (Culvenor and Smith, 1966; Fowler, 1968; Kingsbury, 1964; Schoental, 1963; Warren, 1966). *S. ambrosioides* is shown in Figure 8. Schoental (1963) reports that the hepatotoxic activity is associated with alkaloids of the pyrrolizidine class when they are open or cyclic esters of 1-hydroxymethyl-1,2-dehydro-7-hydroxypyrrolizidine with mono- or dicarboxylic branched-chain acids One of the many active alkaloids (Schoental, 1963) from these plants is retrorsine (Figure 9).

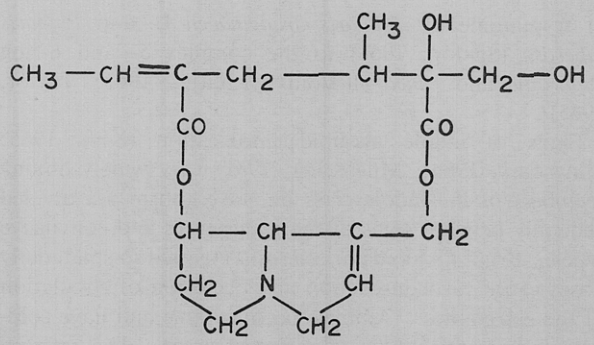

Figure 9. Structure of the pyrrolizidine alkaloid, retrorsine

Excessive doses of plant or alkaloid produce death from acute liver damage and lung edema. Lower doses give rise to the typical hepatotoxicity with acute effects possible some years after ingestion. Livers may be deformed and nodular with varying degrees of fibrosis. Hepatoma or other liver tumors are rather common in this insidious form of the disease (Fowler, 1968; Kingsbury, 1964; Schoental, 1963).

Symptoms of acute poisoning include reduced appetite, depression, yellow discoloration of mucus membranes, a peculiar unpleasant, sweetish odor emanating from the skin, and general debility (Fowler, 1968; Kingsbury, 1964; Schoental, 1963).

INDOLE CLASS

While alkaloids of the indole class occur in a variety of plants, recognized sources of serious proportions for livestock toxicosis are from ingesting grasses or grains parasitized by fungi of the *Claviceps* and possibly *Fusarium* genera (Kingsbury, 1964; Muenscher, 1951). It is the fungi themselves from which the alkaloids are derived. The cultivated and wild grasses parasitized include cultivated wheat, rye, and barley, wild wheat and ryegrasses, various bromes, reed, fescue, and bluegrasses (Kingsbury, 1964; Muenscher, 1951). Structures of alkaloids (Kim, 1965; Saxton, 1968; Schlittler, 1965; Taylor, 1965) from this source are closely related to lysergic acid (Figure 10). Indole alkaloids from all known sources vary remarkably in structure (Kim, 1965) retaining at a minimum only the indole ring system in common with one another (Saxton, 1968) and vary from the simple indole derivative

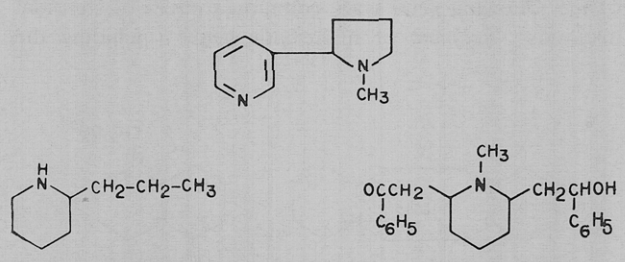

Figure 7. Structures of the pyridine alkaloids nicotine (top), coniine (bottom left), and lobeline (bottom right)

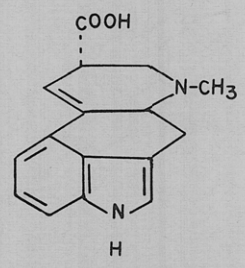

Figure 10. Structure of the indole alkaloid, lysergic acid

Figure 11. *C. caseana*, **a plant containing isoquinoline alkaloids**

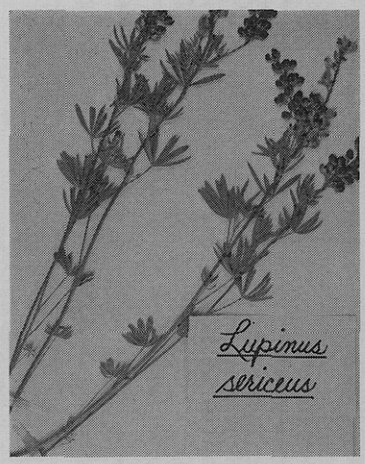

Figure 13. *L. sericeus*, **a plant containing quinolizidine alkaloids**

of tryptamine of *Phalaris arundinacea* L. and *Phalaris tuberosa* (Saxton, 1968) to the complex 5- and 6-ring *Rauwolfia* and *Vinca* alkaloids (Schlitter, 1965; Taylor, 1965).

Signs of indole alkaloid intoxication (Kim, 1965; Kingsbury, 1964; Muenscher, 1951) vary widely among members of the indole class but in ergotism are divided generally between signs of the gangrenous and convulsive types. Ergot alkaloids or sclerotia ingested in small daily doses cause vasoconstriction and occlusion of circulation of the extremities. Animals become lame and have coldness and insensitivity in the affected areas. The reduced circulation ultimately causes gangrene in the affected extremities. Convulsive ergotism apparently results from ingesting larger amounts of ergot daily. The signs are hyperexcitability, belligerency, trembling, and incoordination. Accelerated heart rate, periods of kicking and tetanic rigidity, followed by death within a few days, usually occur in severely poisoned animals.

ISOQUINOLINE CLASS

Alkaloids of the isoquinoline class are found in a wide variety of genera (Jeffs, 1967; Kingsbury, 1964; Manske and Ashford, 1954) of which *Corydalis* and *Dicentra* (fitweed and Dutchman's breeches) are perhaps the most important as plants causing livestock poisonings. *C. caseana* (Figure 11) is a succulent perennial found in watercourses in mountain ranges of the west, but rarely abundantly. It is very palatable and may be sought and eaten freely by livestock. The toxicity of both *Corydalis* and *Dicentra* species is in the alkaloid fraction (Kingsbury, 1964; Miller, 1931), although pharmacologic information on the effect of individual alkaloids on domestic animals is nonexistent. Many isoquinoline alkaloids have been isolated from both species (Jeffs, 1967; Kingsbury, 1964; Manske and Ashford, 1954). Two of the most recent (isolated from *C. caseana*) for which structures have been elucidated are caseamine and caseadine (Chen *et al.*, 1968). The latter is shown in Figure 12. Signs of poisoning include depression, uneasiness, twitching, convulsive clonic spasma, muscular rigidity, variable respiration, and biting movements (Kingsbury, 1964; Muenscher, 1951).

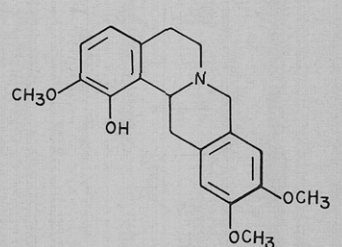

Figure 12. Structure of the isoquinoline alkaloid, caseadine

QUINOLIZIDINE CLASS

Alkaloids of the quinolizidine class are found in members of the *Lupinus*, *Cytisus*, *Baptisa*, *Laburnum*, and *Sophora* genera (Bohlmann and Schuman, 1967; Kim, 1965; Kingsbury, 1964; Leonard, 1960; Muenscher, 1951). Of this group, the lupines are responsible for the most common toxicosis of sheep on western ranges (Kingsbury, 1964). *L. sericeus* is shown in Figure 13. Over 100 species of lupine occur in the grazing areas of the United States. Some of these species are considered desirable forage, but *L. sericeus*, *L. caudatus*, and several others are markedly toxic. The acute toxicoses they produce are due to the quinolizidine alkaloids they contain (Kingsbury, 1964; Muenscher, 1951). Two alkaloids common to members of the lupine genera are lupinine, a single, and sparteine, a double quinolizidine heterocyclic system (Figure 14).

Acute toxicosis (Kingsbury, 1964; Muenscher, 1951) common to the United States varies considerably among the species, evidently because of the variability in the distribution of individual alkaloids among them. Usually, the signs include nervousness, difficult breathing, loss of muscular control, excess salivation, convulsions, coma, and death.

STEROIDAL CLASS

Alkaloids of the steroidal class are found in a variety of botanically-unrelated plants (Fieser and Fieser, 1959; Heftmann, 1967; Kingsbury, 1964; Kupchan and By, 1968; Kupchan *et al.*, 1961; Schreiber, 1968). Species of the following genera are common sources of steroidal alkaloids: *Solanum* or nightshade genus (including the

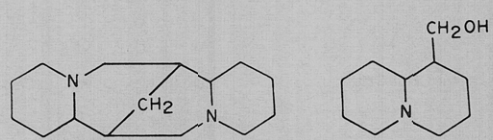

Figure 14. Structures of the quinolizidine alkaloids sparteine (left) and lupinine (right)

Figure 15. *V. californicum*, **a plant containing steroidal alkaloids**

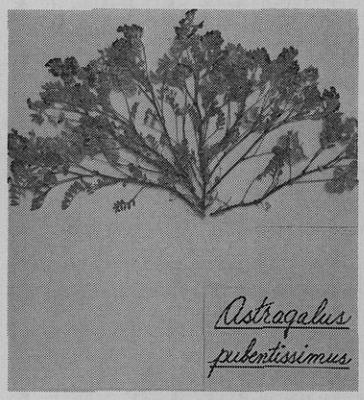

Figure 17. *A. pubentissimus*, **a loco plant**

cultivated potato, *S. Tubersum*), the *Lycopersicon* or tomato genus, the *Veratrum* or false hellebore genus, and the *Zigadenus* or death camas genus. While all have been frequently incriminated in animal poisonings, *Veratrum* (Figure 15) and *Zigadenus* are the most important from the grazing point of view, since they are frequently found in the open range. Over 50 different alkaloids have been isolated from members of the genera producing steroidal alkaloids. They include esters, glycosides, and parent alkamines. Their structures differ widely and, as one might suppose, the pharmacology of the individual alkaloids also differs (Krayer, 1958). Jervine and protoveratrine (Figure 16) are examples of the parent alkamine and ester compounds from *Veratrum*. Both are steroidal, and both have the C-nor-D-homo modified ring system. Toxicity of individual plants varies with alkaloid distribution, but acute toxicosis generally derives from the ester alkaloids whose toxicity is a few orders of magnitude greater on a weight basis than the parent alkamines or glycosides (Krayer, 1958). Signs of acute poisoning (Keeler and Binns, 1967; Kingsbury, 1964), with *V. californicum* include salivation, frequent urination, irregular gait, vomition, prostration, and decreased respiratory and heart rates.

UNKNOWN STRUCTURAL CLASS

The only example of alkaloids of unknown structure in plant genera considered here is that of the reported presence of alkaloids in members of the *Oxytropis* and *Astragalus* genera (loco plants) (Figure 17). These plants produce a very high incidence of livestock toxicosis and deaths in the United States (Kingsbury, 1964; Muenscher, 1951). They have been known as toxic plants since early times, but in spite of this and in spite of the great effort expended in their study, little is known concerning active compounds. Locoed animals appear crazy. The signs (Kingsbury, 1964; Muenscher, 1951) include a peculiar pacing gait, holding the head high, nervousness, and unawareness of surroundings. There have been reports that alkaloids were responsible. Pease and Elderfield (1940) reported the presence of two alkaloids, α- and β-earleine, which later proved to be choline and betaine (Stempel and Elderfield, 1942). Neither can be held to be responsible for the toxicosis. Fraps and Carlyle (1936) purified a fraction from *O. muricata* to the point where they obtained physical constants and other chemical data. Although it was never pure, and no structural or empirical formula information was obtained, they gave it the name locoine, presumed it to be at least closely related to an alkaloid, and reported that it had the ability to induce typical loco toxicosis. Duboshina and Proskurnina (1963) recently isolated an alkaloid *N*-benzoyl phenylaminomethyl carbinol (Figure 18) from *Oxytropis*, but reported no pharmacologic information about it.

HISTORICAL SURVEY OF TERATOGENIC EFFECTS OF ALKALOIDS FROM RANGE PLANTS

A few studies have incriminated certain alkaloids from range plants or the plants themselves as agents which induce teratogenic effects (congenital malformations) in laboratory animals or domestic livestock.

Green and Christie (1961) showed the *Senecio* plants, containing pyrrolizidine alkaloids, produced teratogenic effects in offspring from rats fed plant material during gestation. Some indole alkaloids produced fetal malformations in hamsters. These alkaloids included vinblastine and vincristine from *Vinca rosea* (Ferm, 1963) and from ergot, the lysergic acid derivatives lysergic acid diethylamide and bromolysergic acid diethylamide (Gerber, 1967). Rabbits (Landauer, 1960) and mice (DiPaolo and Kotin, 1966) had fetal resorptions and malformations when given nicotine, a pyridine class alkaloid, from the

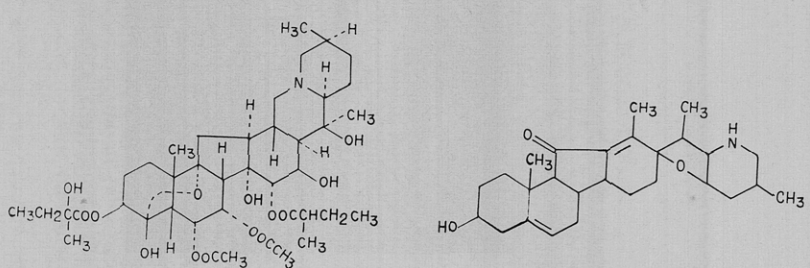

Figure 16. Structures of the steroidal alkaloids protoveratrine (left) and jervine (right)

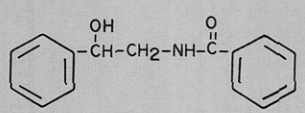

Figure 18. Structure of the loco alkaloid, *N*-benzoyl phenylaminomethylcarbinol

Conium, *Lobelia*, and *Nicotiana* genera. Cytisine, a quinolizidine class alkaloid from *Lupine*, *Cytisus*, and other genera, produced neck abnormalities in chick embryos (Landauer, 1960).

We have demonstrated that lupine plants are responsible for the teratogenic effects known as the crooked calf disease (Shupe *et al.*, 1967, 1968), that loco plants produce certain congenital skeletal deformities in lambs (James *et al.*, 1967), and that *Veratrum* plants produce cyclopic congenital deformities in lambs (Binns *et al.*, 1963, 1965).

CURRENT INVESTIGATIONS ON CHEMISTRY OF POISONOUS PLANT TERATOGENESIS

Loco Plants. The insidious toxicity of loco plants, giving rise to classical locoism, has been the object of study for decades (Kingsbury, 1964; Muenscher, 1951).

Congenital deformities and abortions in sheep are often observed under field conditions in areas where loco plants grow. These are due to ingestion of loco by the pregnant ewe at almost any period during gestation (James *et al.*, 1967). These congenital deformities are characterized by excessive flexure of the carpal joints and by contracted tendons. Some animals have anterior flexure and hypermobility (or looseness) of the hock joint (Figure 19).

These deformities are similar in certain gross respects to congenital deformities reported to be produced in laboratory animals by osteolathyrogens from members of the *Lathyrus* genus. Both loco plants and lathyrus plants are closely related botanically. For these reasons, we wished to ascertain whether alkaloids or lathyrogens were responsible for malformations induced by loco.

Over 200 pregnant ewes have been fed (Keeler *et al.*, 1967) either *Astragalus lentiginosus* and other loco plants, the lathyrogen aminoacetonitrile, a 1% H_2SO_4 extract of the plant, or a polar or nonpolar phase from a 30% ethanol extract of the plant. Both the plant and aminoacetonitrile produced congenital deformities and abortions when fed for 10- to 20-day periods of gestation between the 10th to 120th days. The gross clinical appearance of the lathyrogen-induced malformation was in every way similar to that produced by the plant. Figure 20 shows typical congenitally-deformed lambs born to ewes fed aminoacetonitrile. The 1% H_2SO_4 extract of the plant, after neutralizing with $CaCO_3$ and removing the $CaSO_4$ precipitate, was fed to pregnant ewes, and also was active. Polar and nonpolar phases from a 30% ethanol extract of the plant were prepared by partitioning the partially evaporated extract between aqueous and $CHCl_3$ phases. The polar phase was very active, causing two malformed and two aborted lambs from five ewes. The results do not rule out the very polar locoine of Fraps and Carlyle (1936) as an active teratogen. We can rule out the only alkaloid of known structure from loco plants, *N*-benzoyl phenylaminomethyl carbinol (Duboshina and Proskurnina, 1963) since it is readily soluble in $CHCl_3$. The results, however, strongly suggested that the loco-induced congenital deformities could be of a lathyritic type (Keeler *et al.*, 1967).

We, therefore, attempted to detect by paper chromatography known neuro- and osteolathyrogens in *A. lentiginosus* and *A. pubentissimus* and also in *O. sericeus* (Keeler *et al.*, 1967). Water, 1% acid, 30% ethanol, and acid-ethanol extracts of the plants and plant parts contained no two-dimensional chromatographically detectable β-aminopropionitrile or β-cyanolanine (Figure 21). No γ-glutamyl-β-aminopropionitrile was detected by the colorimetric method of Garbut and Strong (1955) nor by chromatography of the hydrolyzed products. No conclusions were drawn from chromatography regarding the presence or absence of either γ-glutamyl-β-cyanolanine or β-*N*-oxalyl-L-2,3 diaminopropionic acid, since no authentic samples were then available. We provisionally assume the presence of a trace of α,γ-diaminobutyric acid and a significant level of aminoacetonitrile because there were spots in the correct locations and of the correct color for each—mauve for aminoacetonitrile (AAN) and reddish lavendar for α,γ-diaminobutyric acid (α,γ-DABA).

Infrared spectra of sulfate preparations from extracts expected to contain aminoacetonitrile purified by the method of Dasler (1954) for β-aminopropionitrile resembled pure aminoacetonitrile sulfate. However, our purification attempts have not been fully successful as yet.

These purification attempts are currently in progress, and this laboratory is also making a comparison of the proline and hydroxy proline ratios (Haber *et al.*, 1967; Jackson, 1967) and levels of lysine and desmosines (Piez, 1968) in various types of connective tissue from deformed lambs born to ewes fed the plant, the lathyrogen, and the extracts. The results are expected to establish whether the loco-induced congenital malformation of lambs is a lathyritic disease unrelated to alkaloids of the plant.

Figure 19. Congenital malformations resulting from maternal consumption of locoweed

Figure 20. Congenital malformations resulting from maternal consumption of the synthetic lathyrogen, aminoacetonitrile

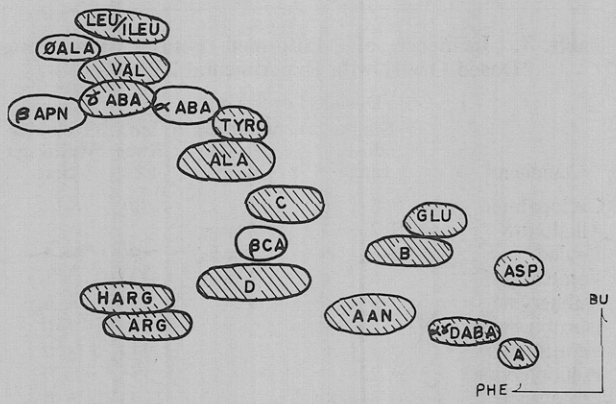

Figure 21. Diagram of a two-dimensional chromatogram of ninhydrin-positive spots from a 30% ethanol extract of *A. pubentissimus* (cross-hatched spots)

All spots except unknowns A, B, C, and D were also the positions of standards.

LUPINE PLANTS

Plants of the *Lupinus* genus are rich in quinolizidine alkaloids and their acute toxicity is well documented (Bohlman and Schuman, 1967; Kingsbury, 1964; Leonard, 1960; Muenscher, 1951). However, the plant also produces the marked congenital deformities known as the crooked calf syndrome (Shupe *et al.*, 1967, 1968), characterized (Figure 22) grossly by congenital deformities of the skeletal system. Arthrogryposis, torticollis, and scoliosis are characteristic. A typical calf with permanently rotated and flexed front limbs is shown in Figure 22. Some of the reported congenital defects of lathyrism (Chang *et al.*, 1955; Herd and Orbison, 1966; Rosenberg, 1957; Stamler, 1955) in laboratory animals are similar to those of the lupine-induced crooked calf disease. Further, lupine (like loco) is also a member of the same botanical family as the *Lathyrus* genus, which may suggest the presence of similar toxic material in each.

Because of these apparent similarities and the knowledge that toxic alkaloids were present in lupine, the following experiments were performed. Effects induced in calves upon maternal feeding of the lupine plant were compared with effects of a polar extract of the plant expected to contain lathyrogens if present in the plant. These were compared with effects produced by a nonpolar phase extract containing the mixed alkaloid fraction, the synthetic lathyrogen aminoacetonitrile, and the commercially available lupine alkaloid sparteine. All were fed at levels just below those giving rise to acute toxicity symptoms.

Typical crooked calves were born to two of four cows fed the plant. One of three cows fed the extract expected to contain lathyrogens produced a crooked calf. Aminoacetonitrile resulted in one crooked calf from three cows fed. Neither the pure alkaloid sparteine nor the mixed alkaloid fraction resulted in unequivocally deformed offspring. All deformed animals were essentially similar in gross clinical appearance. The aminoacetonitrile-produced malformed calf is shown in Figure 23. These preliminary results suggest that perhaps it is not an alkaloid from lupine which produces the crooked calf syndrome, but rather another example of a lathyrogenic effect.

The same spot on paper chromatograms, which we believe to be aminoacetonitrile from loco extracts, can be seen in lupine extracts.

Only histopathologic and biochemical comparisons of the lesions produced in lathyrism and the crooked calf disease and also isolation and positive identification of the active material from *Lupine* will establish the etiology. These studies are presently underway.

VERATRUM PLANTS

We showed that the plant *V. californicum* causes cyclopian and related cephalic malformation in lambs from ewes that ingested the plant (Binns *et al.*, 1963) on the 14th day of gestation (Binns *et al.*, 1965). This teratogenic effect (Figure 24) whose etiology was unknown until Binns *et al.* (1963) demonstrated the role of the plant, occurred in some areas of Idaho in endemic proportions. It is characterized by anatomical deviations varying in the

Figure 23. Congenitally-deformed calf resulting from maternal ingestion of the synthetic lathyrogen aminoacetonitrile

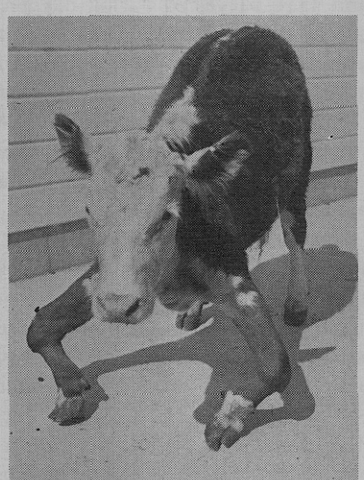

Figure 22. Congenitally-deformed calf resulting from maternal ingestion of *L. sericeus*

Figure 24. Heads of congenitally-malformed lambs resulting from maternal ingestion of *V. californicum*

extreme from a true cyclopia (single median eye), a shortened upper jaw and protruding lower jaw with a peculiar skin covered proboscis above the single eye, to examples of lesser severity with normal eyes and only a shortening of the upper jaw. Identifying the plant as the responsible agent and demonstrating the insult period as the 14th day of gestation have enabled us to design extraction methods and testing procedures that have culminated in the isolation and characterization of the teratogenic compound (Keeler and Binns, 1966a, 1966b, 1968; Keeler, 1968; Keeler, 1969).

Our original premise was that the activity would likely be due to one or more of the steroidal alkaloids of the plant. This premise was attractive because *Veratrum* alkaloids were known to interfere with nuclear and cellular devision in *Allium cepa* (Burroni, 1955; Smith and Hiner, 1960), known to inhibit the growth of *Candida* (Frank, 1959), were effective insecticides (Seiferle *et al.*, 1942), and for various other reasons which all suggested a cell division inhibition potential. During our experimental work, we sought to prepare alkaloid-containing fractions and pure compounds for testing.

The extraction procedures finally adopted and presently used for obtaining the major alkaloids of the plant are shown in Figure 25. During the studies, it became evident that the activity did reside in the alkaloid fractions. Both the benzene-soluble and alcohol-soluble crude alkaloid fractions were active. We, therefore, prepared and/or purchased for comparison all of the alkaloids that we knew were present in major proportions and also certain others. Results of feeding trials to test their activity are shown (Table I). The compounds to which we assigned the names cyclopamine and alkaloid X as well as jervine and veratrosine were all active. Others tested were not. However, only one malformed lamb was obtained from 23 ewes fed veratrosine and none from 21 ewes fed its parent alkamine, veratramine. Since the veratrosine preparation is now known in retrospect to have been significantly contaminated with alkaloid X, we conclude that veratrosine *per se* was not active. Thus, only cyclopamine, alkaloid X, and jervine were unequivocally established as active.

The malformations induced by cyclopamine were typical of natural cases in every respect (Figure 26). Further, since cyclopamine is present in the plant in a much higher concentration than the other teratogens and is more active on a weight basis, it seems probable that naturally-occurring cases are due to the cyclopamine in the plant.

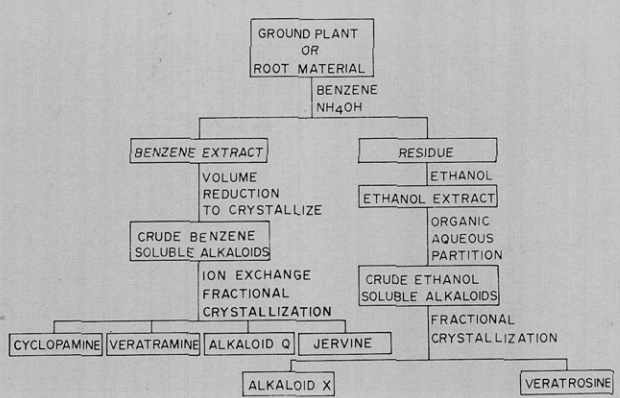

Figure 25. Extraction and purification flow sheet for recovering teratogenic and other alkaloids from *V. californicum*

Table I. Incidence of Malformed Fetuses from Ewes Dosed Orally with Experimental Alkaloids

Compound	Dosage Level, Gm. Single dose range	Minimum effective dose	No. Ewes Fed	Cyclopian Malformations
Cyclopamine	0.8–2.0	1.0	40	12
Alkaloid X	0.7	0.7 twice	2	1
Jervine	0.3–1.2	1.2 twice	9	2
Veratrosine	0.6–1.8	1.2 twice	23	1
Rubijervine	0.8–1.2	...	5	0
Isorubijervine	0.2–1.3	...	8	0
Veratramine	0.3–1.4	...	21	0
Pseudojervine	0.25	...	1	0
Protoverine	0.3–0.8	...	4	0
Cevine	0.6–1.1	...	3	0
Veracevine	0.6–0.7	...	2	0
Alkaloid Q	1.0–1.5	...	5	0
3β,23-*N*-triacetylveratramine	0.9–1.1	...	8	0

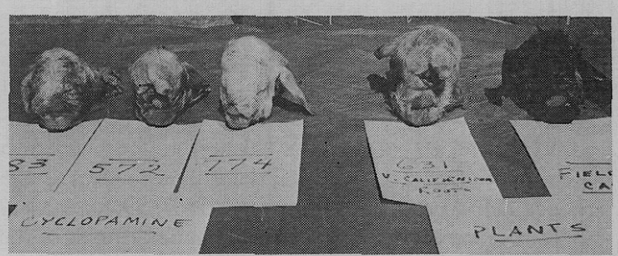

Figure 26. Comparison of gross appearance of cyclopamine-induced malformation and those induced by the natural ingestion and experimental feeding of *V. californicum*

The author therefore sought to determine the structure of cyclopamine. Experimental data clearly established the structural identity of cyclopamine as 11-deoxojervine (Figure 27). This structure is nearly identical to jervine (Figure 27), another of the three active compounds. The third active compound, alkaloid X, appears to be 3-glucosylcyclopamine.

All acquired data were consistent with the assignment of the cyclopamine structure as 11-deoxojervine. Pure cyclopamine produced an infrared spectrum suggesting a Δ^5, 3β-ol steroidal system (1050 to 1057 cm.$^{-1}$), about the same 3500 cm.$^{-1}$ hydroxyl absorption as jervine, and only about one-half that of veratramine. This fact suggested a single rather than two OH groups. Peaks at 927, 984, and 1118 suggested a ring oxygen system (the ether bridge) as with jervine. There was no aromatic ring absorption in the ultraviolet region as for jervine and unlike veratramine (Figure 27). In the latter, ring D is completely unsaturated. The molecular weight from mass spectrometry indicated that the empirical formula should be $C_{27}H_{41}O_2N$. This empirical formula matched well with that from elemental analysis.

Nuclear magnetic resonance spectrometry (Figure 28) provided the following information. Integration suggested about 41 to 42 protons. The following assignments could be made: two overlapping doublets (J = 6.0 and 6.6) and a singlet at 0.88 to 0.96 δ (9 protons — 3-methyl groups at C_{19}, C_{21}, and C_{26}), a singlet at 1.63 δ (3 protons — 1 methyl group at C_{18}), a multiplet (triplet?) at 5.35 δ (1 proton — C_6 olefinic proton), a multiplet at 3.4 δ (1 proton — C_3 proton). The foregoing assignments are consistent with Shoolery's rules (Shoolery and Rogers, 1958) and with assignments made for verarine (Tomko

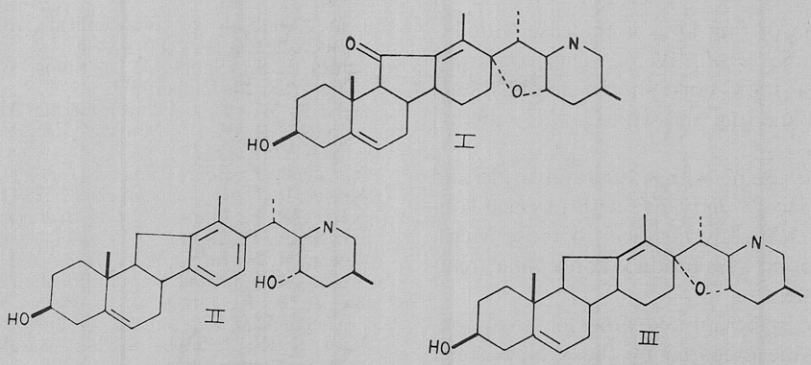

Figure 27. Structures of jervine (I), veratramine (II), and 11-deoxojervine (III)

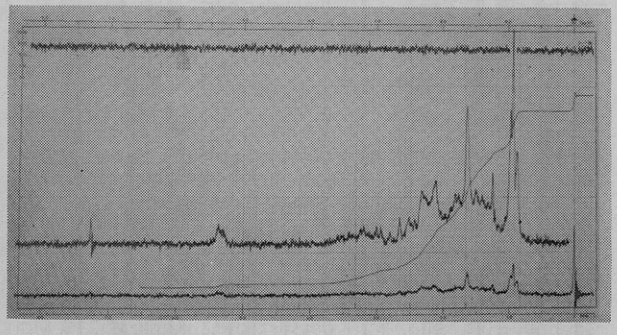

Figure 28. Nuclear magnetic resonance spectrum of cyclopamine

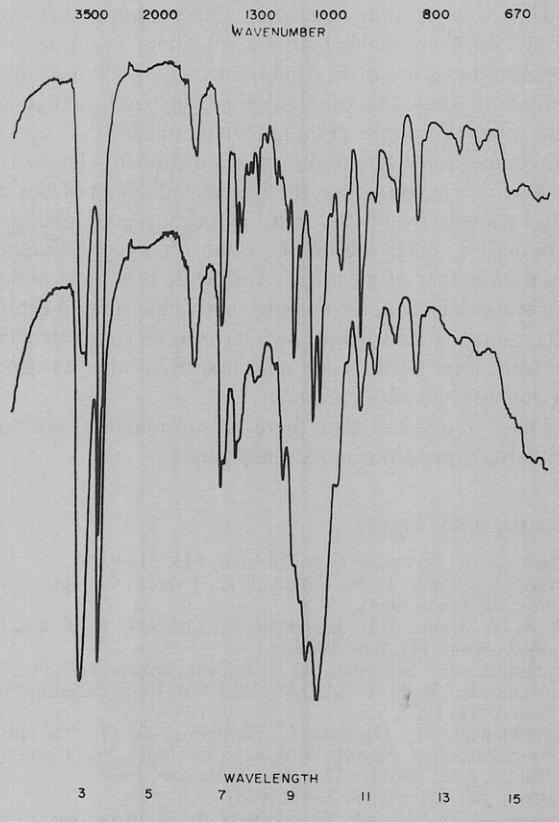

Figure 29. Comparative infrared spectra of cyclopamine (top) and alkaloid X (bottom)

Table II. Principal Ion Fragments of Cyclopamine between Mass 413 and 110[a]

m/e	Relative Abundance[b]
413	0.6
412	2.5
411	6.6
397	8.3
396	27
340	1.2
326	1.2
310	2.4
298	5.9
145	9.5
131	5.9
126	35.8
125	100
124	99
114	22.6
113	3.6
110	95.0

[a] Fragments of abundance less than 5 omitted except those for which an assignment is made.
[b] Percentage of base peak (125).

and Bauer, 1964), veralkamine (Tomko *et al.*, 1967), jervine (Masamune *et al.*, 1964), and jervane derivatives (Masamune *et al.*, 1967).

The physical constants of cyclopamine (Keeler, 1969) included m.p. 237–8° C., $[\alpha]_D^{25}$ −48 [C = 1% in methanol: $CHCl_3$ (2:1)]; UV had a 250-nm. shoulder with strong end absorption. It had a single spot on TLC. The IR in KBr pellet had γ max 3400, 3250, 2900, 2860, 1450, 1118, 1067, 1060, 1042, 984, 927, and 809.

Found: C, 78.96; H, 10.29; N, 2.96; Calcd. for $C_{27}H_{41}O_2N$: C, 78.78; H, 10.04; N, 3.40%.

Calculation of "double bonds and rings" by the Soffer (1958) method gave 8 for cyclopamine, which is consistent with the 11-deoxojervine structure. Mass spectrometry fragmentation analysis (Table II) was consistent with the 11-deoxojervine structure. The fragmentation assignment from the data of Table II are as follows. The 411 is the parent peak with 412 and 413 as P + 1 and P + 2, respectively. The *m/e* 396 peak is 411 minus a CH_3 group. The single 340 peak is 411 minus an $NH\text{-}CH_2\text{-}CH(CH_3)\text{-}CH_2$ group. The *m/e* 326 is the 411 minus a $CH_2\text{-}NH\text{-}CH_2\text{-}CH(CH_3)\text{-}CH_2$ group. The 310 is the 411 minus a $CHO\text{-}CH_2\text{-}CH(CH_3)\text{-}CH_2\text{-}NH$ group. The 298 is the 411 minus a 2,3 epoxy-4-methyl piperidine group. The 125 peak results from the opening of the tetrahydrofuran ring, producing a 2-ethyl-2-ene-5-methyl piperidine-1-yl radical ion which is further stabilized by loss of a hydrogen to give the 124 peak or of a methyl group to give the 110 peak. These fragmentation assignments are consistent with that reported for jervine (Budzikiewicz *et al.*, 1964) and expected for cyclopamine.

Treatment of cyclopamine with 0.6% HCl at 38° C. for 24 hours produced veratramine by cleavage of the ether

bridge and dehydration of ring D as is the case with 11-deoxojervine (Masamune *et al.*, 1965). This confirmed that the ring system was the C-nor-D-homo steroid system with a terminal piperidine ring and with $C_{18,19,21,26}$ methyl groups.

Acetylation of cyclopamine with acetic anhydride and pyridine produced a diacetyl derivative with physical constants (Keeler, 1969) essentially identical to those Masamune *et al.* (1965) reported were produced on similar treatment of 11-deoxojervine.

Finally, the infrared spectrum and other physical constants of 11-deoxojervine prepared by the Wolf-Kishner reduction of jervine and those of isolated cyclopamine were identical (Masamune *et al.*, 1965).

Thus, cyclopamine, the compound responsible for prenatal cyclopian malformations in lambs resulting from maternal ingestion of *V. californicum* is 11-deoxojervine.

It seems likely that alkaloid X is 3-glycosylcyclopamine. The infrared spectra (Figure 29) of alkaloid X and cyclopamine differ essentially in absorption in the 1000 to 1100 $cm.^{-1}$ region where the primary alcohols of the glucosyl moiety would be seen. Vigorous acid hydrolysis produced a sugar whose R_f value on paper chromatograms was that of glucose. And while it is most difficult to recover 11-deoxojervine after even mild acid hydrolysis because of its acid lability, we have some suggestive TLC evidence after weak acetic acid hydrolysis that 11-deoxojervine was one of the products.

Thus, it appears that there is considerable structural similarity among the active compounds.

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