Teratogenic Compounds of *Veratrum californicum* (Durand). XIV. Limb Deformities Produced by Cyclopamine (37225)

RICHARD F. KEELER

U.S. Department of Agriculture, Agricultural Research Service, Poisonous Plant Research Laboratory, 1150 East 14th North, Logan, Utah 84321

We have described cyclopic and related cephalic congenital deformities in lambs born to ewes ingesting the plant *Veratrum californicum* on Day 14 of gestation (1). We characterized the teratogen as a steroidal alkaloid to which we gave the name cyclopamine (2). Other deformities, resulting from maternal ingestion of the plant, have included certain limb malformations such as hypoplasia of the metacarpal or metatarsal bones (3). These limb deformities arose from experimental feeding of the plant, primarily during the fifth week of gestation (3).

This report describes the experimental feeding to pregnant ewes of four predominant alkaloids isolated from *Veratrum californicum*—veratramine, veratrosine, cyclopamine, and muldamine—during the fifth week of gestation to determine if any were responsible for inducing the limb deformities.

Materials and Methods. Preparation of alkaloids. The four alkaloids—veratramine, veratrosine, muldamine and cyclopamine (Fig. 1)—were isolated from *Veratrum californicum* roots by modification of methods previously described (2, 4). Finely-ground *Veratrum californicum* root and rhizome material was moistened with 1% NH_4OH and extracted by very slow percolation with ethanol. The plant residue after ethanol extraction was then extracted with benzene by percolation in a similar fashion. About 4 vol of solvent was used for each volume of plant material.

Veratrosine was obtained from the ethanol extract by previously described methods (4). The benzene extract was the source of the other three alkaloids. The benzene was removed by evaporation. When sufficiently concentrated, the alkaloids in the benzene phase generally crystallized to yield a mixed crystalline preparation of alkaloids. The predominant alkaloids present were muldamine, cyclopamine, and veratramine. The mixed crystalline material was dissolved in boiling acetone. The first crop of crystals obtained upon cooling was largely cyclopamine, the middle crop was largely veratramine, and the final crop was largely muldamine. Each was recrystallized to yield pure compounds using acetone, acetone–water, methanol–water, and ethanol–water systems. Purification was followed and assessed by infrared spectrophotometry. Heights of the 1725 and 1260 cm^{-1} carbonyl peaks were used to assess the purity of muldamine; the 927, 984, and 1118 cm^{-1} ring oxygen peaks to assess the purity of cyclopamine; and the unassigned 963 cm^{-1} peak to assess the purity of veratramine. Relative proportions in the original mixture were determined in this way also. Final identification was by methods previously described (2, 4–6).

In some preparations, direct crystallization from the benzene extract did not occur, perhaps because of low concentration of alkaloids. In those instances, the solids after benzene removal were triturated with petroleum ether and filtered. The residue was dried, macerated, taken up in boiling 95% ethanol (about 200 g/liter), and filtered. To 20 vol of the ethanol filtrate was added 5 vol of petroleum ether and 60 vol of 1% NH_4OH to about pH 10.00 to precipitate the alkaloids, which were then recovered by filtration. The dried material could then be successfully subjected to the same crystallization from hot acetone used in preparations where the crude mixed alkaloids crystallized directly from the benzene extract (above).

Treatment of animals. All ewes were of mixed breed averaging 55-kg weight. The

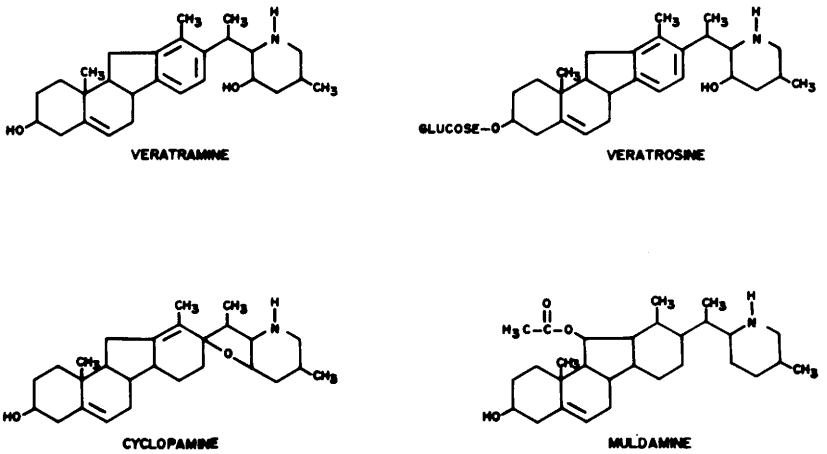

FIG. 1. Structures of veratramine, veratrosine, cyclopamine, and muldamine.

day of breeding was recorded as Day 0 of gestation. The experimental compounds were administered orally by capsule on the gestational days indicated in Table I. The ewes were maintained on a diet of hay and salt with water free choice. The dosage level of experimental alkaloids was 1.5 g/animal/day, administered in a single dose. The levels were such that the ewes had few signs of toxicosis except with veratramine. The latter is quite toxic, and seven ewes died from overdose. Resulting lambs were examined for limb deformities, particularly the shortening of metacarpal and metatarsal bones characteristic of deformities produced by *V. californicum* (3). Some 200 other pregnant ewes on the premises maintained on a similar diet served as controls. None of their lambs had metacarpal or metatarsal hypoplasia.

Results and discussion. Among the four alkaloids (Fig. 1) fed to ewes during the fifth week of gestation, only cyclopamine produced limb deformities (Table I). Four ewes from among 22 pregnant ewes so fed had lambs with congenital defects. The data suggest that the date of insult was probably Day 30 of gestation. Of the 12 pregnant ewes fed on Day 30 alone or in combination with other days, 4 had lambs with limb deformities for an incidence of 36%.

The defects ranged from a very modest shortening of all four limbs (No. 1302) and moderately relaxed pasterns in all four limbs (No. 1319) to unquestionably severe and typical shortening of metacarpals (No. 2168) and also both metacrapals and metatarsals (No. 2141). Figure 2 shows the latter two lambs and describes the deformities. The metacarpal and metatarsal hypoplasia induced by cyclopamine (Fig. 3) was identical to that produced by feeding the plant *Veratrum californicum* during the same period (3).

The lack of activity of veratramine, its glycoside veratrosine, and muldamine was not unexpected. They do not produce the cyclopia and related cephalic deformities produced by cyclopamine (7). We cannot unequivocally rule out these three compounds as teratogenic because of the restricted numbers of animals in the experimental groups. But the possibility seems remote since we had an incidence of 36% in those fed cyclopamine on Day 30 of gestation. There were seven normal lambs born to dams fed on Day 30 in each of the three groups except veratramine. But the latter is the aglycone of veratrosine and would be expected to possess similar activity.

Only cyclopamine, of the four, has a fused furanopiperidine system in rings E and F. Our previous results suggest that an intact, fused furanopiperidine system is essential to produce cyclopia in sheep and rabbits (7). The data reported herein suggest a similar conclusion for limb deformities. There may be a rather general teratological significance to the fused furanopiperidine system. Various congenital defects are induced by cyclopamine (and possibly other furanopiperi-

TABLE I. Pregnancy Consequences in Ewes Fed the Experimental Alkaloids.

Alkaloid	No. of ewes fed	Gestational days fed	Results
veratramine	3	30,31	2 ewes died (overdose), 1 ewe delivered normal twins
veratramine	2	30	1 ewe died at 110 days gestation—(twin fetuses, both normal), 1 ewe died (overdose)
veratramine	1	31	1 ewe delivered normal twins
veratramine	2	31,32	2 ewes died (overdose)
veratramine	4	32	3 ewes delivered normal singles, 1 ewe died (overdose)
veratramine	5	33	3 ewes delivered normal singles, 1 ewe delivered normal twins, 1 ewe died (overdose)
veratrosine	3	30,31	2 ewes delivered normal twins, 1 ewe nonpregnant
veratrosine	2	30	1 ewe delivered normal single, 1 ewe delivered normal twins
veratrosine	2	31	1 ewe delivered normal single, 1 ewe delivered normal twins
muldamine	3	30,31	2 ewes delivered normal twins, 1 ewe delivered normal single
muldamine	2	30	1 ewe aborted, 1 ewe delivered normal twins
muldamine	2	31	1 ewe died (overdose), 1 ewe delivered normal twins
muldamine	2	31,32	2 ewes delivered normal singles
muldamine	1	32	normal single
muldamine	1	33	normal twins
muldamine	2	32,33	2 ewes delivered normal singles
cyclopamine	4	28,29,30	2 ewes delivered normal singles, 2 ewes delivered abnormal singles. Lamb from ewe 2141 had short metacarpals and metatarsals. Lamb from ewe 2168 had short metacarpals in front legs, relaxed pasterns in front legs, and corkscrew tail.
cyclopamine	2	30	2 ewes delivered normal twins
cyclopamine	2	31	1 ewe delivered normal twins, 1 ewe nonpregnant
cyclopamine	6	30,31	2 ewes delivered normal twins, 1 ewe delivered normal single, 1 ewe non-pregnant, 2 ewes delivered abnormal twins. The female lamb from ewe 1302 had all four limbs shorter than twin. Both lambs from ewe 1319 had relaxed pasterns in front and rear limbs.
cyclopamine	2	32	1 ewe delivered normal single, 1 ewe nonpregnant
cyclopamine	2	33	2 ewes delivered normal singles
cyclopamine	2	31,32	2 ewes delivered normal singles
cyclopamine	2	32,33	2 ewes delivered normal singles

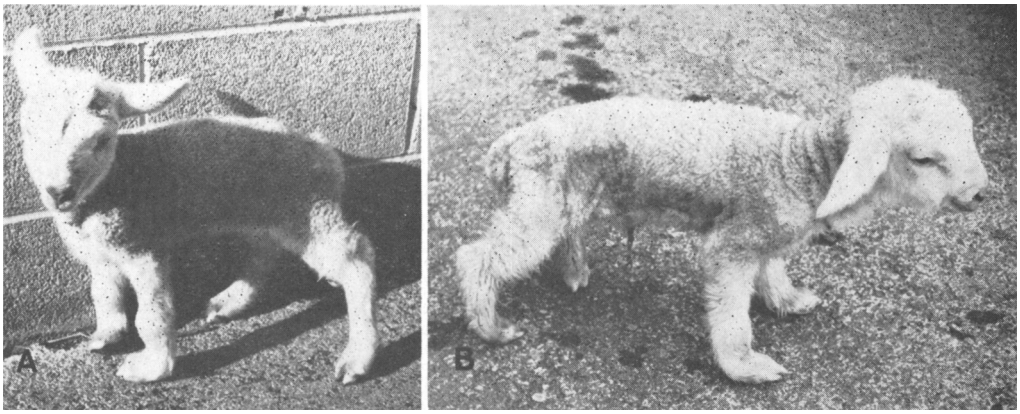

FIG. 2. (A) Lamb born to ewe 2168 fed cyclopamine on Days 28, 29, and 30 of gestation. Lamb had shortened metacarpal bones, relaxed and rotated pasterns in the front foot, and twisted phalangeal bones. Rear limbs were normal. (B) Lamb born to ewe 2141 fed cyclopamine on Days 28, 29, and 30 of gestation. Lamb had shortened metacarpals and metatarsals with some pastern involvement.

dine-containing compounds) depending on individual susceptability and gestational insult period.

Renwick (8) hypothesized the probable role of blighted potatoes in production of anencephalia and spina bifida in humans. Epidemological criteria suggested to him that potatoes may be responsible for many thousands of such births yearly. Among possible teratogens, he suggested potato alkaloids as one possibility and discussed in some detail variations in solanine and solanidine content possibly related to incidence.

The possibility that potato or tomato alkaloids were teratogenic had occurred to us many years ago, based on structural similarity to veratrum alkaloids (7). We fed both potatoes and tomatoes selected for high total alkaloid content to ewes on Day 14 of gestation, but no cyclopic or related deformities were produced (7).

Nonetheless, it does seem possible that certain potato alkaloids could be teratogenic. Our experience with veratrum alkaloids would suggest that a potato alkaloid with a fused furanopiperidine functional group

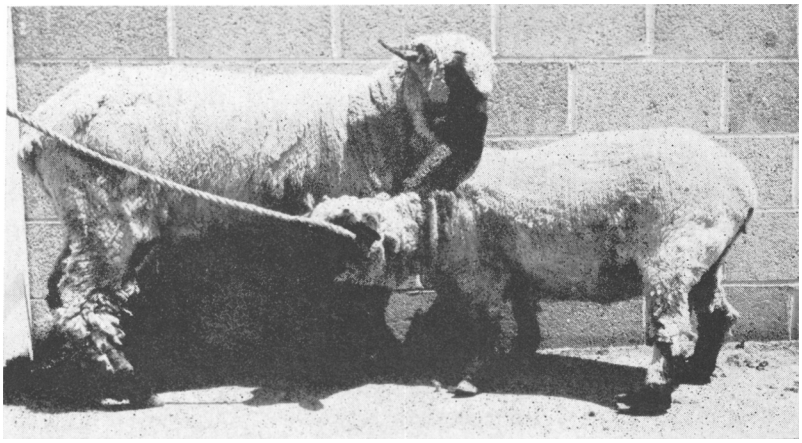

FIG. 3. Two adult sheep. A normal on the left and one with metacarpal and metatarsal hypoplasia induced by maternal feeding of *V. californicum* during the fifth week of gestation on the right.

might be highly suspected. Such compounds have not yet been found to our knowledge, although potato and tomato alkaloids with furan ring E fused to the steroidal position rather than the piperidine portion of the molecule are known (9).

Clearly, further experimental work will be required to assess the general teratogenic significance of fused furanopiperidines. But the data reported here suggest wider specificity for cyclopamine than originally assumed.

Summary. Four major alkaloids isolated from *Veratrum californicum*—veratramine, veratrosine, cyclopamine, and muldamine—were fed to pregnant ewes during the fifth week of gestation. Cyclopamine, when administered during Days 28, 29, and 30 of gestation, produced the same congenital deformities of limbs previously produced by whole plants. These deformities included shortening of the metacarpal or metatarsal bones.

1. Binns, W., Shupe, J. L., Keeler, R. F., and James, L. F., J. Amer. Vet. Med. Ass. **147**, 839 (1968).

2. Keeler, R. F., Phytochemistry **7**, 303 (1968).

3. Binns, W., Keeler, R. F., and Balls, L. D., Clin. Toxicol. **5**, 235 (1972).

4. Keeler, R. F., and Binns, W., Can. J. Biochem. **44**, 819 (1966).

5. Keeler, R. F., Phytochemistry **8**, 223 (1969).

6. Keeler, R. F., Steroids **18**, 741 (1971).

7. Keeler, R. F., Teratology **3**, 169 (1970).

8. Renwick, J. H., Brit. J. Prev. Soc. Med. **26**, 67 (1972).

9. Schreiber, L., *in* "The Alkaloids" (R. H. F. Manske, ed.), Vol. 10, Chap. 1, p. 1. Academic Press, New York (1968).

Received Nov. 10, 1972. P.S.E.B.M., 1973, Vol. 142.