Teratogenic Compounds of *Veratrum californicum* (Durand) XI. Gestational Chronology and Compound Specificity in Rabbits (35453)

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Maternal ingestion of *Veratrum californicum* by ruminant animals produces cyclopia and related cephalic malformations in their offspring (1, 2). The teratogen cyclopamine, whose structure we have elucidated, is responsible for natural outbreaks of the disease in sheep (3).

Among veratrum alkaloids and a variety of related compounds, only cyclopamine, its glycoside cycloposine, and jervine produced this teratogenic effect experimentally in sheep (4). The rabbit, a nonruminant, is also susceptible to the teratogenic effects of cyclopamine provided the compound is not structurally altered to veratramine before it enters the vascular system(5). Ingestion of cyclopamine by pregnant rabbits for 4 successive days (days 6–9) of gestation produced what appeared to be the same teratogenic effect as seen in sheep (5).

Further experimental work on this effect would be more easily accomplished using rabbits as test animals rather than sheep, provided: (i) the precise gestational insult period was known, (ii) there was certainty that similar compound specificity was involved, and (iii) there was assurance that the lesions were similar morphologically and biochemically. This report elucidates the gestation insult period and natural alkaloid specificity in rabbits.

Materials and Methods. Alkaloids. The compounds cyclopamine, veratramine, and alkaloid Q were isolated from *Veratrum californicum* by methods previously described (6). Jervine,[1] rubijervine,[1] and veratrine,[2] were obtained commercially.

Administration of compounds. Compounds were administered by stomach tube at dose levels shown in Tables I and II suspended in about 20 ml of H_2O. Where noted, 3 g $CaCO_3$ and 3 ml of corn oil in some instances were added to the cyclopamine suspension to prevent stomach acid-induced conversion of the compound to veratramine (5). Except for compounds with high direct toxicity in the ingesting dam, all were administered in the 100–300-mg range which is perhaps ⅓ more than is necessary to produce the effect with active compounds. Veratramine and veratrine were excessively toxic at doses higher than 75 mg and 15–20 mg, respectively. Some deaths occurred from overdose in animals fed veratramine and veratrine at these levels.

Experimental animals. All rabbits were of mixed breed weighing an average of 4.5 kg. They were purchased from a local supplier. Breeding was accomplished by placing fresh bucks with the does in the morning and then rebreeding with different bucks in the afternoon to insure a high conception rate. Experimental treatments are shown in Tables I and II. All rabbits were killed on day 28 of gestation and fetuses were examined for evidence of malformations (the day of breeding was day 0).

The rabbits were maintained in an animal room whose temperature fluctuated between 21 and 26°. They were kept in commercial metal rabbit cages with a mesh wire floor. Feces and urine were trapped in a 1 in. layer of fresh shavings in the droppings pan. Cages were cleaned 2 times/week. The rabbits were fed *ad libitum* daily with a commercial feed[3] and watered twice daily. On the days of dosing and for 1 to 2 days thereafter, the rabbits decreased their feed intake very slightly.

Gross morphology of lesions. The gross external morphology of the malformations in the rabbits in this experiment was compared

[1] S. B. Penick & Company.

[2] Mann Research Laboratories.

[3] Pillsbury's Best 16% Rabbit Hopperettes.

to malformed lambs from the same cause observed in our laboratory over several years' period. These lambs were field cases of the disease derived from ewes pastured in heavy stands of *Veratrum californicum*, were cases derived from experimental feeding of the plant, and were cases derived from experimental feeding of the pure teratogens.

TABLE I. Gestational Insult Period in Rabbits by Cyclopamine Ingestion.

Rabbit no.	Cyclopamine dose (mg/day)	Gestation day	Results[c]
1	200[a]	6–9	1 normal, 6 nose and lip deformities (3 hydrocephalic), 1 cyclops
2	200[a]	6–9	9 nose and lip deformities (4 cyclopic, 2 cebocephalic, 1 cebocephalic-dead)
3	150[a]	6–9	15 normal
4	150[a]	6–9	9 normal, 3 very small normal
21	200[a]	8–9	8 normal, 1 crooked spine
22	200[a]	8–9	5 normal
23	200[a]	8–9	4 normal
24	200[a]	6–7	5 normal
25	200[a]	6–7	Aborted (day 20 of gestation)
26	200[a]	6–7	7 normal, 1 hydrocephalic
27	200[a]	7	10 normal
28	200[a]	7	5 normal
29	200[a]	7	Fetal death or aborted
30	200[a]	8	7 normal
31	200[a]	8	8 normal, 1 dead in utero
32	200[a]	8	9 normal, 1 very small normal
33	260[b]	7–8	Aborted or fetal death
34	260[b]	7–8	7 cyclopic, 1 cyclopic dead in utero
35	260[b]	7–8	2 cyclopic, 1 cyclopic-dead, 1 hydrocephalic, 1 cebocephalic, 2 dead in utero
36	260[b]	6–7	5 normal, 1 cebocephalic
37	260[b]	6–7	5 cyclopic, 3 nose deformity (2 hydrocephalic)
45	300[b]	7	2 normal, 5 hydrocephalic (1 lip deformity), 1 no head with external viscera
46	300[b]	7	2 hydrocephalus (1 nose deformity), 1 tailless, 1 dead
49	250[b]	7	6 normal, 1 hydrocephalus
60	200 a.m. and 200 p.m.[b]	7	Fetal death
61	200 a.m. and 200 p.m.[b]	7	1 normal, 3 hydrocephalus (1 lip and nose deformity)
62	200 a.m. and 200 p.m.[b]	7	Fetal death
78	300[b]	7	4 normal, 2 hydrocephalus
80	300[b]	7	1 hydrocephalus with nose and lip deformity, 6 partially resorbed
81	300[b]	7	7 normal
82	300[b]	7	8 normal, 2 nose and lip deformity (1 hydrocephalic)

[a] With oil and buffer.

[b] With buffer.

[c] Normal rabbits had no apparent gross external malformations and were of normal size. Rabbits with nose and lip deformities had single, central or closely spaced double nostrils and a "beaklike" upper lip. Other deformities are self explanatory.

Results. The results in Table I indicate that day 7 of gestation is the date of insult in rabbits for production of cyclopia and related cephalic malformations by cyclopamine (Fig. 1). Among rabbits fed from days 6 through 9 of gestation, only those fed on day 7 or day 7 in combination with other days had malformed offspring except for one with

TABLE II. Specificity of Naturally Occurring Veratrum Alkaloids on Day 7 Other Than Cyclopamine.

Animal no.	Dose (mg/day)	Compound	Result
57	75	Veratramine	6 normal
58	75	Veratramine	Fetal death
59	75	Veratramine	5 normal
55	75	Veratramine	3 normal, 2 dead partly resorbed (normal appearance)
98	75	Veratramine	6 normal, 1 dead (normal appearance)
100	75	Veratramine	8 normal
101	75	Veratramine	9 normal
94	75	Veratramine	Died from overdose
95	75	Veratramine	Died from overdose
96	75	Veratramine	Died from overdose
99	75	Veratramine	Died from overdose
102	75	Veratramine	Died from overdose
40	250	Jervine	5 normal, 2 hydrocephalic, 1 dead (normal appearance)
41	250	Jervine	3 normal, 2 cyclopic and hydrocephalic, 1 hydrocephalic and nose deformity, 1 dead
63	300	Jervine	6 normal
73	300	Jervine	Fetal death
74	300	Jervine	10 normal
76	300	Jervine	11 normal
88	300	Jervine	3 normal
89	300	Jervine	2 normal, 2 dead (1 external viscera)
90	300	Jervine	Aborted
51	300	Alkaloid Q	6 normal
52	300	Alkaloid Q	6 normal
53	300	Alkaloid Q	6 normal, 1 dead (looked normal)
93	300	Alkaloid Q	10 normal
97	300	Alkaloid Q	7 normal
68	300	Rubijervine	6 normal
69	300	Rubijervine	Fetal death
71	300	Rubijervine	8 normal
83	300	Rubijervine	3 normal
84	300	Rubijervine	5 normal
85	300	Rubijervine	5 normal, 1 dead (normal appearance)
86	300	Rubijervine	5 normal
87	300	Rubijervine	7 normal
105	30	Veratrine	3 normal, 2 dead (partly resorbed)
107	20	Veratrine	4 normal, 4 dead (partly resorbed)
114	15	Veratrine	10 normal
115	15	Veratrine	7 normal
116	15	Veratrine	5 normal, 1 dead (partly resorbed)
103	30	Veratrine	Died from overdose
106	20	Veratrine	Died from overdose

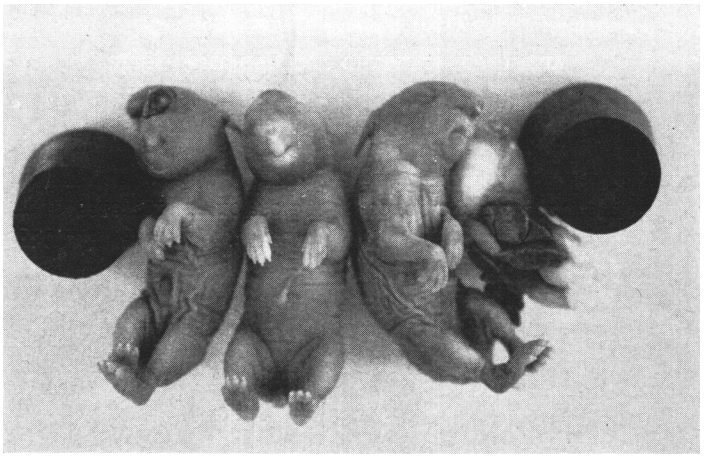

FIG. 1. Examples of the cyclopic and related cephalic malformations produced in rabbits by cyclopamine: (left to right) a cyclopic, a hydrocephalic with nose and lip deformities, a cebocephalic, and a headless with external viscera.

a crooked spine born to doe 21. Six control does given corn oil and buffer in water from days 6 through 9 of gestation gave birth to 38 normal offspring.

In common with sheep (4), rabbits were malformed when their dams ingested cyclopamine or jervine and were not malformed in the cephalic region by other veratrum alkaloids devoid of the tetrahydrofurylpiperidine function of rings E and F (Table II). Only jervine and cyclopamine among the alkaloids tested here possess this structural feature (Fig. 2).

Our observations of many hundreds of malformed lambs over a number of years from natural occurrence and from experimental induction, both with plant material and pure compounds has shown a wide range of lesion severity. The extremes included a true cyclopia, anopthalmia, or microphthalmia. A marked shortening of the upper jaw and a skin-covered proboscis approximately 1 × 5 cm arising in the median plane dorsal to the eyes with a fibrous core were common in extreme examples. The cyclopia has varied from a true cyclopia with a single globe, a more cebocephalic variation with a single eye composed of two cornea in a single distorted sclera with a common eyelid in a single median bony orbit, another cebocephalic type in which there were two eyes of more or less normal appearance with separate eyelids but upon dissection had fused sclera, and finally to a type with normal eyes and only a shortening of the upper jaw. The shortened upper jaw was common regardless of severity. The maxillae were shortened anteriorly with curvature in the dental arches, vestigial premaxillae and shortened nasal bones. The mandibles had an exaggerated upward curvature presumably resulting from the lack of prenatal opposition from the shortened upper jaw. The tongues of normal length commonly protruded because of the shortened upper jaw. In extreme examples possessing the proboscis, the nasal cavity was not patent and no nostrils were present. An externally evident hydrocephalus has been a common observation. Other gross anatomical and pathological details of the condition in sheep have been published by Binns *et al.* (7).

The morphologic deviations in rabbits paralleled those of sheep in many ways. The extreme cyclopia with a single globe which occurs with moderate frequency in sheep was not seen in any of the malformed rabbits included here nor in those of earlier experiments (5). As with sheep, a cyclopia with two globes in a central orbit and the typical proboscis was very common in rabbits. Even more typically a cebocephalia was present in which there was a single or closely spaced double nostril as with sheep but in which a "beaklike" upper lip was evident. The latter characteristic was not common in sheep. A cleft upper lip was occasionally seen in field

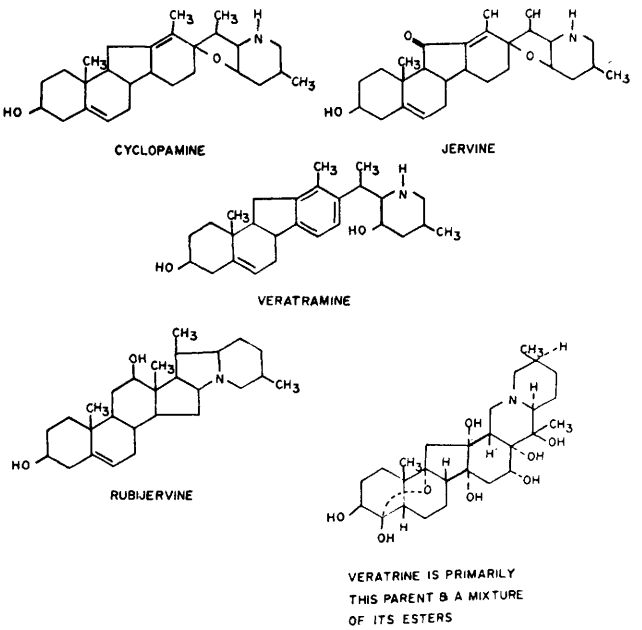

FIG. 2. Structures of the Veratrum alkaloids fed to rabbits: The ether bridge of cyclopamine and jervine along with the nitrogen-containing ring comprise the substituted tetrahydrofurylpiperidine ring system found only in the active compounds. (The structure of alkaloid Q, one of the major alkaloids of *Veratrum californicum*, has not been elucidated, but infrared analysis suggests the absence of a tetrahydrofurylpiperidine system).

cases in lambs and occasionally occurred in rabbits. An externally evident hydrocephalus was common in rabbits as with sheep. Maxillary, premaxillary, and nasal bone shortening was not pronounced in rabbits although common in sheep. Further, the anophthalmia and microphthalmia preseent at very low incidence in sheep was not seen in rabbits.

Discussion. These data suggest that the rabbit will indeed be a useful test animal for the further study of veratrum alkaloid-induced cyclopia. The rabbit system is promising because a single oral dose on day 7 using only about one tenth the amount of teratogen used in sheep, produced malformations. Secondarily, multiple offspring provide better statistical probability of acceptable incidence. Thirdly, the compound specificity appears to be the same as in sheep. Finally, there appears to be considerable similarity in essential aspects of the external gross morphology of the lesion in rabbits and sheep.

A rather high incidence of fetal deaths was observed particularly in rabbits fed veratramine and veratrine. We have observed similar effects in sheep with veratramine and various mixed ester preparations such as veratrine (4, 8). The very marked toxic effect in the dam (as evidenced by the high mortality rate at these doses) may account for this. Fraser and Fainstat (9) suggest that a compound producing fetal deaths at one dose should be a teratogen at lower doses. Neither veratramine nor veratrine produce cyclopia in sheep at any dose (8), and we suppose they will not in rabbits at lower doses. Since approximately 30 normal and no malformed offspring were born to does at high doses of each compound, it seems improbable that lower doses would be teratogenic.

Production of malformations in rabbits with these teratogens by routes other than oral is under investigation in our laboratory. Preliminary results are encouraging.

Summary. Fetal rabbits become malformed in ways grossly similar to lambs upon maternal ingestion of cyclopamine and jervine, naturally-occurring teratogens of *Veratrum californicum*. Other veratrum alkaloids tested were inactive. The cyclopia and related

cephalic malformations occurred when ingestion took place on the 7th day of gestation.

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Received Sept. 30, 1970. P.S.E.B.M., 1971, Vol. 136.