Chemical Compounds of *Veratrum californicum* Related to Congenital Ovine Cyclopian Malformations: Extraction of Active Material. (29178)

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Binns and co-workers(1,2) have established that the congenital cyclopian malformation of lambs observed to occur spontaneously under certain conditions(3) is due to ingestion of the plant *Veratrum californicum* by the pregnant ewe. The malformation may reflect an inhibition of cell division during fetal development. Feeding trials have established(4) that the malformation can be produced if dried, ground plant material is ingested late in the second and early in the third week of gestation.

Plants of the *Veratrum* genus synthesize a series of complex steroidal alkaloids which have been of interest pharmacologically because of their hypotensive activity, and therefore have been rather thoroughly characterized chemically by a number of workers(5). *Veratrum* alkaloids interfere with nuclear and cellular division in the onion as shown by Burroni(6) and by Smith and Hiner(7). *Veratrum* has been reported to contain an alkaloid which inhibits growth of Candida(8) perhaps by inhibition of cell division. Alkaloids of this series are well known for their insecticidal characteristics(9) although this action may be unrelated to cell division. Experimental work to be described elsewhere has shown that certain *Veratrum* series alkaloids produce malformation in chick embryos. Further Korte and Weitkamp(10) have reported that the glycoside condurangin, isolated from the plant *Marsdenia condurango*, and structurally related to some of the *Veratrum* alkaloids(5), has cytostatic activity against normal and cancerous mammalian cells.

Because of these biological properties and structural considerations associated with the *Veratrum* alkaloids, our first premise has been that one or more of this class of compounds would prove to be the teratogen.

To study the mechanism whereby fetal development is arrested or altered in this deformity, we are attempting to extract, isolate and characterize the teratogenic compound(s) from the plant. Procedures for extraction of plant material were designed to provide a good yield of alkaloid while allowing extraction of other classes of compounds to be separated in subsequent steps.

The present report describes the extraction of the active principle(s), preliminary experimental work on purification, and the significance of the extractability and solubility of the active principle(s) in various solvents. Inferences as to the chemical nature of the material drawn from the experimental work and literature reports are discussed.

Materials and methods. Collection and preparation of plant material. Veratrum californicum plants growing in Utah and Idaho were cut about 10 to 20 cm above the ground when they had attained a height of 1 to 1½ meters and when 30% had reached the flowering stage. The stalks and leaves were chopped and dried at a temperature not exceeding 60°C. The dried pieces were then finely ground. Root and rhizome material was chopped and dried similarly after washing.

Extraction and purification of plant material. Extraction was accomplished by slight modifications of the methods of Jacobs and Craig(11,12) and of Fried, White and Wintersteiner(13).

In the first experiment 75 kg of ground stalks and leaves were extracted by percolation 3 times with sufficient 95% ethanol to cover the plant material. The residue was dried and re-extracted 3 times with H_2O. The alcohol was removed by vacuum distillation (temperature $< 60°$) leaving the syrupy initial alcohol extract. The initial alcohol extract, the water extract and the residue were all stored at —20°C until used for feeding trials.

In the second experiment 25 to 50 kg lots

of the stalks and leaves were extracted first with benzene and ammonia, then secondarily with ethanol in the manner described by Jacobs and Craig(11,12).

Purified alkaloid fractions were prepared from dried ethanol and benzene extracts by a double partition between aqueous acid and chloroform phases(11) (into 1% H_2SO_4 from $CHCl_3$, back into $CHCl_3$ from the alkalinized aqueous phase). The final chloroform phase was dried *in vacuo* and this residue was the purified alkaloid preparation. The yield of the latter in various preparations approximated 1 to 5 g/kg dry weight of original plant material.

The initial alcohol and the initial benzene extractions provide the alkamine and the ester forms of the *Veratrum* series alkaloids in good yield(12,13). With alcohol as solvent, a secondary extraction after the benzene extraction provides the glycosidic forms in good yield somewhat free of alkamine and ester (11). Most of the esters and alkamines are more organophilic while the glycosides and a very few alkamines and esters are somewhat more hydrophilic. The water extract was included to cover other compound classes.

Bioassays and methods of feeding. Ewes were force-fed the experimental preparations by stomach tube, except where otherwise noted, at the levels and schedules indicated below. Fifty days after breeding, the ewes were killed and examined for evidence of nonpregnancy, pregnancy, or pregnancy with fetal death. All fetuses were examined for malformations. Fetal deaths, which were evidenced by intrautero changes including alteration in cotyledon pigmentation and tissue turgidity and by late recycling for breeding, were considered equivalent to malformations and were thought to result from excessively high doses of the teratogen.

Levels of extracts fed in the first experiment were governed by the toxicity of the most toxic preparation. The most toxic preparation was fed at the highest possible nonlethal level and the levels of other preparations were fed on an equivalent plant weight basis. Preparations in the second experiment and purified materials were fed usually at as high a level as possible governed by their toxicity. In the first experiment the ethanol extract, the water extract, the residue and a recombination of all 3 were fed at levels equivalent to 212 g of original plant material per dose. A purified alkaloid preparation from the ethanol extract was administered intraperitoneally at 230 gram plant equivalents per dose. In the second experiment the benzene extract was fed at 460 g plant equivlents, the residue at 230 g plant equivalents and the ethanol extract at 2 different levels, 616 and 220 g plant equivalents per dose. At the 616-g level the extract was fed once per day on the 12th, 13th and 14th days only. The other preparations in Exp. 1 and 2 were fed or administered once per day from the 7th through the 15th days of gestation.

Groups of 8 to 12 animals were fed unfractionated plant material from the 7th through the 15th days as controls on fractionation losses. They were fed at levels of 150 to 200 g dry weight of plant material per dose per day, the standard level required to produce malformations. Other groups of 8 to 12 were fed similarly with plant material from widely separated areas which had been assayed for total alkaloid content. This allowed a comparison of total alkaloid content, toxicity in feeding and ability to produce malformations. Groups of 2 to 3 ewes were fed protoveratrines A and B* at 70 to 100 mg levels per dose twice daily, veratrine alkaloids of Sabadilla† at 160 to 180 mg levels per dose twice daily, and mixed ester alkaloid preparations‡ at 100 to 160 mg levels per dose twice daily on the 12th, 13th and 14th days of gestation to examine teratogenicity of *Veratrum* ester alkaloids.

Chemical assay. Total alkaloid content of fractions during preparation and assay of alkaloid content in plants from various sources was followed semiquantitatively by turbidimetry after addition of the Mayer reagent(14) to an aqueous acid phase containing the alkaloids. Paper chromatographic

* California Biochemical Corp., Protoveratrine, C grade, Catalog #5397.

† Mann Research Labs., Veratrine alkaloid, Catalog #6156.

‡ Irwin Neisler & Co., Cryptenamine, and Riker Laboratories, Veriloid®.

examination of various preparations was done by the method of Hegi and Flück(15) using pH 5.4 water saturated ethyl acetate as the developing solvent. Thin layer chromatography was employed using silica gel as adsorbent with $CHCl_3$:methanol (6:1) as the solvent. Alkaloids were detected in all cases by bromphenol blue spray followed by sufficient acid to turn the background yellow. The purified alkaloid material from the alcohol extract was subjected to methanolic base and acid hydrolysis(11,16) to determine if hydrolysis of sugar groups by the latter or ester groups by the former could be demonstrated by Rf changes in the spots. Crystallization techniques to recover pure alkaloids were those of Jacobs and Craig(11,12).

Results. In the first experiment neither the ethanol nor the water extract produced malformations in offspring from 20 ewes. The residue produced malformed twins from a single ewe of 10. The recombined extracts and residue produced 4 malformed lambs and 2 fetal deaths from 5 of 10 ewes. This was nearly the same incidence produced by two-thirds the weight of unfractionated stalks and leaves (6 malformed and 4 fetal deaths from 8 of 12 ewes fed). And finally the purified ester alkaloids from the ethanol extract produced no malformations.

In the second experiment neither the benzene extract fed 5 pregnant ewes nor the residue fed 7 pregnant ewes produced fetal deaths or malformations in the offspring. The ethanol extract was relatively free of toxicity since the ester alkaloids had been largely removed by the benzene extraction and in part remained in the residue. This material could therefore be fed at a higher level. It produced one malformation and one fetal death from 2 of 3 ewes when fed at a level equivalent to 616 g original plant material per day on the 12th, 13th and 14th days of gestation. It failed to produce malformations when fed to 5 pregnant ewes at 220 g plant equivalents.

Limited trials failed to produce malformations with pure mixed protoveratrines A and B, veratrine alkaloids of Sabadilla, or with ester alkaloid preparations from *Veratrum viride* root and rhizome.

Experiments in which plant material collected from widely separated areas was fed pregnant ewes indicated that there was little or no relationship between total alkaloid content of the plant and its ability to produce malformations. Further there seemed to be little or no relationship between the toxicity (*i.e.*, level of ester alkaloids) and ability to produce malformations.

Partial results from other experiments presently under way, to be reported elsewhere, have verified the ready extractability of the teratogenic material by ethanol. Six additional malformations and fetal deaths have been observed thus far from ewes fed similar ethanol extracts. In addition, a purified alkaloid preparation from a secondary ethanol extraction of plant material after an initial benzene extraction has thus far produced 3 malformations in fetuses from 2 of 3 ewes when fed at levels which progressively increased from 200 to 800 g equivalents of original plant material per dose on the 12th, 13th and 14th days of gestation. Yield of the teratogen (activity) during purification was at a minimum one-fourth of the total present in the original plant material, while weight yield was about 1 g per 1000. Thus specific activity increase in the purified preparations was at least 250-fold.

Separation by paper chromatography of the alkaloids from the various extracts revealed the following information. The benzene extract contained approximately a dozen alkaloids with Rf values distributed from near zero up to approximately 0.95. The initial ethanol extract contained about the same number although the distribution and intensities differed somewhat. The secondary ethanol extract and its purified product, which produced the malformations, contained 5 or 6 detectable alkaloids all with rather low Rf values indicating the more hydrophilic nature of the alkaloids present. Changes in Rf values on thin layer chromatograms of the latter material were evident after both acid and base hydrolysis. On the basis of these results, there appeared to be 1 ester (Rf change in basic hydrolysis) and 2 or 3 glycosidic alkaloids (Rf changes in acid hydrolysis) in this fraction along with 2 alkamines

(no Rf change). One of the glycosides was tentatively identified as veratrosine and one of the alkamines as jervine by thin layer chromatographic behavior.

These two alkaloids were readily isolated by crystallization from the purified alkaloids of the ethanol extract of root and rhizome material from this plant. The root and rhizome material had a much higher alkaloid concentration, appeared rather similar on chromatography to leaf and stem material and also produced malformations. The alkamine jervine was isolated and identified after 2 recrystallizations in ethanol-water by a melting point of 244 to 246°C, by optical rotation of $a_D^{25} = -150°$, (C = 5.0 in $CHCl_3$:ethanol, 1:1), by its picrate, MP 273° C, and hydrochloride, MP 325°C (dec). The glycosidic alkaloid veratrosine, after a single recrystallization from methanol-water, was identified by MP 230 to 235°C, and optical rotation of $a_D^{25} = -55°$ (C = 2.5 in $CHCl_3$:ethanol, 1:1).

Discussion. The experimental work lends support to our first premise that one or more of the *Veratrum* series alkaloids will prove to be the active teratogen.

In the first experiment the active material evidently had been extracted, probably by the ethanol. It appears that we failed to produce malformed fetuses because it was not possible to feed the extract at a high enough level due to the toxicity of the ester forms of the alkaloids present in that extract.

In the second extraction experiment, the initial benzene extraction had removed an appreciable quantity of the toxic esters. This made it possible to feed the alcohol extract at a level sufficient to produce the malformation and fetal death which resulted.

The fact that no extract or purified preparation rich in ester alkaloids and no commercial ester alkaloid preparation produced malformations would tend to suggest that if the teratogenic material is of the *Veratrum* alkaloid class, it is probably a hydrophilic glycoside or alkamine form. It has not been possible to determine if high levels of ester forms would produce fetal malformations because of the lethal toxicity to the ewe at even low levels. The known glycosidic alkaloids of the *Veratrum* series are more readily extracted with ethanol than with benzene(11). The secondary ethanol extract described here which produced malformations contained at least 2 or 3 glycosidic alkaloids, one of which was identified as veratrosine. It also contained the alkamine jervine. The latter two along with veratramine, the parent alkamine of veratrosine, and pseudojervine, the glycoside of jervine, possess ring structural similarities to that of the cytostatic compound condurangin. Selenium dehydrogenation of the aglycone of the latter and of veratramine(5) both yield the Jacob's hydrocarbon. Jervine differs significantly from veratramine only in having a carbonyl oxygen at position 11 and an ether bridge between carbon atoms 17 and 23(5).

The fetus appears to be susceptible to the teratogen only during a very brief period of gestation. It would be desirable to determine what factors influence this susceptibility and why only those cells associated with the clinical manifestations of this malformation are evidently affected. Until the teratogen(s) is identified, it will be difficult to postulate a mechanism for the malformation at the cellular or molecular level.

It would be profitable to be able to use intravenous or intraperitoneal injection of purified materials for convenience and to conserve limited quantities available provided rumen microorganisms play no role in this process such as production of the teratogen from a plant precursor.

Experiments presently under way are designed to establish whether rumen microorganisms are involved. Other experiments under way will investigate the teratogenicity of various plant parts, plants obtained from other locations, other *Veratrum* species and purified alkaloids. We are also attempting to identify the active principle(s) in the secondary ethanol extract and its purified alkaloid fraction by further purification and isolation as well as by structural alteration experiments.

Summary. The teratogenic material from *Veratrum californicum* has been extracted from dried plant material by an ethanol extraction of the plant residue remaining after

an initial benzene extraction in presence of ammonia. A 250-fold purified preparation from this extract also produced the malformations. It is theorized that the teratogenic material may be an alkaloid of the *Veratrum* series, likely a glycoside or parent alkamine.

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Effect of Injected DNA on a Mild Hypercholesterolemia in Rabbits.* (29179)

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It has been noted in this laboratory that a single injection of purified, undenatured deoxyribonucleic acid (DNA) produced in rabbits a prolonged decrease in serum lipids, with no return to control levels for at least 2 years(1,2). DNA preparations from heterologous, homologous and autologous mammalian tissues were all effective, indicating that no new, specific, transferable, genetic information was introduced into the animals by the DNA(3). The lowered mean serum cholesterol levels largely reflected the significant decrease in those rabbits which maintained high normal values. Varying the amount of DNA injected over a 50-fold range showed a dose-response relationship in length of time required for the changes to appear(2).

It would be interesting to know what effect the DNA injections produce during an experimental dietary hypercholesterolemia. One such attempt has been reported from this laboratory(4). The feeding of a usually innocuous 0.5% cholesterol diet to rabbits, injected with DNA 4 months before, resulted in death of 100% of the DNA-injected animals. This unexpected lethal effect was not understood but it was hoped that a milder form of hypercholesterolemia might permit experimental study.

Several investigators have induced significant hypercholesterolemias by feeding rabbits saturated fats along with synthetic or semi-synthetic diets(5,6). Steiner and his colleagues(7) produced a more moderate elevation of serum lipids in rabbits by feeding them a standard laboratory diet (non-synthetic, containing small amounts of fat,

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