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TERATOGENIC COMPOUNDS OF *VERATRUM CALIFORNICUM* (DURAND)—IV.

FIRST ISOLATION OF VERATRAMINE AND ALKALOID Q AND A RELIABLE METHOD FOR ISOLATION OF CYCLOPAMINE

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Abstract—The known steroidal, teratogenic alkaloid veratramine and another alkaloid, designated alkaloid Q with as yet unknown structure and biological activity, were isolated for the first time from *Veratrum californicum*, a plant that induces teratogenic effects in sheep. A more reliable method than previously available is also described for isolation of cyclopamine, the alkaloid that induces cyclopian teratogenic effects in sheep.

THE plant *Veratrum californicum* induces cyclopian and related central nervous system and facial malformations in offspring from ewes that ingest the plant on the 14th day of gestation.[1] We previously isolated from the plant and demonstrated that the responsible teratogens were certain glycosidic and parent alkamine steroidal alkaloids, including a new alkaloid, then designated alkaloid V and for which we now propose the trivial name cyclopamine.[2,3] Another parent alkamine of apparently similar structure,[2] veratramine, induces congenital abnormalities although of a noncyclopian type.[4] They are characterized by slight lateral or medial bowing of the front legs, slight to marked flexure of the knee joints, looseness (hypermobility) of the hock and stifle joints, or complete lack of skeletal muscular control.[4] *V. californicum* plant or root induces these same congenital abnormalities,[5] which suggested the presence of veratramine in the plant. Further, we previously isolated veratrosine, the glycoside of veratramine, from the plant[2,6] which suggested the presence of the latter in the plant as a precursor.

We describe here the first isolation from *V. californicum* of veratramine and also another alkaloid designated alkaloid Q, an alkaloid of as yet unknown biologic effect and structure. We further describe here a convenient and reliable method for isolation of pure cyclopamine, the cyclopia inducing alkaloid, previously obtained in preparations contaminated[2] with what is now known to have been veratramine. Included are various physical constants for the three alkaloids.

Evaporation of the benzene-extractable alkaloids from *V. californicum* roots resulted in crystallization of a colored product, which upon washing in cold benzene proved by TLC to

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[3] R. F. KEELER and W. BINNS, *Can. J. Biochem.* **44**, 829 (1966).
[4] R. F. KEELER and W. BINNS, *Proc. Soc. Exptl Biol. Med.* **123**, 921 (1966).
[5] R. F. KEELER and W. BINNS, manuscript in review.
[6] R. F. KEELER and W. BINNS, *Proc. Soc. Exptl Biol. Med.* **116**, 123 (1964).

be a mixture of some eight to ten alkaloids. Recrystallization of this product from acetone–water and methanol–water yielded a preparation enriched with respect to three alkaloids. One of these, the middle spot on TLC chromatograms, proved to be cyclopamine; the slower running spot had an R_f identical to veratramine; the faster spot was unlike previously isolated and available authentic alkaloids of the steroidal veratrum class and was designated alkaloid Q.

Column chromatography on silica gel (Fig. 1) and pooling of appropriate effluent tubes provided the six fractions (A–F in Fig. 1). Nearly pure cyclopamine was obtained from fractions E and F from which the pure compound was obtained by crystallization from

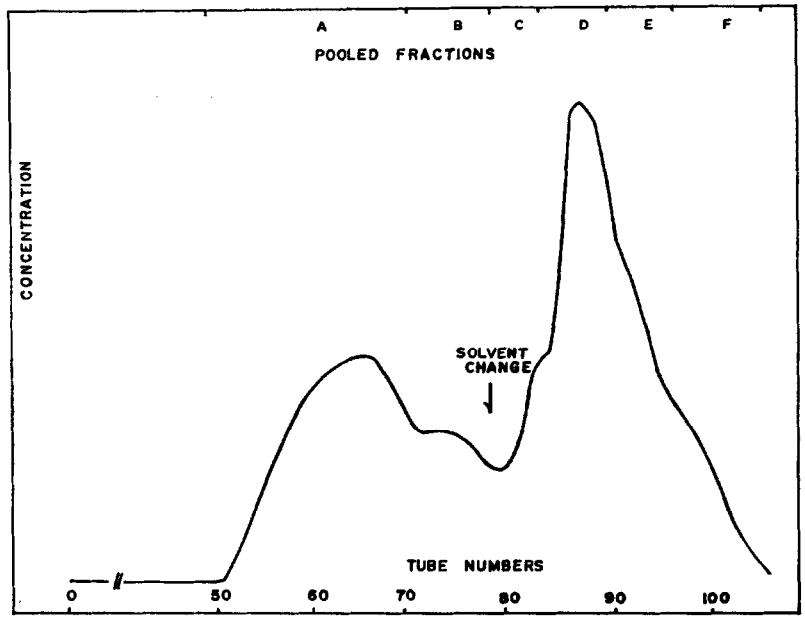

FIG. 1. SEPARATION PATTERN OF RECRYSTALLIZED BENZENE EXTRACTABLE ALKALOIDS CHROMATOGRAPHED ON SILICA GEL SHOWING A SMOOTH PLOT OF THE RELATIVE ALKALOID CONCENTRATION RELATED TO EFFLUENT TUBE NUMBERS AND SUBSEQUENTLY POOLED FRACTIONS. THE POSITION OF THE SOLVENT CHANGE FROM 60:1 TO 20:1 (BENZENE:METHANOL) IS INDICATED.

ethanol–water. Enriched preparations of veratramine (fraction C) and alkaloid Q (fraction A) were obtained. Pure samples of the latter two alkaloids were obtained by rechromatography and subsequent crystallization.

Characterization of the preparation presumed to be veratramine by melting point, optical rotation, molecular weight, carbon–hydrogen–nitrogen composition, u.v. and i.r. spectra proved its identity with authentic veratramine.[7,8] Characterization of cyclopamine, similarly verified its identity with previously isolated cyclopamine, then designated alkaloid V.[2] Characterization of the alkaloid labeled Q by similar means failed to reveal its identity with any known veratrum alkaloid.

Infrared spectra of the three isolated alkaloids are recorded in Fig. 2.

[7] K. SAITO, *Bull. Chem. Soc. Japan* **15**, 22 (1940).
[8] W. A. JACOBS and L. C. CRAIG, *J. Biol. Chem.* **160**, 555 (1945).

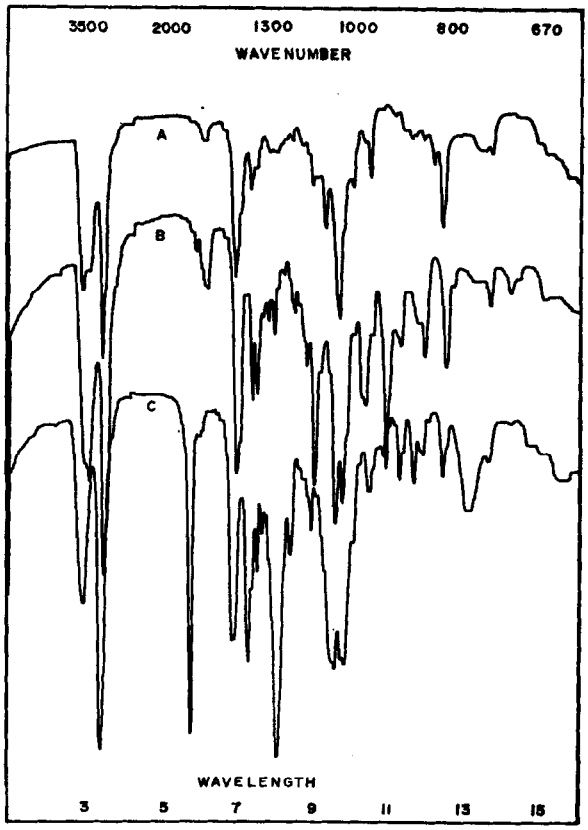

FIG. 2. INFRARED PATTERNS OF THE THREE ISOLATED ALKALOIDS. A IS VERATRAMINE, B IS CYCLOPAMINE, C IS ALKALOID Q.

EXPERIMENTAL

Extraction of Plant Material

The plant roots used were harvested in July, 1965, in Franklin Basin, Idaho. The roots were washed, chopped, air dried in the sun, and then ground to a fine consistency in a hammer mill. Because of additional use for biologic studies, rather large quantities were extracted. Typically, 150 kg was extracted by covering it with benzene: 5 per cent NH_4OH (3:1, v/v). After 24–48 hr, the benzene was allowed to drain through. The material was re-extracted similarly three or four times with fresh benzene. The pooled extracts were evaporated at reduced pressure and about 50–60° to about 1/100 original volume, whereupon crystallization of a mixed alkaloid fraction occurred.

Fractionation and Crystallization

The benzene extractable crystallized, mixed alkaloid preparation was washed in cold benzene and recrystallized in acetone–water and then in methanol–water. This purified preparation was then subjected to column chromatography on silica gel. 300 mg of the preparation was brought into solution in 3 ml benzene: methanol (10:1) and loaded on the silica gel column previously packed from a slurry of silica gel in benzene: methanol (60:1). The column packing was 20 × 250 mm. Elution was accomplished by the addition of 400 ml benzene:methanol (60:1) and then 200 ml of benzene:methanol (20:1). 5-cm^3 samples of the eluate were collected, a suitable aliquot shaken into 1 per cent H_2SO_4, an aliquot therefrom treated with the Mayer reagent and the relative alkaloid concentration determined turbidimetrically.[2] The column elution pattern is shown in Fig. 1. The eluted solvents containing alkaloid were pooled on the basis of the plot of alkaloid concentration to yield six pooled fractions designated A–F in Fig. 1. The collected pooled fractions from three separate column runs were treated as follows: Pooled fractions E and F (0·1816 g) were recrystallized

thrice from ethanol–water and yielded 0·083 g of pure cyclopamine. Fraction A (0·2409 g) was rechromatographed on a scaled-down silica gel column and recrystallized twice from methanol–water. This procedure yielded 0·1844 g of pure alkaloid Q. Veratramine was obtained by methanol–water recrystallization of 0·2448 g of fraction C, subsequent rechromatography, and final crystallization of the resulting fraction C in methanol–water carefully to yield three crops. The third crop (0·042 g) was pure veratramine.

Characterization

Veratramine. It was positive to the Mayer reagent, had m.p. 203–205, $[\alpha]_D^{25}$ −59° [C = 1 per cent in ethanol: $CHCl_3$ (3:1)]; λ_{max}, 265 nm. TLC revealed a single spot with R_f identical to that of authentic veratramine. The i.r. spectrum is shown in Fig. 2. It had a molecular weight of 394.

(Found: C, 76·88; H, 9·42; N, 3·09. Calc. for $C_{27}H_{39}O_2N.H_2O$: C, 75·83; H, 9·66; N, 3·28 per cent.) The analytical results, physical constants and spectrum of the isolated compound were essentially identical to authentic veratramine, but analytical results differed from calculated values for anhydrous veratramine presumably because of the hygroscopic nature of the compound.[7]

Cyclopamine. It was positive to the Mayer reagent, had m.p. 232–234, $[\alpha]_D^{25}$ −48° [C = 1 per cent in methanol: $CHCl_3$ (2:1)]; λ_{max} 248 nm. TLC revealed a single spot. The i.r. spectrum is shown in Fig. 2. It had a molecular weight of 423.

(Found: C, 77·50; H, 10·04; N, 2·96. $C_{27}H_{43}O_2N$ required: C, 78·39; H, 10·48; N, 3·39 per cent.)

The i.r. spectrum was similar to that previously obtained[2] but melting point, u.v. spectrum and optical rotation were somewhat different, presumably due to the contamination of previously prepared samples with veratramine.[2]

Alkaloid Q. It was positive to the Mayer reagent, had m.p. 209–210, $[\alpha]_D^{25}$ −95° [C = 1 per cent in ethanol: $CHCl_3$ (3:1)]; λ_{max}, 278–290 (broad shoulder) nm. TLC revealed a single spot. The i.r. spectrum is shown in Fig. 2. It had a molecular weight of 513.

(Found: C, 75·39; H, 10·41; N, 2·98. $C_{32}H_{53}O_4N$ required: C, 74·52; H, 10·36; N, 2·72 per cent.)

The analytical results, physical constants and spectra of the isolated compound were unlike known veratrum alkaloids.[9–11]

Methodology. Melting points were determined on a Fisher–Johns apparatus and were uncorrected. Optical rotations were determined on a Kern polarimeter. U.v. spectra were obtained in ethanol on a Beckman model DB spectrophotometer. TLC was done on alumina plates, developed in benzene: methanol (6:1 v/v) and successively sprayed with bromphenol blue in acetone (20 mg/100 ml) and then with 0·5 M phosphate–citrate buffer at pH 3·95. The plates were photographed rapidly before fading occurred. Molecular weights were determined cryoscopically in camphor. Nitrogen was determined by the micro Kjeldahl method. Carbon and hydrogen were determined as previously.[2] I.r. spectra were obtained from samples in KBr disks with a Beckman IR-4 spectrophotometer, 2 × standard slit, period 2, gain 2·5 per cent with a chart speed of 2 μ/min, but with the scale compressed to yield spectra of 5 × 10 in. in dimension. The spectra were stacked with the use of a special device designed for this purpose.[12] Authentic veratramine was obtained from the S. B. Pennick Co.

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