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Title: A PHYTOCHEMICAL STUDY OF ACHLYS TRIPHYLLA (SMITH) DC., (BERBERIDACEAE)

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Achlys triphylla, a member of a medicinally active family containing alkaloids, was examined phytochemically to determine its possible value as a source of medicinal agents.

Pharmacological testing of the crude ethanol extract exhibited a competitive inhibition of acetylcholine and histamine. Purification of alkaloid 'A' from the crude ethanol extract and subsequent pharmacological testing showed a lack of any biological activity.

The alkaloid responded to typical alkaloid reagents and was isolated by means of alumina column chromatography. Preliminary data indicated that the alkaloid's identity was magnoflorine, a quaternary alkaloid commonly occurring in the plants of this family. However, its IR spectrum was different from that of magnoflorine and it could be precipitated from its acidic solution by ammonium hydroxide. Similarly, its iodide salt could not be prepared by the

method commonly used for preparing the derivative for quaternary bases. The data suggested the alkaloid may be the tertiary analog of magnoflorine, namely, corytuberine. Co-chromatography and the UV spectrum of alkaloid 'A' and authentic corytuberine showed that the two compounds were not identical. The empirical formula $C_{18}H_{19}O_4N$ was determined for alkaloid 'A' (melting point 243-5°C. decomp.) from elemental analysis and available spectral data.

The presence of the simple quaternary base, choline, in the ethanol extract was noted by a characteristic purple coloration with Dragendorff's reagent. This simple base was not isolated, but its identity was established by means of extensive co-spotting experiments. Four different TLC developing systems (three acidic and one alkaline) and four different visualizing reagents were used to identify the choline spots.

A carbohydrate was isolated from the ethanol extract. Its identity was concluded to be sucrose from its melting point, its behavior on acidic and enzymatic hydrolysis, and the melting points of its osazone and octa-acetate derivatives.

The commonly occurring phytosterol, β-sitosterol, was isolated from the roots and rhizomes. Melting point, mixed melting point, IR spectra, and co-chromatography in three solvent systems confirmed its identity.

A PHYTOCHEMICAL STUDY OF *ACHLYS TRIPHYLLA* (SMITH) DC., (BERBERIDACEAE)

by

Alvin Edmund Born

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A PHYTOCHEMICAL STUDY OF ACHLYS TRIPHYLLA (SMITH) DC., (BERBERIDACEAE)

I. INTRODUCTION

Achlys triphylla (Smith) DC., commonly known as vanilla leaf, is a member of the medicinally important family Berberidaceae. This plant family is comprised of several genera including Berberis, Podophyllum, and Caulophyllum which yield various medicinally useful agents. The genus Achlys, composed of only two species, has received very little attention.

Berberidaceous plants, although known primarily as alkaloid bearing plants, do produce other agents of medicinal importance. The perennial herb Podophyllum peltatum has long been used as a cathartic because of its resin content. The plant has even been claimed to correct certain liver dysfunctions (3, p. 213). Recently, a constituent of this plant, podophyllotoxin, has been shown to possess certain tumor-inhibiting properties (9). Podophyllum resin is presently incorporated in Compound Colocynth and Jalap Pills and Compound Pills of Cascara (Hinkle's Pills). The United States Pharmacopeia XVII (14, p. 481-2) officially lists podophyllum resin as a 25% dispersion in compound benzoin tincture, or as an ethanolic solution to be used as a caustic agent for certain papillomas.

Extracts of the fruit, bark, roots and rhizomes of numerous

Berberis species have produced many alkaloids. Much of the biological activity attributed to these plants is probably due to their alkaloid content. In moderate doses (usual dose--0.3-0.6 ml of fluid extract) they have been used not only as tonics, diuretics, and anti-periodics, but also to cure uterine diseases, eczema, and psoriasis. In larger doses they have been used as cathartics or hepatic stimulants (3, p. 214-5; 5, p. 317; 6, p. 34). French (4) reported that some of the Indian tribes of the Pacific Northwest used various Berberis species as a source of pigment to color manufactured objects.

The Indians of the Northeastern United States administered an infusion of the roots and rhizomes of Caulophyllum thalictroides to aid in childbirth, often drinking it for several weeks prior to labor (3, p. 214). This usage of the plant by the Indians was reflected in its common names, Papoose Root or Squaw Root.

Old materia medica literature often referred to the herb Achlys triphylla (Berberidaceae), but its medicinal value was never established (5, p. 318; 17, p. 20). Stuhr (17, p. 20) suggested the need for further study of this herb. A survey of the literature disclosed only one report on the chemical constituents of A. triphylla. Coumarin, in a 0.2% yield, was isolated by Bradley (2) from an aqueous extract of the air-dried plant.

Achlys triphylla belongs to a medicinally active and an alkaloid-bearing family. Since it is generally accepted that plants related phylogenetically are usually related chemically, the present investigation was undertaken to determine if the Berberidaceous alkaloids could be identified in a related species. A secondary objective was to determine if any other useful medicinal agents were present in A. triphylla.

Botanical Classification and Description

Berberidaceae, commonly referred to as the Barberry family, contains about 12 genera and 500 species, mostly found in the Northern Hemisphere. Oregon Grape, Oregon's state flower and Podophyllum peltatum, whose resin is often used as a hydrogogue cathartic, are two well known members of this family (3, p. 213; 8, p. 413; 13, p. 346).

The genus Achlys contains only two closely related species. Achlys japonica Maxim. is found only in Japan with distribution restricted to the islands of Hokkaido and the Northern District of Honshu (12, p. 465). Achlys triphylla is found growing on the western slopes of the Cascade Mountains of the Pacific Northwest. Its distribution ranges from British Columbia, in the north, to northwestern California in the south. The plant is usually found growing in the deep woods, but also may be located in more open areas,

especially along streams. The perennial herb grows in such close proximity to one another that it often forms a canopy of leaves about six inches above the ground. Hitchock (8, p. 413) describes the species as follows:

> Scapose perennial herb, spreading widely by rhizomes, glabrous throughout; leaves with petioles 10-30 cm long, the blades 5-20 cm broad, 3 foliated, the segments flabelliform, coarsely sinuatedentate; scapes 20-40 cm long; spikes 2.5-5 cm long; fruits reddish-purple, 3-4 mm long, puberulent, the ventral side incurved (concaved), but with a very prominent cartilaginous ridge, the dorsal surface strongly convex, with a slight ridge.

The scape is slender and is equal to or surpassing the leaf in length. The dense spike bears many small flowers which bloom in April to July. This plant, due to its inconspicuous flowers, was named Achlys, after the Greek goddess of night or obscurity.

The plant is known by several synonyms, i.e. deer foot, wild vanilla, and vanilla leaf; the last two synonyms being due to the pleasant smell of the dried leaves (8, p. 413; 13, p. 348; 17, p. 20).

II. EXPERIMENTAL

Collection and Storage

The whole plant of Achlys triphylla used in this study was collected from the western slopes of the Cascade Mountains, east of Eugene, Oregon. Collections were made during the summers of 1965 and 1966 in sections 14 and 16 of Township 16 S., Range 6 E. of the Willamette meridian. The plant's identity was authenticated by Dr. Kenton L. Chambers, Director of the Herbarium, Oregon State University and a voucher specimen was deposited therein.

The plant material was separated into two groups, (1) leaves and stems and (2) roots and rhizomes. The two groups, after having been dried in a forced-air drier at 40°C. for 18 hours, were ground to a coarse powder in an Abbe' mill. The powdered plant parts were then stored, in the dark, in air-tight plastic bags until time for analysis. For this investigation, only the roots and rhizomes were examined.

Thin-layer Chromatography--Methodology

Thin-layer plates were prepared by thoroughly mixing 30 Gm of silica gel G [1] with 60 ml of distilled water for 90 seconds. Using a

[1] E. Merck AG, Darmstadt (Germany). U. S. distributors, Brinkmann Instruments, Inc., Cantiague Road, Westbury, New York.

DeSaga apparatus the slurry was spread uniformly on 20 x 20 cm glass plates to a thickness of 250 μ. The coated plates were activated by heating in a 110°C. oven for 30 minutes and were subsequently stored in a desiccator.

For chromatography, 1 cm wide channels were etched in the silica gel G layer. The extracts were applied with a capillary tube 1.5 cm from the bottom of the plate. The plates were developed in a closed, filter paper lined, saturated chamber and the solvent front was allowed to ascend a minimum of 10 cm. The compounds were visualized by viewing under UV light and/or spraying with the desired spray reagent, as was necessary.

Unless otherwise stipulated, thin-layer chromatography (TLC) was carried out according to the above procedure.

Preliminary Investigation

A 30 gram sample of the powdered root and rhizome material was exhaustively extracted in a Soxhlet extractor by a succession of four solvents. The extraction process was initiated with petroleum ether (90-95°C.), followed by chloroform, NF, alcohol, USP, and terminated with distilled water. Each extract was evaporated to ca. 5 ml and was subsequently analyzed for various classes of compounds by means of thin-layer chromatography (TLC).

Detection of Steroids/Triterpenes

The concentrated extracts were chromatographed using chloroform-acetone (CA, 9:1 v/v) as a solvent system. The compounds on the plates were visualized using antimony trichloride (10% w/w in chloroform) as a spray reagent and heating the plates in an oven at 110°C. for 3-5 minutes (16, p. 486). Pink to reddish-violet spots indicated the presence of sterols/triterpenes in the petroleum ether extract of the roots and rhizomes.

Additional evidence was obtained by the Liebermann-Burchard (L-B) color reaction. To about 5 mg of the dried petroleum ether extract was added two drops of acetic anhydride followed by two drops of sulfuric acid. Instant reddish-violet color indicated the presence of sterols/triterpenes in the petroleum ether extract of the roots and rhizomes of A. triphylla.

Detection of Alkaloids

The concentrated extracts were again chromatographed using n-butanol-glacial acetic acid-water (BAW, 4:1:5 v/v, upper phase) as a solvent system. The compounds on the plate were visualized using UV light [2] and Dragendorff's spray reagent. [3] The ethanol

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[2] Long wave length filter.

[3] Solution A: 0.8 Gm of bismuth subnitrate, 10 ml glacial acetic acid, 40 ml distilled water; Solution B: 40% (w/v) aqueous potassium iodide. Spray reagent: mix 5 ml of solution A and B with 20 ml of glacial acetic acid and dilute to 100 ml with distilled water (1, p. 361).

extract indicated the presence of one major alkaloid (UV gave a light blue fluorescence and a red-orange spot with Dragendorff's reagent).

Further evidence was established by means of Mayer's reagent.[4] To about 5 mg of the dried ethanol extract was added 1 ml of hydrochloric acid (10%) followed by two or three drops of Mayer's reagent. Instant formation of a white precipitate indicated the presence of an alkaloid in the ethanol extract of the roots and rhizomes of Achlys triphylla.

Extraction and Purification of the Roots and Rhizomes

Eight hundred and eighty grams of ground root and rhizome material was exhaustively extracted in a Soxhlet extractor by a succession of three solvents. Extraction was initiated with petroleum ether (90-95°C.) for 18 hours, followed by chloroform, NF, for 42 hours and terminated after 88 hours of extraction with alcohol, USP. This is summarized in Figure 1.

The petroleum ether and chloroform extracts were individually reduced to dryness in vacuo, at 55°C. in a rotary flash evaporator. The residue was placed aside for later study. The ethanol extract was reduced to a thick syrupy consistency in vacuo, at 55°C. in a rotary flash evaporator. To the reddish-brown colored fluid was

[4] 1.36 Gm mercury bichloride, 5 Gm potassium iodide dissolved in 100 ml of distilled water (10, p. 1174).

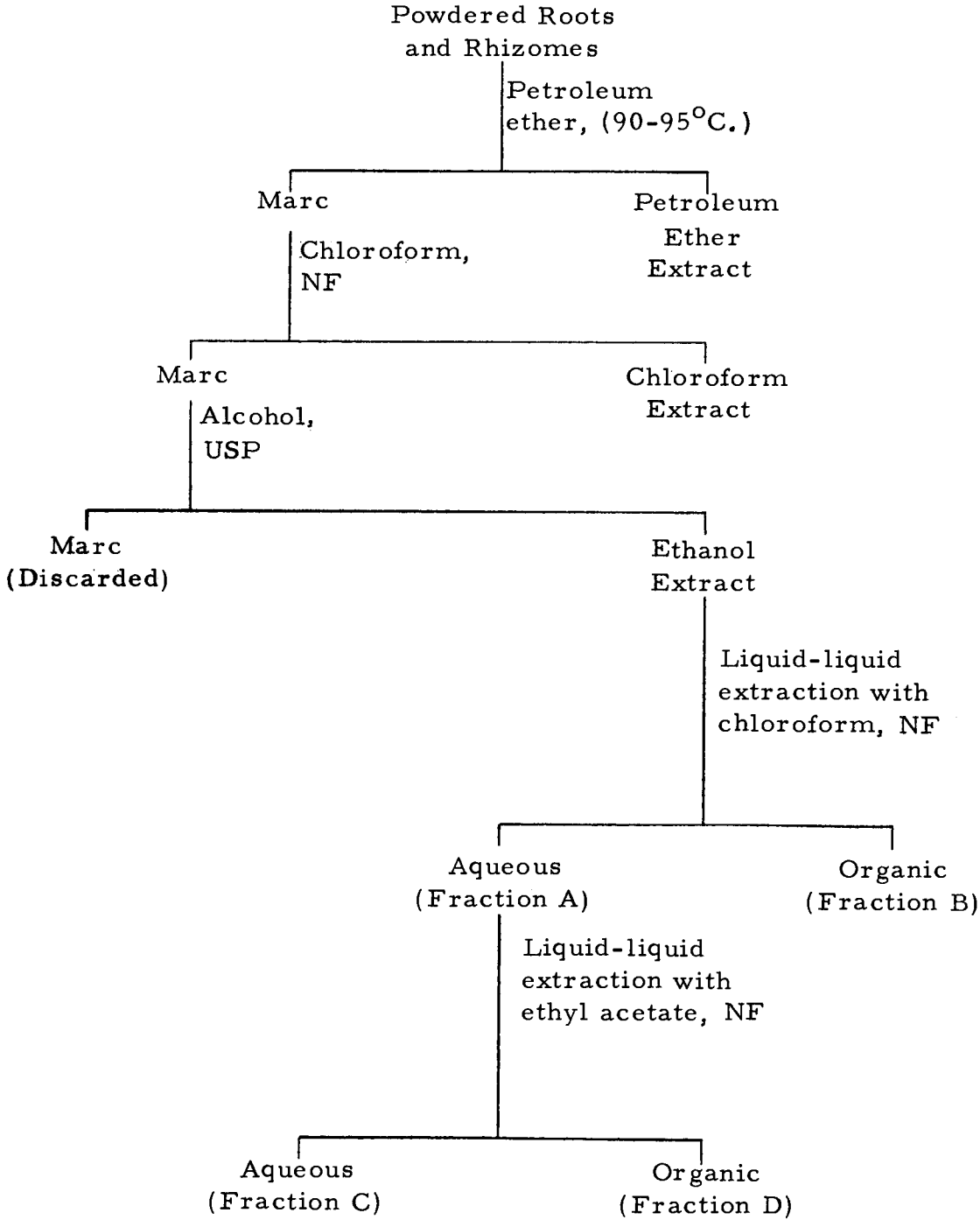

Figure 1. Extraction scheme for Achlys triphylla.

added 250 ml of distilled water giving a total volume of approximately 400 ml.

TLC plates of the ethanol extract were developed in BAW (4:1:5) and PAW (n-propanol-28% ammonia-water, 2:1:1 v/v). Red-orange spots developed upon spraying with Dragendorff's spray reagent with R_f = 0.20 in BAW and R_f = 0.62 in PAW.

The reddish-brown aqueous solution was subjected to 20 hours of continuous liquid-liquid extraction with chloroform, NF. The progress of the extraction was tested in the following manner. A filter paper strip was spotted with two or three drops of the extract and then allowed to dry. It was then sprayed with Dragendorff's reagent. A red-orange spot indicated the presence of the alkaloid in the extract. The aqueous extract (fraction A) had retained almost the entire quantity of the alkaloid while the chloroform extract (fraction B) had a negligible amount. No further work was done on fraction B.

The aqueous phase was separated and continuously extracted with ethyl acetate, NF, for 20 hours. The aqueous layer (fraction C) continued to retain the alkaloid. Fraction C was separated and permitted to evaporate. The resulting viscous fluid (ca. 250 ml) was adsorbed on 200 Gm of activated alumina, air-dried, and pulverized.

Column Chromatography

Three hundred grams of activated alumina[5] was packed dry inside a glass chromatographic column, 4.5 cm in diameter. The 42 cm high alumina column was flushed with chloroform, NF, for 30 minutes. Forty grams of the crude extract-adsorbed alumina mixture was deposited on top of the column and covered with 1.5 cm of Ottawa sand. The column was developed as shown in Table 1.

Table 1. Chromatographic development of Column A.

Fraction	Composition	Volume (ml)
1	chloroform-methanol (19:1)	2 x 1000
2	chloroform-methanol (9:1)	4 x 500
3	chloroform-methanol (5:1)	4 x 500
4	chloroform-methanol (3:1)	5 x 500
5	chloroform-methanol (1:1)	5 x 500
6	methanol	2 x 500

Each fraction was reduced to approximately 25 ml volume, *in vacuo*, at 50°C. in a rotary flash evaporator. To monitor the progress of the column, each fraction was chromatographed in BAW (4:1:5) and sprayed with Dragendorff's reagent. Fractions 3, 4, and 5 showed the presence of an alkaloid; they were combined, and evaporated to dryness. The amber colored residue was washed

[5] Chromatographic grade, 80-200 mesh. Matheson Coleman and Bell, Norwood, Ohio.

with chloroform, which removed some of the color. The residue was redissolved in 200 ml of hot ethanol, filtered, and refrigerated overnight. A light-buff colored, crystalline material settled out and was collected on a Büchner funnel. This substance when chromatographed developed a single alkaloid spot.

The crystalline material was dissolved in about 5 ml of 2% hydrochloric acid and filtered to remove suspended impurities. The alkaloid was precipitated from the filtrate by the drop-wise addition of 28% ammonium hydroxide. The bone-white colored precipitate was collected on a Büchner funnel and recrystallized from 30 ml of ethanol. About 145 mg of the compound (alkaloid A), melting point 243-5°C. (decomp.), was obtained.

Co-chromatography

Numerous reports (19, 21, 22, 24) in the literature cite the presence of magnoflorine in many Berberidaceous plants. Based on preliminary chromatography and UV analysis of alkaloid 'A', it appeared that the alkaloid may be identical to magnoflorine. Hence, alkaloid 'A' was co-chromatographed with magnoflorine iodide[6] in two difference solvent systems.

A 0.5% solution of 'A' in methanol was spotted in two different

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[6] Obtained through the courtesy of Prof. Jack L. Beal, School of Pharmacy, Ohio State University, Columbus, Ohio.

channels of a chromatoplate. A 0.5% methanolic solution of reference magnoflorine iodide was also spotted in one channel containing the isolated alkaloid and in one additional channel. The plate was developed in BAW (4:1:5). Examination of the plates under UV light and subsequent spraying with Dragendorff's reagent (blue spot under UV; red-orange spot with Dragendorff's) revealed that alkaloid 'A', reference magnoflorine iodide and the co-spot of alkaloid 'A' and magnoflorine iodide gave one zone with identical R_f values. Similarly, the isolated alkaloid and authentic magnoflorine iodide were co-chromatographed in PAW (2:1:1); again there was only a single zone with identical R_f values in the three channels. In both instances there was no separation of the co-spot. The results are summarized in Table 2.

Table 2. Co-spotting of reference magnoflorine iodide and alkaloid 'A'.

Compound	R_f values[1]	
	BAW[2]	PAW[2]
1. Magnoflorine iodide	0.20	0.62
2. Alkaloid 'A'	0.20	0.62
3. Co-spot (1 and 2)	0.20	0.62

[1] Detection with UV light and Dragendorff's spray reagent.
[2] BAW, n-butanol-glacial acetic acid-water (4:1:5, upper phase); PAW, n-propanol-28% ammonium hydroxide-water (2:1:1).

Preparation of the Iodide

Co-chromatographic results indicated that alkaloid 'A' could be magnoflorine. To confirm this, the conversion of the alkaloid into its quarternary iodide salt was attempted.

A 49 mg sample of alkaloid 'A' was dissolved in 90 ml of hot methanol to which was added 3 ml of a saturated aqueous solution of sodium iodide and the mixture was placed in the refrigerator. The yellow crystalline material which slowly formed (over a seven day period) was filtered and washed with cold acetone, leaving 30 mg of a bone-white colored material with a melting point of 243-5°C. (decomp.).

Approximately 3 mg of the material was dissolved in 0.5 ml of water containing two drops of 10% nitric acid. This was tested for iodide ions by the addition of two drops of dilute silver nitrate solution. The test for iodide ions was negative. A comparable test on authentic magnoflorine iodide was positive. Thus, alkaloid 'A' could not be converted into its iodide salt by means of the method employed for converting quarternary bases into their iodide salts.

Ultra-violet Analysis of Alkaloid 'A'

The ultra-violet spectrum[7] of alkaloid 'A', in alcohol, USP,

[7] The UV spectra were taken on a Beckman DB Spectrophotometer.

showed three absorption maxima which were different from those of magnoflorine iodide. However, the differences in absorption maxima disappeared in the UV spectra taken following the addition of either acid or base. The results are shown in Figures 2, 3, and 4. The data are summarized in Table 3. The similarity of the UV spectrum of alkaloid 'A' to that of magnoflorine suggested the alkaloid to be an aporphrine base.

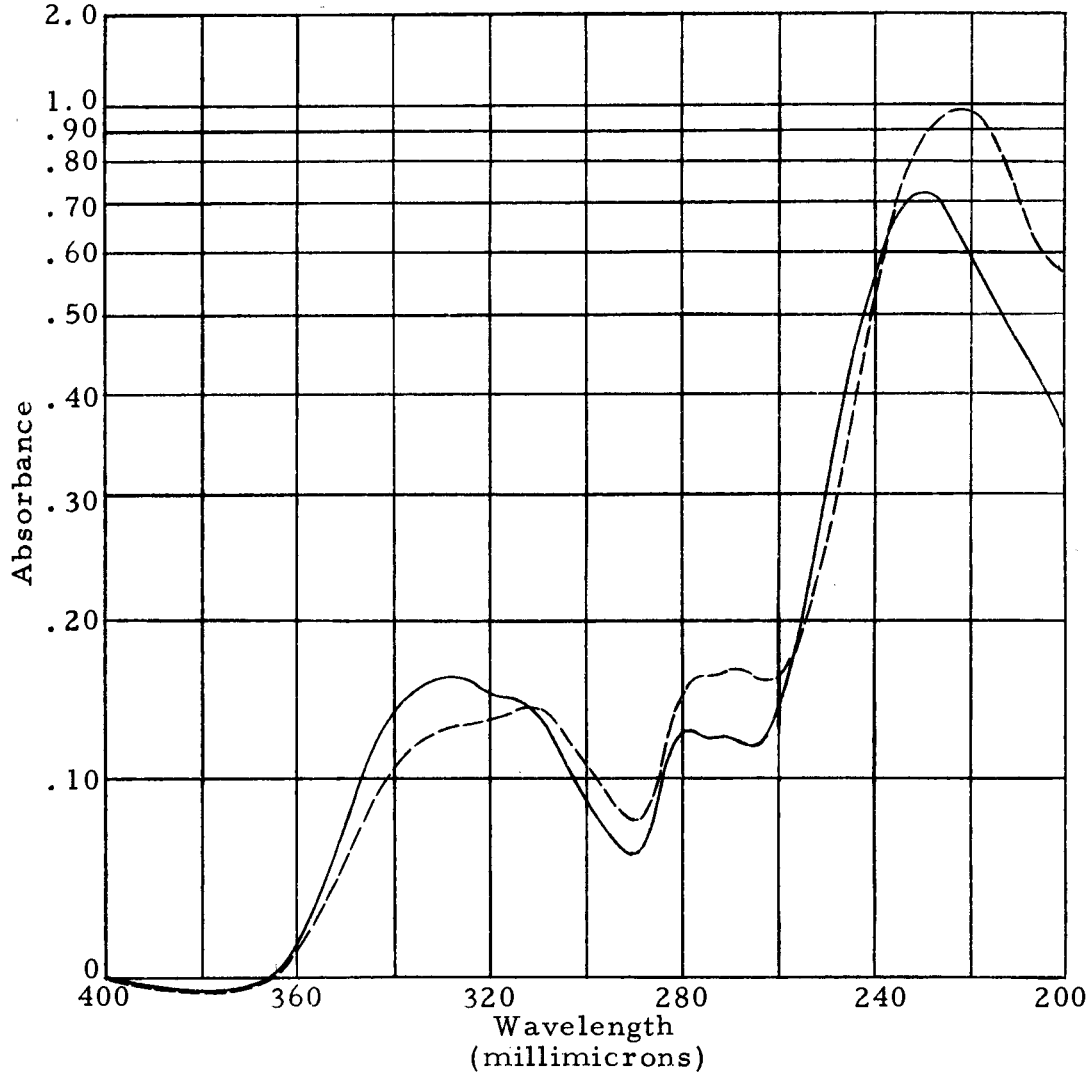
Table 3. Ultra-violet analysis.

Compound	Neutral		Acid		Base	
	λ max.	Log E	λ max.	Log E	λ max.	Log E
Magnoflorine iodide	222	4.68	221	4.62	224	4.63
	268	3.92	267	4.15	278	3.77
	310	3.82	302	3.84	328	3.88
Alkaloid 'A'	229	4.46	221	4.52	230	4.46
	278	3.71	266	4.03	278	3.74
	328	3.80	302	3.71	328	3.82

Infrared Analysis of Alkaloid 'A'

The infrared spectrum of alkaloid 'A' (KBr pellet)[8] (Figure 5) revealed several structural features. The following bands of the spectrum were ascribed to specific functions in the structure.

[8] The author is grateful to Prof. Jack Beal, College of Pharmacy, Ohio State University, for carrying out the infrared analysis.



----- Magnoflorine Iodide

——— Alkaloid 'A'

Figure 2. Ultra-violet spectra--neutral.

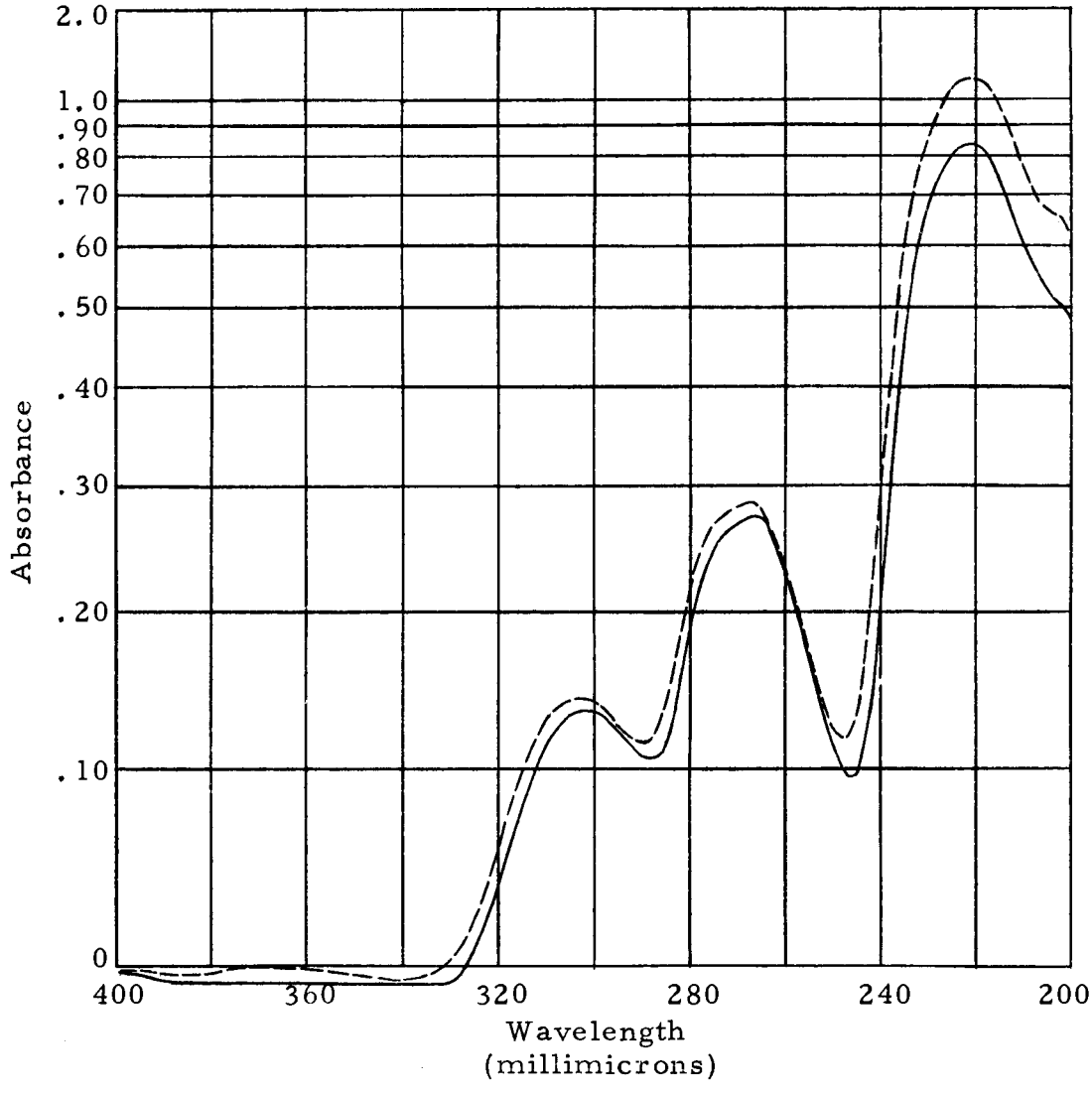


Figure 3. Ultra-violet spectra--acid.

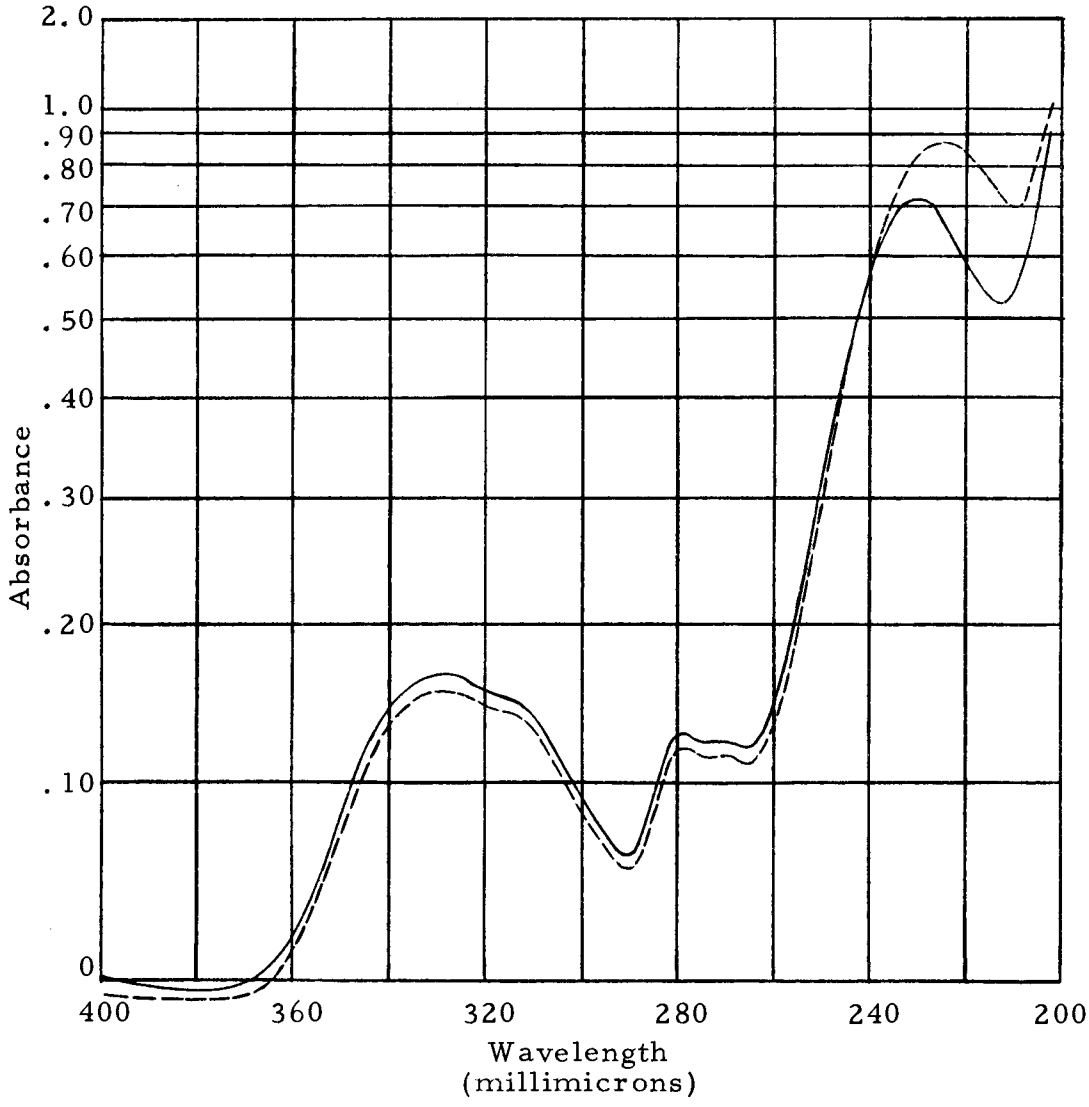


Figure 4. Ultra-violet spectra--alkaline.

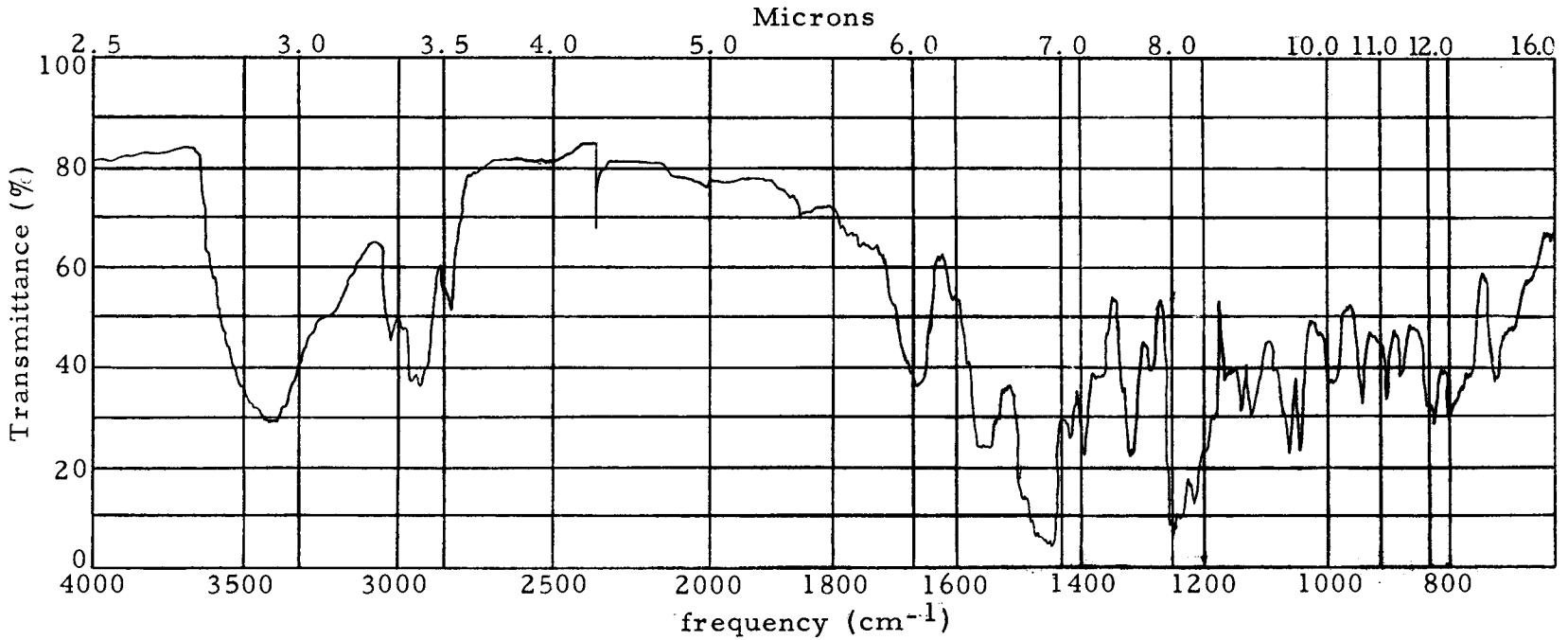


Figure 5. Infrared spectrum of alkaloid 'A'.

See Table 4. The spectrum lacked any absorption in the carbonyl region, thus eliminating the presence of aldehyde, keto, and ester functions in the structure (11, p. 20-48).

Table 4. Infrared spectrum data.

Wave number (cm^{-1})	Functionality
3420 broad	polymeric hydroxyl
3030 and several bands in the 2000 to 1660 region	aromatic nucleus
2850	aromatic methoxy
1250	aromatic ether linkage

Elemental Analysis

The elemental analysis[9] and the methoxy group determination provided the results expressed in Table 5. A value of 1.5 methoxy groups per molecule was determined for alkaloid 'A'. This was interpreted to mean that alkaloid 'A' contained two methoxy groups. The UV spectrum indicated the compound to be an aporphine (C_{16}) derivative. These two facts would require the alkaloid to have a C_{18} structure. Thus, the formula $C_{18}H_{19}O_4N$ seems to best accommodate the available spectral and chemical data.

[9]Performed by Galbraith Laboratories, Inc., Knoxville, Tennessee.

Table 5. Elemental analysis.

	%C	%H	%O	%N	$\%OCH_3$
Observed	67.39	7.18	20.88*	4.55	15.49
Calculated for					
$C_{17}H_{17}O_4N$	68.23	5.68	21.40	4.68	10.37
$C_{17}H_{21}O_4N$	67.33	6.94	21.21	4.62	10.23
$C_{18}H_{19}O_4N$	69.01	6.07	20.45	4.60	19.80
$C_{18}H_{21}O_4N$	68.57	6.67	20.31	4.44	20.31

*Obtained by difference.

III. IDENTIFICATION OF CHOLINE

Preliminary Investigation

During the column chromatography of the crude alkaloid fraction (cf. page 11), it became apparent that while alkaloid 'A' constituted the major portion of the mixture, there were also present trace quantities of other compounds. Fraction 5 (Table 1) was particularly rich in a compound which gave a very characteristic purple spot with Dragendorff's spray reagent. This chromogenic response is known to be characteristic of simple quarternary bases like choline. Further evidence was obtained by subsequent chromatographic comparison with a standard sample of choline chloride.[10] Identical results were obtained when the plates were developed in BAW (4:1:5) and sprayed with Dragendorff's spray reagent. Based on this preliminary information, extensive co-chromatography experiments were designed.

Co-chromatography

A search of recent literature revealed that two groups of workers have devised ways to separate and identify choline, betaine, and their derivatives by means of thin-layer chromatography (18, 20). In the opinion of these workers, the separation of these simple quarternary

[10] Merck and Company, Inc., Rahway, New Jersey.

salts is best obtained with the use of chromatoplates made with aluminum oxide G rather than the customary silica gel G. Hence, in the following experiments, aluminum oxide G coated chromatoplates were used.

Thin-layer plates were prepared by thoroughly mixing 30 Gm of aluminum oxide G [11] with 45 ml of distilled water. A uniform 250 μ layer was applied to 5 x 20 cm glass plates. The plates were activated by heating in an oven at 110°C. for two hours and subsequently stored in a desiccator until further use.

Co-chromatography of the fraction suspected to contain choline was carried out using a reference sample of choline chloride. The method employed for co-chromatography was the same as that employed for alkaloid 'A' (cf. page 12). Three acidic systems and one alkaline system were used for developing the chromatograms as shown in Table 6.

Table 6. Co-chromatography developing systems.

1. MCA, absolute methanol-carbon tetrachloride-glacial acetic acid (28:12:1).
2. BWF, n-butanol-distilled water-formic acid (12:7:1), upper phase.
3. BAW, n-butanol-glacial acetic acid-distilled water (4:1:1).
4. BWD, n-butanol-distilled water-N, N-Dimethylformamide (7:2:1).

[11] E. Merck AG, Darmstadt (Germany), U. S. distributors: Brinkmann Instruments, Inc., Cantiague Road, Westbury, New York.

The following four spray reagents were used to visualize the spots of choline (see Table 7).

Table 7. Spray reagents for choline.

1. Potassium iodoplatinate (16, p. 493), 3 ml of 10% platinum chloride solution mixed with 97 ml of water to which is added 100 ml of 6% aqueous solution of potassium iodide.
   Choline salts appear as blue-grey spots.

2. Iodine (20), 1% iodine in chloroform.
   Choline salts show as yellow-brown spots.

3. Dipicrylamine (20), 0.2% in a 50% aqueous-acetone solution.
   Choline salts appear as red-orange spots.

4. Dragendorff's spray reagent, (cf. page 8).
   Choline salts show as violet spots.

In the case of the chromatograms developed in the alkaline system (BWD), the plates had to be sprayed with 2% hydrochloric acid followed by either Dragendorff's spray reagent or dipicrylamine reagent in order to obtain an immediate, clear, chromogenic response. This procedure was not necessary for the chromogenic response when visualizing with the other two spray reagents. In none of the instances was there any separation of the co-spots. The results are summarized in Table 8. The chromogenic responses to the four spray reagents of the reference choline, as well as the fraction suspected to contain choline, were not only similar but also the

same as that recorded by Taylor (20). Thus, it was concluded that fraction 5 contained choline.

Table 8. Co-spotting of choline chloride and fraction 5.

Compound	R_f values [1]			
	MCA[1]	BWF[1]	BAW[1]	BWD[1]
1. Choline chloride	0.48	0.36	0.69	0.34
2. Fraction 5	0.46	0.36	0.70	0.34
3. Co-spot (1 and 2)	0.48	0.35	0.69	0.34

[1] For detection and solvent systems, see text pages 22 and 23.

IV. IDENTIFICATION OF SUCROSE

Preliminary Investigation

Fraction 5 (cf. page 11) upon reduction in volume to approximately 200 ml and kept at room temperature for three days, deposited an amber crystalline material. The supernatant liquid was decanted and the crystals were washed with chloroform. The sweet tasting crystals emitted a carmel-like fragrance when ignited. The compound was very soluble in water and insoluble in organic solvents. The slightly amber colored crystals were recrystallized from a minimum quantity of water. The monoclinic crystals, upon drying in a desiccator, melted at 178-180°C. (decomp.). The compound failed to reduce Benedict's reagent.[12]

Acid Hydrolysis

To 4 mg of the isolated compound dissolved in 0.5 ml of distilled water was added two drops of 10% hydrochloric acid. The mixture was heated in a boiling water bath for 30 minutes. A drop

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[12] Dissolve 17.3 Gm of sodium citrate and 10 Gm anhyd. sodium carbonate in about 60 ml water and dilute to 85 ml with water; dissolve 1.73 Gm copper sulfate ($CuSO_4 \cdot 5\ H_2O$) in about 10 ml water and dilute to 15 ml, this solution is added to the citrate carbonate solution with constant stirring (10, p. 1172).

of Benedict's reagent was added and the mixture again heated in the boiling water bath. The appearance of a brick-red colored precipitate indicated that the Benedict's reagent was reduced. Thus, the compound upon acid hydrolysis liberated a reducing compound.

Enzymatic Hydrolysis

Four milligrams of the isolated compound was dissolved in 0.5 ml of distilled water and two crystals of invertase [13] were added. A few crystals of emulsin [14] were added to a similar solution in another test tube. Both tubes were incubated overnight at 37°C. Following incubation, one drop of Benedict's reagent was added to each test tube and heated in a boiling water bath. The solution to which the invertase was added gave the characteristic brick-red colored precipitate produced in the presence of a reducing sugar. No change occurred in the solution containing the emulsin. Since the compound, upon hydrolysis by invertase, was capable of reducing Benedict's reagent, the presence of an α-glucosidic linkage in the compound was indicated.

Preparation of the Osazone

The osazone was prepared according to the method outlined by

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[13] City Chemical Corporation, New York.
[14] K & K Laboratories, Inc., Plainview, New York.

Vogel (23, p. 455). One hundred milligrams of the compound, 200 mg of phenylhydrazine HCl (white, reagent grade), and 300 mg of sodium acetate were dissolved in 2 ml of distilled water. The lightly corked test tube was placed in a boiling water bath. The time lapse between the immersion of the test tube and the beginning of osazone formation was noted. The reaction mixture was refrigerated overnight. The greenish-yellow, long needle-like crystals were then collected in a Büchner funnel and recrystallized twice from 60% ethanol.

The osazone of an authentic sample of sucrose was made simultaneously and in an identical procedure. The time end-point for authentic sucrose was 29 minutes in contrast to 27 minutes for the isolated compound. Sucrose, in order to form the osazone, must first hydrolyze into glucose and fructose. This accounts for the additional time required for osazone formation than would ordinarily be expected for glucose or fructose. The osazone of the isolated sugar melted at 191-2° C. (decomp.) while the osazone prepared from reference sucrose melted at 193-4° C. (decomp.). Vogel (23, p. 457) reported a melting point of 205° C. (decomp.) for glucosazone and fructosazone. It was impossible to distinguish the crystalline pattern of the osazones formed from the isolated compound and reference sucrose.

Preparation of the Octa-acetate

One hundred milligrams of the isolated compound were mixed with 2 ml of pyridine and 4 ml of acetic anhydride and refluxed for two hours. The reaction mixture was poured over about 20 ml of crushed ice and refrigerated overnight. Long, white, needle-shaped crystals growing in rosettes were formed. They were recrystallized once from alcohol, USP.

The octa-acetate of reference sucrose was prepared in an identical manner. Sucrose octa-acetate melted at 86-7° C. while the octa-acetate of the isolated compound melted at 85-7° C. The literature (15, p. 341) reports 89° C. as the melting point of sucrose octa-acetate. The melting point of an admixture of equal parts of both octa-acetates was undepressed at 84-6° C. Thus, the presence of sucrose in the roots of Achlys triphylla was confirmed.

V. IDENTIFICATION OF β-SITOSTEROL

Isolation

The original petroleum ether extract (cf. page 8) (ca. 15 Gm) was redissolved in 10 ml of petroleum ether, (90-95° C.), and adsorbed on 10 Gm of activated alumina, evaporated, and pulverized. The powdered material was placed on top of a 300 Gm (4.5 x 20 cm) column of activated alumina previously flushed with hexane. A 2 cm layer of Ottawa sand was subsequently added. The column was developed as shown in Table 9.

Table 9. Chromatographic development of Column B.

Fraction	Composition	Volume (ml)
1	Hexane	1000
2	Benzene	1300
3	Benzene-chloroform (3:1)	4 x 250
4	Benzene-chloroform (1:1)	4 x 250
5	Benzene-chloroform (1:3)	4 x 250
6	Chloroform	5 x 300
7	Chloroform-methanol (3:1)	4 x 250
8	Chloroform-methanol (1:1)	4 x 250
9	Chloroform-methanol (1:3)	4 x 250

Each fraction, after concentration in vacuo, was chromatographically examined for sterols/triterpenes, using chloroform-acetone (9:1) as a solvent system and antimony trichloride (10% w/w in chloroform) as the visualizing reagent (cf. page 7). Fraction 6 revealed the presence of two compounds (R_f = 0.46 and 0.60) in

this manner. This fraction also gave a strongly positive L-B test (cf. page 7) indicating the presence of sterols/triterpenes. Fraction 6 was evaporated to dryness *in vacuo* at 50°C. in a rotary flash evaporator. The residue (510 mg) was redissovled in 10 ml of chloroform, NF, and adsorbed on to 1.2 Gm of activated alumina. The dried and pulverized material was rechromatographed using a column containing 100 Gm of activated alumina (2.5 x 21 cm). The column was developed as shown in Table 10.

Table 10. Chromatographic development of Column C.

Fraction	Composition	Volume (ml)
1'	Hexane	250
2'	Chloroform	4 x 250
3'	Chloroform-ether (9:1)	4 x 125
4'	Chloroform-ether (3:1)	4 x 125
5'	Chloroform-ether (1:1)	4 x 125
6'	Ether	4 x 125

Each fraction was concentrated to approximately 10 ml and examined chromatographically for steroidal components. Excellent separation of spots resulted from this column. Fraction 4' contained a single component (R_f = 0.46 in CA 9:1). The fraction also gave a positive L-B test. The fraction was evaporated to dryness and the residue was dissolved in about 10 ml of hot ethanol. Long, white, needle-shaped crystals were obtained with overnight refrigeration. Recrystallizing from alcohol, USP, yielded 31 mg of compound,

mp 135-7° C. Its melting point was undepressed when mixed with an equal part of reference β-sitosterol[15] (mp 136-7° C.). Heilbron (8, p. 361) reported 136-7° C. as the melting point of β-sitosterol.

Identification of β-sitosterol by co-chromatography

The isolated compound and the reference β-sitosterol were co-chromatographed in exactly the same manner as that described for alkaloid 'A' (cf. page 12). In three different solvent systems, the R_f values of the isolate and reference compound were in very close agreement. Furthermore, there was no separation of the co-spot. The results are summarized in Table 11.

Table 11. Co-spotting of reference β-sitosterol and the isolated compound.

Compound	R_f values[1]		
	CM[2]	SE[2]	BE[2]
1. β-sitosterol	0.23	0.16	0.44
2. Isolated compound	0.22	0.16	0.43
3. Co-spot (1 and 2)	0.22	0.16	0.44

[1] Detected with antimony trichloride (10% w/w in chloroform) and heating the plates at 110° C. for 3-5 minutes.

[2] CM, chloroform-methanol (99:1); SE, Skelly B-ethyl acetate (85:15); BE, benzene-ethyl acetate (3:1).

[15] Nutritional B iochemicals Corp., Cleveland, Ohio.

The IR spectrum of the isolated compound and the spectrum of reference β-sitosterol in chloroform were superimposable, thus confirming the identity of the isolate as β-sitosterol.

VI. DISCUSSION

Prior to the present work, there was only one report of a phytochemical study of Achlys triphylla (2). The absence of such work maybe due to the plant's limited habitat and the difficulty in collecting a sufficient quantity of plant material for study. Since A. triphylla belongs to a medicinally useful and alkaloid containing plant family, its phytochemical investigation was warranted.

The investigation of A. triphylla was also motivated by the preliminary pharmacological screening [16] of certain extracts prepared from the plant. The crude ethanol extract, which contained an alkaloidal material, was found to competitively inhibit the effect of acetylcholine and histamine on the isolated rabbit ileum.

During purification of the crude ethanol extract, the pharmacological activity was retained in those fractions containing the alkaloid. However, after alkaloid 'A' was isolated and tested pharmacologically it was found to be devoid of any biological activity. Thus, the activity associated with the crude ethanol extract was not due to the alkaloid.

The alkaloid contained in the ethanol extract responded in a positive manner to typical alkaloid reagents and ultimately was

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[16] The author is grateful to Dr. Robert Brummett, Department of Pharmacology, University of Oregon Medical School, for carrying out the pharmacological screening.

isolated in a pure form by means of alumina column chromatography.

Initial chromatographic analysis indicated that alkaloid 'A' was identical to magnoflorine, an alkaloid commonly occuring in the plants of this family. This tentative conclusion was further substantiated by comparing the UV spectra (maxima) and the melting points (243-5°C. decomp.) of the two compounds. However, the IR spectrum of alkaloid 'A' was different from that of magnoflorine. Furthermore, alkaloid 'A' could not be a quaternary base since it was precipitated from an acidic solution by ammonium hydroxide. Similarly, an iodide salt of the alkaloid could not be prepared by the method commonly used for preparing such derivatives from quaternary bases. The very similar UV spectrum suggested that the isolated alkaloid may be a tertiary analog of magnoflorine, namely corytuberine. See Figure 6.

Co-chromatography with an authentic sample of corytuberine[17] indicated that alkaloid 'A' and corytuberine were not the same compound. The UV spectra of the two compounds were also different.

A determination of the number of methoxy groups of a compound invariably yields a low value because of the drastic conditions used during the analysis. A value of 1.5 methoxy groups per molecule was

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[17] Obtained through the courtesy of Dr. R. H. F. Manske, Dominion Rubber Research Laboratory, Guelph, Ontario, Canada.

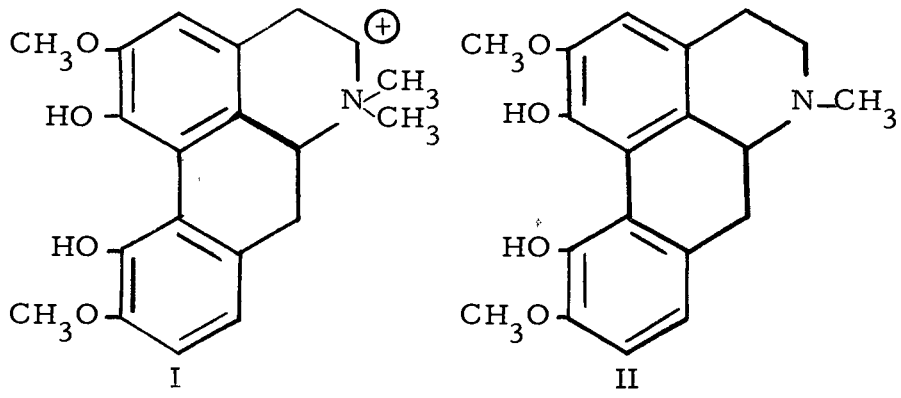

Figure 6. Structures of magnoflorine (I) and corytuberine (II).

obtained for alkaloid 'A'. Hence, it must be assumed that alkaloid 'A' contained two methoxy groups. The UV spectrum indicated the compound to be an aporphine (C_{16}) derivative. These two facts would require the alkaloid to have a C_{18} structure. Thus, the formula $C_{18}H_{19}O_4N$ seems to best accommodate the available spectral and chemical data. The low concentration of alkaloid 'A' in the plant coupled with the relatively small amount of plant material prevented a more extensive investigation at this stage.

The presence of a simple quaternary base in the crude alkaloidal extract was detected by a characteristic purple coloration with Dragendorff's reagent. Such a response is characteristic of choline. This simple base was not isolated, but its identity was established as choline by means of extensive co-spotting experiments.

Four solvent systems, three acidic and one alkaline, were employed. Recent workers (19, 20) had not developed an alkaline TLC system for the separation of simple quaternary bases. They depended solely upon closely related acidic systems. The alkaline system used herein gave sharp, clear zones of choline with no tailing.

Four different visualizing reagents were used to detect the choline spots. The chromogenic response of choline and the fraction suspected to contain choline was identical in all four reagents. This fact coupled with the excellent agreement between the R_f values of the two compounds and their co-spots confirmed the presence of choline in A. triphylla.

Sucrose is a highly mobile disaccharide found abundantly in the free state, particularly in plant storage organs. Sucrose was encountered in this study. Its identity was concluded from its melting point, its behavior on acidic and enzymatic hydrolysis and the melting points of its osazone and octa-acetate derivatives.

The phytosterols occur in complex mixtures and isolation of the pure individual components is often difficult. The advancement of chromatographic technique, particularly column chromatography, has greatly simplified this task. In this study, the commonly occurring phytosterol, β-sitosterol, was isolated. Melting point, mixed melting point, and co-chromatography in three different solvent systems confirmed its identity as β-sitosterol. In addition the IR

spectra of the isolated and the reference compound were entirely superimposable.

Several aspects of the present investigation need to be resolved. The limited quantity of alkaloid 'A' (0.016%) prevented the complete determination of its structure. Since alkaloid 'A' was not responsible for the observed pharmacological activity of the crude extract, the activity must reside in some other component.

BIBLIOGRAPHY

1. Block, Richard J., Emmett L. Durrum and Guther Zweig. A manual of paper chromatography and paper electrophoresis. 2d ed. New York, Academic Press, 1958. 710 p.

2. Bradley, C. E. The occurrence of coumarin in Achlys triphylla. Journal of the American Chemical Society 29:606-607. 1907.

3. Culbreth, David M. R. A manual of materia medica and pharmacology. 7th ed. Philadelphia, Lea and Febiger, 1927. 855 p.

4. French, David H. Ethnobotany of the Pacific Northwest Indians. Economic Botany 19:378-382. 1965.

5. Hare, Hobart Amory, Charles Caspari, Jr. and Henry H. Rusby. The national standard dispensatory. 3d ed. Philadelphia, Lea and Febiger, 1916. 2081 p.

6. Hatcher, Robert A. and Torald Sallmann. A textbook of materia medica. Philadelphia, Saunders, 1904. 411 p.

7. Heilbron, Sir Ivan (ed.) Dictionary of organic compounds. Vol. 4. New York, Oxford University Press, 1953. 694 p.

8. Hitchock, Leo C., Arthur Cronquist, Marion Ownbey and J. W. Thompson. Vascular plants of the Pacific Northwest. Vol. 2. Seattle, University of Washington Press, 1964. 597 p.

9. Kier, Lemont B., Dorothea B. Fitzgerald and Shirley Burgett. Isolation of podophyllotoxin from Callitrus drummondii. Journal of Pharmaceutical Sciences 52:502-503. 1963.

10. Lang, Norbert A. and Gordon M. Forker. Handbook of chemistry. 7th ed. Sandusky, Ohio, Handbook Publishers, 1949. 1920 p.

11. Nakanishi, Koji. Infrared absorption spectroscopy. San Francisco, Holden-Day, 1964. 233 p.

12. Ohwi, Jisaburo. Flora of Japan, ed. by Frederick G. Meyer and Egbert H. Walker. Washington, D. C., Smithsonian Institute, 1965. 1067 p.