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A CHEMICAL INVESTIGATION OF AMERICAN VERATRUM

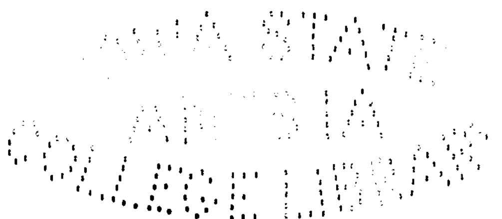
by

Edwin J. Seiferle

A Thesis Submitted to the Graduate Faculty
for the Degree of

DOCTOR OF PHILOSOPHY

Major Subject Plant Chemistry



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INTRODUCTION AND STATEMENT OF PROBLEM

The use of Veratrum viride Aiton both in insect control and in medicine has declined greatly in the last twenty or thirty years. This decline is the result of a number of factors, chief among which are the nonuniformity of samples obtainable in the market, the lack of an efficient method of assay, and the scarcity of information on the active constituents of the plant.

Since the plant has been proved to have several desirable pharmacologic properties and is effective in the control of certain insects, it became apparent that information should be gathered with the ultimate object of determining whether the properties of the plant could be sufficiently standardized to warrant continued recommendation of Veratrum viride, or whether its use should be discontinued. With this objective in mind the problem was attacked with the following purposes: to examine the procedures for chemical assay of the plant, to compare the chemical with the biological assay on insects, to separate the crude, physiologically active mixture into its constituents, and to test them for toxicity to insects.

REVIEW OF LITERATURE

Botanical

Veratrum viride Aiton (1) is a member of the family Liliaceae. It is a stout, broad-leafed perennial, two to seven feet tall, with simple stems growing from a thickened base producing coarse, fibrous roots. The yellowish-green, sometimes drooping flowers are arranged in dense, spike-like racemes; they bloom in summer. The plant is distributed generally over the continent, but especially in eastern North America, west to Minnesota, south to Georgia and north to Alaska. Its preferred habitat is swamps and wet woods and along borders of mountain streams (29, 35, 59).

Veratrum viride is known commonly under a multitude of names, many of which result in confusion: green hellebore, American white hellebore, American false-hellebore, swamp hellebore, American Veratrum, Indian poke, itch weed, poke root, and "nieswurz" or sneeze root. The names containing the word hellebore are especially ill-advised for they bring about immediate confusion with the plants belonging to the genus *Helleborus* which are entirely unrelated to the Veratrums.

To complicate matters further, another plant known to the medical profession as sabadilla seeds, *Schoenocaulon*

officinale Gray, was once considered to be a member of the Veratrums and was known as Veratrum officinale and V. sabadilla. Its literature is consequently intermingled with that of V. viride.

Commercial supplies of the crude drug are obtained principally from North Carolina, Virginia, Illinois and Michigan. The roots and rhizomes (underground, fleshy stems), in which most of the active principles are found, are collected in the autumn after the leaves have died. They are washed, and dried either whole or after slicing (35, 59, 99). Due to the difficulty in distinguishing commercial samples of Veratrum viride from its common adulterants, V. album L. and V. californicum Dur., Bastin (2), Denniston (22), Viehoever, Keenan and Clevenger (91) and Meyer (52) have made studies of the microscopic characters of these plants. The consensus of opinion of these investigators is that the characters so overlap that distinction is difficult.

Chemical

The literature relating to the chemistry of Veratrum viride is decidedly dependent on that of the closely related, European species, V. album, which has been investigated rather thoroughly. For this reason the literature of V.

viride will be reviewed in its entirety and the other pertinent literature will be added.

The first recorded chemical investigation of Veratrum viride is that of Worthington in 1838 (103). His color tests and precipitation experiments indicated that the plant contained "an alkaloid substance, identical with veratria." Veratria (veratrine) had previously been isolated from Schoenocaulon officinale by Meissner (51) in 1819 and independently from this plant and V. album by Pelletier and Caventou (61) in 1820. Richardson (74) in 1857, also by color and precipitation tests, obtained results which led him to confirm the statement of Worthington as to the identity of veratria and the alkaloid of V. viride. Further confirmation of these claims is found in the work of Scattergood (82) in 1862 and of Percy (62) in 1864. Scattergood obtained a resin and an amorphous material which possessed the characteristics of veratria, and suggested the presence of another alkaloid, jervia. Jervia (jervine) had been isolated previously, in crystalline form, from V. album by Simon (84) in 1837. Percy proceeded in a manner similar to that followed by Richardson, and obtained a semi-crystalline solid which he termed veratria.

In 1865 and 1866 Bullock (7) isolated from the powdered drug, besides an alkaloid-containing resin, two noncrystalline principles, one soluble in ether and the other insoluble.

The soluble alkaloid was later called veratroidia and the insoluble viridia by Wood (95). Neither of these alkaloids resembled the veratria of commerce. Peugnet (63) in 1872 also separated veratroidia and viridia from _Veratrum viride_ and stated that the latter was identical with jervia. Mitchell (53, 54) confirmed these findings completely in 1874 as did Bullock (8) in 1875. A reversion to the older ideas was suggested in 1876 when Wormley (102) claimed the isolation of veratria and jervia only from the fluid extract of _V. viride_. In the same year Bullock (9) decided that jervia was the only alkaloid in _V. viride_, veratroidia being a mixture of jervia and resin. He wondered, however, at the difference in physiological action of jervia and the veratroidia-resin, for the latter was more active. Robbins (75) published a paper in 1877 in which he claimed the presence of veratria, jervia and possibly a new, third principle, veratridia, in _V. viride_. The claims were based on color tests. Tobien (87) in 1878 isolated veratroidia and jervia from _V. lobelianum_ and _V. album_.

The most important contribution to the chemical study of _Veratrum viride_ was made in 1879 when the third of a series of papers on the alkaloids of the Veratrums was published by Wright (104). (Parts I and II, on _V. sabadilla_ and _V. album_, respectively, were by Wright and Luff (105, 106).) From _V. viride_ were isolated in crystalline form

three alkaloids, jervine, pseudojervine and rubijervine, the latter in very small quantity. In addition, amorphous fractions were obtained which were thought to contain a trace of veratrine, a trace of veratralbine, and considerable cevadine. A large amount of amorphous veratralbine had been found in V. album and much crystalline cevadine in V. sabadilla.

Bullock (10) then, in 1879, reworked several of his resinous materials, obtaining alkaloids which, he felt, coincided with the rubijervine and pseudojervine of Wright.

No further work has been done in investigation of the alkaloidal constituents of Veratrum viride but there are several valuable studies on the alkaloids of V. album which have a direct bearing on the present work.

Salzberger (81) in 1890 developed two procedures for the separation of the alkaloid mixture from Veratrum album. By one, the baryta method, he obtained in crystalline form considerable quantities of jervine, rubijervine and protoveratridine; by the other, the metaphosphoric acid method, he produced mainly protoveratrine and pseudojervine with a little jervine and rubijervine. Protoveratridine was not considered to be present originally in the crude drug but was thought, wrongly as will be seen later, to be a degradation product of protoveratrine formed in the baryta procedure.

In 1906 Bredemann (5) applied some of Salzberger's methods to his study on Veratrum album and isolated jervine, pseudojervine, rubijervine and protoveratrine.

Saito, Suginome and Takaoka (79) in 1934 investigated Veratrum grandiflorum Loes, fil. (=V. album), collected in Japan and obtained only jervine in crystalline condition. Two years later Saito and Suginome (78) published a paper in which they again isolated only jervine from the resinous materials previously obtained. A third paper by these authors (80) reported experiments on the constitution of jervine.

In a series of papers published in 1937 and 1938, Poethke (68, 69, 70) reported the discovery in Veratrum album of a new alkaloid, germerine, in large quantity. In addition he also isolated jervine, rubijervine, pseudojervine, protoveratrine and protoveratridine. He studied the interrelationships of the alkaloids and observed that germerine on treatment with alcoholic potassium hydroxide yielded a crystalline base, germine, and two acids, methylethylacetic and methylethylglycolic acids. Protoveratridine by the same treatment yielded germine and methylethylacetic acid. Germerine on treatment with barium hydroxide was converted into protoveratridine and methylethylglycolic acid. Thus germerine is the methylethylglycolate of protoveratridine which is the methylethylacetate of germine.

Protoveratrine when treated with alcoholic potassium hydroxide yielded acetic, methylethylacetic and methylethylglycolic acids and an uncrystallized base called protoverine. Jervine, pseudojervine and rubijervine are not ester alkaloids.

In a fourth paper Poethke (71) discussed his experiments on the amorphous alkaloid fraction which constituted about fifty per cent of the crude alkaloids. In repeated attempts at crystallization no success was attained. However, on saponification by alcoholic potassium hydroxide, considerable amounts of germine were obtained, as well as appreciable amounts of acetic acid and methylethylacetic acid. Poethke did not believe this germine could have been present in the amorphous fraction as germerine or protoveratridine for these alkaloids are readily crystallized from crude alkaloid mixtures.

The chemical formulas of the pure, crystalline alkaloids thus far isolated from the Veratrums, and of their known degradation products, are listed with their discoverers and the dates of these discoveries:

Jervine	$C_{26}H_{37}O_3N$	Simon (84)	1837
Pseudojervine	$C_{23}H_{39}O_8N$	Wright and Luff (106)	1879
Rubijervine	$C_{26}H_{43}O_2N$	Wright and Luff (106)	1879
Protoveratrine	$C_{40}H_{63}O_{14}N$	Salzberger (81)	1890
Germerine	$C_{36}H_{57}O_{11}N$	Poethke (68)	1937

Protoveratridine	$C_{31}H_{49}O_9N$	Salzberger (81)	1890
Germine	$C_{26}H_{41}O_8N$	Poethke (69)	1937

Pharmacological and Physiological

Josselyn (43), who visited North America in 1638-1671, noted that the active properties of *Veratrum viride* were known to the aborigines of the continent before their intercourse with Europeans, since they used it as a vomit in a type of ordeal in the choice of chiefs. It was also employed by the colonists as a purgative and antiscorbutic.

Osgood (57), who in 1835 first called to the attention of the medical profession the therapeutic properties of the drug, concluded from his studies that *V. viride* did not contain veratria.

Oulmont (58) in 1868 compared the pharmacological actions of *V. viride*, *V. album* and veratria and concluded that the alkaloid veratria was not the true active principle of *Veratrum*, and that *V. viride* was a cardiac poison analogous to digitalis but more reactive. Scattergood (82) tested his resin from *V. viride* and veratria on dogs and found them both to have similar therapeutic effects, the resin appearing to be more active than veratria.

H. C. Wood (96) tested in 1870 the alkaloids and resin prepared by Bullock (7) in 1865 and 1866. He found that there was some difference in the action on cats and dogs of

these impure samples of veratroidia and viridia, and that the resin was almost inactive. Wood and Berens (100) in 1874 compared the veratroidia of Mitchell (54) and of Bullock (7) and found them to produce the same physiological action, while Bullock's viridia and Mitchell's jervia were considered to be one for the same effects were given by each.

Peugnet (63) examined in 1872 his own preparations of veratroidia, viridia, jervia and resin from V. viride and V. album, comparing them with veratria. He concluded that veratroidia was distinct from veratria, that jervia and viridia were identical, and that the resin of V. album contained a principle resembling veratria in its action while that of V. viride was inactive. To this latter difference he attributed the essential deviations in the action of the two species. He also concluded that veratroidia was the active sedative principle of V. viride.

Following the discovery of protoveratrine in V. album by Salzberger (81) its action was extensively investigated. Wood (98) in 1908 and Waller (92, 93) both claimed that the actions of veratrine and protoveratrine were distinct, the former acting mainly on muscle and the latter on nerve. These claims are disputed by the work of several other investigators, although it is generally agreed that veratrine may be eliminated from consideration as the active principle

of the Veratrums. Eden (23) in 1892 made a careful study of the physiological effects of protoveratrine and compared it with cevadine (veratrine), which had previously been carefully studied by Lissauer (47). Eden found that the pharmacological action of protoveratrine was characterized by a specific central action on the medulla oblongata and the spinal cord, an action on the striped muscle, and a paralytic effect on the peripheral sensitive nerves. The results of the central action in frogs were suspension of respiration and depression of the reflexes, and on mammals similar respiratory disturbances, vomiting in carnivores, cramps and paralysis. He noted a qualitative difference between cevadine and protoveratrine, in that the characteristic "veratrine effect" (lag) in muscle contraction was lacking in protoveratrine and that cevadine did not exert the energetic action of protoveratrine on the peripheral nervous system nor its extreme vagus paralysis. He concluded that protoveratrine belonged in the group with cevadine, aconitine and delphinine.

Boehm (3) also compared the effects of these two alkaloids and showed that the intensity of action of protoveratrine on the frog heart was much greater than that of cevadine, and that on nerves and skeletal muscle it was less active, although the effects were substantially the same in kind. Boehm (4) later gave a complete review of the work

on these two alkaloids. Pilcher and Sollmann (66) studied the effects of V. viride and cevadine on the vasomotor center and concluded that there was no direct action, the sharp fall of blood pressure being due to stimulation of the vagus center.

In the U. S. Pharmacopoeia VI (1880) and VII (1890) only V. viride was recognized, the use of V. album in official medicinal preparations being excluded. In U. S. P. VIII (1900) both species were sanctioned. This was a direct result of the investigations of H. C. Wood and H. C. Wood, Jr., (101) in 1899, who stated that no clinical differences were likely to be observed in the action of therapeutic doses of the two species, since symptoms qualitatively and quantitatively similar were found in frogs, dogs and rabbits. Wood, Jr., (97) in 1906 re-examined preparations from the two species and reversed the previous decision as to their identity, the same alkaloids "not being necessarily present in both, at least not to the same extent or proportions." He concluded that neither veratrine nor rubijervine (veratroidine) was present in sufficient amount to be responsible for the activity of the plant, that protoveratrine differed essentially in its effects from Veratrum and thus cannot be the active principle, that jervine most nearly corresponds physiologically to the crude drug but is present in too small amounts in proportion to its toxicity to be

the active principle, and finally that there must be in *V. viride* some undiscovered active principle to account for the action of the whole drug. As a result of Wood's work *V. album* was deleted from U. S. P. IX (1910) and is still unrecognized officially in America.

Comparison of the clinical effects of the two *Veratrum* species was made in experiments on human patients by Collins (16) and Collins and Hanzlik (17) using the tincture of *V. album*, and by Hewlett (36) using the fluid extract of *V. viride*. These experiments showed that both drugs produced a fall in blood pressure and slowing of the pulse rate, but that the effective dose of *V. viride* was considerably larger than that of *V. album*.

The pharmacological effects of a commercial fluid extract of *V. viride* were studied by Houghton and Hamilton (37). They showed in experiments on dogs that small therapeutic doses slowed and deepened respiration, decreased pulse rate and produced a fall in blood pressure. Toxic doses produced momentary stimulation of the respiratory center, followed by respiratory paralysis and death from asphyxia.

Wood (98) in 1908 also found *V. viride* to produce decreased pulse rate and blood pressure when administered in small doses. The general effects were quite similar to those of Eden (23) for protoveratrine, and Wood concluded that protoveratrine was the active principle of *Veratrum*.

A very careful study of the action of V. viride was made by Cramer (21) in 1915. Experimenting on anesthetized cats and dogs he showed that the drug in small doses lowered blood pressure, slowed or stopped respiration and generally slowed the heart rate. He stated:

> "It follows then that the drug in small doses exercises its effect on blood-pressure neither peripherally through an action on the vessel walls or the vaso-motor nerve-endings nor through a direct action on the vaso-motor centre or the heart, but reflexly through stimulation of the afferent vagus fibres. The action on the respiration is also produced reflexly. With regard to the slowing of the rate of the heart beat it can be stated with certainty only that it is not due to a stimulation of the cardio-inhibitory nerve-endings or to a direct action on the heart muscle...
>
> "The drug after having stimulated the afferent nerve-endings of the vagus, paralyses them so that a second or third dose is without effect.
>
> "With larger doses the drug in addition to the effects just mentioned, paralyses the cardio-inhibitory nerve-endings of the vagus and has also a direct action on the medullary centres leading to vaso-constriction and to paralysis of respiration. These additional effects are not dependent on the integrity of the vagus nerves...
>
> "It is now possible to understand why large doses of the drug give such irregular and apparently paradoxical results. For in such doses veratrum viride may produce any of the following effects. A stoppage of respiration and a dilatation of the blood-vessels through the afferent vagus nerve-endings; or it may at once paralyse them. It may slow the heart through stimulating the cardio-inhibitory centre or may paralyse the cardio-inhibitory nerve-endings and then increase the heart-beat and raise the blood-pressure. And lastly with such doses it will act directly on the medullary centres. It is not surprising that the resultant effect of so many antagonistic actions is irregular."

He stated further:

> "The effect of small doses of veratrum viride is quite different from the action of protoveratrine as described by Watts Eden and of veratrine as described by Lissauer. On the other hand the accounts of these observers of the action of veratrine and of protoveratrine respectively on the circulation might be taken as a fairly accurate description of the action of large doses of veratrum viride, when one considers that the effect of such doses is very irregular. Compared with the amounts of the crude extracts of veratrum viride used in these experiments, of which the active principle is of course only a fraction, the doses used by Lissauer and Watts Eden in their investigations are indeed large."

Cramer concluded that before the identity or nonidentity of the active principles of _V. viride_ with either cevadine or protoveratrine could be decided a reinvestigation of the two last-named alkaloids was needed.

The extensive chemical studies of _V. album_ recently reported by Poethke (68, 69, 70, 71) have furnished the impetus for this re-examination. Poethke isolated besides protoveratrine a new, active alkaloid which he called germerine, and further showed both to be ester alkaloids. Germerine on partial hydrolysis yielded protoveratridine, first discovered by Salzberger (81), and on complete hydrolysis another new alkaloid, germine. These four alkaloids were investigated by Haas (31), and the work of Lissauer (47) and Boehm (3) on cevadine was compared. Haas's experiments showed that there was a great similarity in the type of action of germerine, protoveratrine and

cevadine, although there were more or less large quantitative differences between them. Protoveratridine and germine, the products of hydrolysis of germerine, showed a much weaker action than the parent alkaloid. The median lethal dose for germerine on the frog was 0.9 mg. per 100 g. and on the rat, subcutaneously, was 0.37 mg. per 100 g. Germine was lethal to the frog only at 50 mg. per 100 g. and to the rat at 200 mg. per 100 g. Whereas germerine acted predominantly by progressive paralysis, as did protoveratrine, the hydrolysis products were characterized by an irritant effect.

V. viride has not yet been proved to contain either protoveratrine or germerine, but the fact that these alkaloids are the predominant toxic components of V. album and that the two species have very similar pharmacological effects allows of at least theoretical extension of the results of Haas to American Veratrum.

Of the other alkaloids of V. viride, jervine, pseudojervine and rubijervine, only jervine has a slight activity, differing somewhat from that of protoveratrine, according to Lissauer (48).

Hanzlik and DeEds (32) examined V. californicum, the western American species, and concluded that it was qualitatively identical with the other Veratrums and stood between them in toxicity.

Although, as stated above, the activity of V. viride had long been known, the popularization of the plant as a drug did not come until after 1851, as the result of the efforts of the physician Norwood (55, 56) who used "Norwood's Tincture" to treat pneumonia and typhoid fever. Other early physicians used it in the treatment of inflammatory rheumatism and puerperal fever.

The drug is at present little used, but there are occasional reports of its successful application, to lower the pulse rate in the treatment of tachycardia, to treat auricular fibrillation and pneumonia, and to lower the blood pressure and produce vasodilatation in puerperal eclampsia (65, 99).

By far the most widespread of these uses is in the treatment of eclampsia. This affliction is the most severe type of the "toxemias" of late pregnancy which rank along with sepsis as a cause of maternal death. Recent statistics have shown that over twenty per cent of women afflicted with eclampsia die. Hypertension is one of the outstanding and constant clinical findings in eclampsia, and is a sign of increased vasoconstriction. This vasoconstriction gives rise to a train of pathologic events. A hypertension develops which throws an added strain on the heart and blood vessels, either of which may give way, with disastrous results. Furthermore, the vasoconstriction itself leads to

anemia of the tissues, with suboxidation, retention of the waste products of metabolism, and eventually edema. Diagnosis of eclampsia (6) is based upon: 1) pregnancy of at least five months duration; 2) hypertension; 3) albuminuria; 4) convulsions; 5) coma following the convulsions.

Haultain (33, 34) in 1913 and 1914 reported the treatment of twelve cases of eclampsia, with success in all but one case. He used the commercial preparation of V. viride, Veratrone, of Parke, Davis and Co., described by Houghton and Hamilton (37).

More recently, Bryant (6) in 1935 recorded his results in the treatment at the Cincinnati General Hospital of 127 consecutive cases of eclampsia, with Veratrone as the principal therapeutic agent. The gross mortality rate for this series was 9.45 per cent, a very favorable record, and there had not been a death among the last 56 patients treated. Bryant showed that the mode of action of Veratrone was the production of a lasting vasodilatation, in agreement with the findings of Cramer (21). This dilatation resulted in an increased blood supply to the various organs, thus effecting a more normal exchange of metabolites and allowing more ready access of drugs such as dehydrators and alkalies (part of Bryant's treatment). The edema of the brain, skin and kidneys diminished, resulting in cessation of convulsions, return of consciousness, diuresis and diaphoresis.

Cramer had said that the suppression of urine in eclampsia was presumably due to extreme vasoconstriction in which the blood vessels of the kidney participated. When this condition was relieved as the result of the vasodilatation produced by the drug the conditions for secretion of urine were re-established.

Bryant (6) pointed out further that proper individualization of dosage was an essential factor in this treatment, for there was marked individual susceptibility to the drug. This point was also made by Chisholm (12).

Assays

Much work has been done in attempts to devise both chemical and biological assays for the Veratrums and to correlate the results obtained by the two techniques.

The first recorded attempt to determine total crude alkaloids in *Veratrum album* was made by Pehkschen (60) in 1890. He obtained a yield of 0.08 per cent with alcohol as original solvent.

LaWall (46) first applied the chemical method for total alkaloid determination to American Veratrum in 1897. His method was based on one of the earliest of such procedures, that developed by Keller (45). This procedure employed a chloroform-ether mixture as extractant.

Bredemann (5) assayed several commercial samples of *V. album* using a gravimetric method which was essentially that employed by LaWall. He also developed a volumetric assay method based on the acid-neutralizing powers of the alkaloids.

The most recent chemical study of assay methods for the Veratrums was made in 1922 by Viehoever and Clevenger (89). These authors greatly shortened the extraction time and made several other modifications in Bredemann's gravimetric method.

Houghton and Hamilton (37) in 1905 made the first definite attempt to work out a biological assay method for the Veratrums. They tested their aqueous preparation, Veratrone, on dogs and frogs and concluded that the frog method was a convenient and reliable indication of the potency of their preparations. Pilcher (64) made similar tests on frogs, guinea pigs and cats and concluded that the fatal dose for frogs was a satisfactory standard for *Veratrum* preparations. Rowe (77) in 1925 recommended the white mouse method over the frog method, claiming advantages of rapidity and definiteness for this technique.

Testing six samples of *Veratrum*, Githens and Vanderkleed (28) compared assays based on the lethal dose for guinea pigs with chemical assays and observed a fairly close agreement in the results from the two methods. On

the contrary, Pilcher (64) used his frog method as a check on chemical assays and found a wide deviation in the results thus obtained. This discrepancy was also noted by Pittenger (67) who reported in 1923 the results on a series of comparative assays of 32 samples made over a fourteen-year period. He used the minimum lethal dose for guinea pigs as his criterion for biological measurement. Further proof of the lack of correlation between chemical assays and bioassays was put forward by Swanson and Hargreaves (85, 86). They used Rowe's lethal white mouse method and concluded that this procedure gave reliable results while the chemical method was unreliable.

Christensen and McLean (13) in 1936 developed a method for assaying _Veratrum viride_ by determining the minimum emetic dose for pigeons. They showed later (14) that this method of bioassay did not give data comparable with those obtained from minimum lethal dose determinations on mice, and further that the latter did not parallel closely the physiological activity of _Veratrum_ preparations, as indicated by blood pressure effects produced on cats and dogs. They also showed that determination of the alkaloid content by the method of Viehoever and Clevenger (89) gave no indication of the physiological activity of _V. viride_ preparations.

Another method for bioassay of _Veratrum_ has recently been developed, by Viehoever and Cohen (90) in 1939. These

authors used the small, transparent crustacean, *Daphnia magna*, as experimental animal and as the criterion of effect the changes in swimming characteristics produced by the drug. The information gathered by this method as to toxicity and depressant action of *Veratrum* preparations was verified by toxicity tests and similar observations on albino rats, guinea pigs and rabbits. Comparative results on two samples of *V. viride* were obtained by this method, and the relative toxicities were reported by Cohen (15).

Entomological

As a member of the large group of plant insecticides *Veratrum viride* is commonly known as green hellebore or American hellebore, and is not generally distinguished from the related European species, *V. album*, known as white hellebore. This distinction is probably of questionable necessity since both species appear to have similar insecticidal properties.

The earliest record of the use of *V. viride* as an insecticide is that of Josselyn (43) who visited the continent in 1638 to 1671. Peter Kalm (44), a Swedish scientist who visited America in 1748 and 1749, referred to the use of an extract of hellebore root when the children "are plagued with vermin." Another early report of its use was its recommendation in 1775 by an author, W. W., for the control

of flies (94). Later Groom (30) recommended it as a remedy for gooseberry worms (Nematus spp.), in answer to a query by the editors of the Gardener's Chronicle, London.

In America Todd (88) in 1864 recommended white hellebore for gooseberry and currant worm control. Fitch (26) found white hellebore as a dust or spray to be specific for the currant worm, and it was he who first suggested that the native V. viride might be as effective an insecticide as V. album.

From that time to relatively recent years hellebore had been rather commonly applied as a dust against larval pests such as the currant worm. It had the advantage of losing its effectiveness rapidly upon exposure to air, thus being safely applied to small fruits near the picking time (83).

In 1916 Cook and Hutchinson (18) reported excellent success with V. album in the treatment of manure to kill housefly larvae. Howard and Bishopp (38), however, later reported hellebore to be inferior to borax for this purpose, and Fenton and Bieberdorf (24) found it to be ineffective in laboratory and field tests against housefly larvae.

McIndoo and Sievers (50) found V. album as a dust to be effective but slow in acting against roaches and silkworms, and to have a slight effect on bees, tent caterpillars and aphids. As a spray it was ineffective against

aphids, but as a stomach poison it was effective but slow against grasshoppers and silkworms.

Richardson (72) in 1933 sprayed housefly adults with a kerosene extract of hellebore with no success. He later (73) obtained considerable toxicity to gladiolus thrips with a spray of V. album.

Fisher (25) in 1938 found that an aqueous extract of V. viride was highly toxic when sprayed on adult houseflies, Musca domestica, but had no effect on the aphids, Aphis rumicis and Myzus persicae.

Fisher also reported experiments in which various crude alkaloid fractions from Veratrum viride were tested as toxic components of poison baits on the American cockroach (Periplaneta americana (L.)). These alkaloid fractions were obtained by following a regular assay procedure: the crude drug was extracted with chloroform-ether (equal parts by volume) using a lime-water suspension to release the alkaloids from the drug; the chloroform-ether solution was extracted with dilute acetic acid, which, after being made alkaline with ammonium hydroxide, was in turn extracted with chloroform-ether; the solvent was evaporated and the dried residue used as "total alkaloids." This procedure was followed on other subsamples of powder, but here the alkaline mixture was first extracted with ether until the ether extracts yielded no precipitate with Mayer's

reagent, and then with chloroform until the alkaloid extraction was complete. Evaporation of the solvents left fractions which were termed "ether-soluble alkaloids" and "ether-insoluble alkaloids" respectively. These three fractions were obtained from two different samples of crude drug.

The "ether-insoluble alkaloids" had no toxic effect on cockroaches at the largest doses given. The "ether-soluble alkaloids" from both samples were significantly more toxic than the respective "total alkaloids" as was to be expected, because of the nontoxicity of the "ether-insoluble" fractions. The mortalities from the "total alkaloids" of the two samples also differed significantly, but the "ether-soluble alkaloids" did not show a significant difference. These statements are based on median lethal doses calculated from the dosage-mortality curves. The median lethal doses are presented in Table I.

Table I. Median lethal doses, in milligrams per gram of body weight, of _Veratrum viride_ extracts tested on _Periplaneta americana_. Data of Fisher (25).

Fraction	M. L. D. (mg./g.)	
	Sample A	Sample B
"Ether-soluble alkaloids"	0.256	0.307
"Total alkaloids"	0.334	0.521
"Ether-insoluble alkaloids"	>3.87	>4.74

Although the "total alkaloids" and "ether-soluble alkaloids" contents of the two samples were approximately the same, sample A was apparently more toxic than sample B.

MATERIALS

Plant Materials

The samples of _Veratrum viride_ used in this investigation were in all cases purchased from wholesale drug supply houses, a total of six different samples being used in the experiments described herein. Two were obtained in powdered form: one from J. L. Hopkins and Co. as "powdered American hellebore," and the other from the Des Moines Drug Co. as "Pennsylvania white hellebore." The other four samples were purchased as the "whole" drug: one from J. L. Hopkins and Co. as "Amer. hellebore U. S. P.," another from the Murray and Nickell Manufacturing Co., and two from S. B. Penick and Co. as "hellebore root" and "hellebore root, American," respectively.

The "whole" drug is the rhizome with attached roots that has been cut, generally in quarters, before drying. In three of the four samples the whole drug was preponderantly rhizome, but in the fourth the rhizomes were very small and much of the root was left attached.

For use the whole drug was ground in a Wiley mill to pass a 20-mesh screen, and for special purposes such as assay determinations it was ground to 40-mesh.

Insects Used

The American cockroach (Periplaneta americana (L.)) was used in the toxicological tests, adults being employed exclusively. These insects were trapped in buildings on the campus of Iowa State College and were kept either in a large, screened cage or in glass aquarium jars at room temperature. They were fed bananas, rolled oats, dried milk, a special salt mixture and dried brewers' yeast, and water was available to them at all times.

ASSAY EXPERIMENTS

Methods

The chemical assay method first adopted was that of Viehoever and Clevenger (89), which was only slightly altered from the one described by Bredemann (5). This method may be outlined briefly as follows:

> A 15-gram sample, powdered to 40-mesh, is allowed to stand for 10 minutes with 150 cc. of chloroform-ether (equal parts). Then 10 cc. of 10 per cent ammonium hydroxide is added and the mixture is shaken frequently for an hour. Then 10 cc. of water is added. The mixture is then filtered through cotton into a flask containing 0.25 g. of calcined magnesia. After shaking, the mixture is filtered into a graduated cylinder, and 80 cc. of the filtrate (corresponding to 8 g. of drug) is transferred to a separatory funnel. This filtrate is extracted with 10 per cent acetic acid solution, first with 20-cc. portions and then with 10-cc. portions. These extracts are combined in a separatory funnel, made alkaline with 10 per cent ammonium hydroxide and in turn extracted with chloroform-ether (equal parts) in 20- and then 10-cc. portions. The chloroform-ether extracts are combined in a tared weighing bottle, evaporated in a current of air, and dried to almost constant weight at 100°C.

This procedure was tried on several different samples of the crude drug and then a few modifications were made to obviate the necessity for extreme care in preventing solvent evaporation. The changes made consisted in using a 10.0-g.

sample, filtering and washing with more solvent the crude drug after the hour's extraction, and combining the filtrate and washings for the acetic acid extraction.

Viehoever and Clevenger (89) had determined that ammonium hydroxide or sodium hydroxide were equally satisfactory for liberating the alkaloids from the crude drug. It was noted, however, that treatment with these solutions made the drug somewhat glutinous and that difficulty was encountered by formation of emulsions in the acetic acid extraction. Therefore duplicate assays were made using 10 per cent solutions of ammonium hydroxide and sodium hydroxide respectively, a 10 per cent suspension of calcium hydroxide, and finally no base. Results indicated the advantages to be gained in using hydrated lime so this variation was incorporated in further study of assay techniques.

The assay procedure as modified is outlined:

> To a 10.0-g. sample of crude drug ground to 40-mesh add 150 cc. of chloroform-ether mixture (equal parts by volume), and allow to stand 10 minutes. Then add 1 g. powdered calcium hydroxide and 10 cc. water and let stand one hour with frequent agitation. Filter, and wash with chloroform-ether mixture until the washings give no precipitate with Mayer's reagent; about 50 cc. of solvent in 4 portions are sufficient. Combine filtrate and washings in a flask containing 0.5 g. powdered magnesium oxide. Shake thoroughly, filter into a separatory funnel and wash the magnesia with more solvent. Extract the filtrate with three 20-cc. portions of 10 per cent acetic acid, and then with 10-cc. portions until the last gives no precipitate with Mayer's reagent.

> Make the acid extract alkaline with concentrated ammonium hydroxide, and extract with three 20-cc. portions of chloroform-ether, and then with 10-cc. portions until a few drops of the alkaline solution, on acidification with acetic acid, give no precipitate with Mayer's reagent. Combine the extracts in a tared flask and evaporate off the solvent under a current of air at 80°C.

In all previous work it was noted that the final chloroform-ether extract and the residual total alkaloids after removal of solvent were more or less amber-colored. Since no alkaloid heretofore isolated from the Veratrums is colored, it was obvious that some impurities were being carried through the procedure. Hence a series of experiments was made on one sample of powdered drug with a view to overcoming this difficulty. Since the chloroform-ether mixture is known to have very high solvent powers, ether alone and chloroform alone were tried; in some experiments ether was used first, followed by chloroform, and in others the order was reversed. The first solvent was used until the last extract gave no precipitate with Mayer's reagent, whereupon the change was made. The chloroform-ether mixture was also used as a control.

Results

A blank assay was made according to the method of Viehoever and Clevenger (89), outlined above. The blank seemed necessary in order to ascertain whether inorganic

salts, especially ammonium salts, might be carried through the procedure. This determination showed that no blank correction was necessary.

The duplicate assays made by the same method on three different samples of crude Veratrum viride gave somewhat inconsistent results. The percentages of total alkaloids extracted by this method are given in Table II.

Table II. Results of chemical assay of Veratrum viride according to method of Viehoever and Clevenger.

Sample	Total alkaloids, per cent	
	Assay No. 1	Assay No. 2
A	0.17	0.17
B	1.09	0.73
C	1.41	1.11

The experiments with the modified procedure, using a 10.0-g. sample and filtering and washing the crude drug after extraction gave more concordant results, when put into use to determine the effectiveness of different alkaline agents in freeing the alkaloids from the crude drug. The data, presented in Table III, show the percentage total alkaloids extracted from subsamples of one sample using the several reagents. They indicate that ammonium hydroxide, sodium hydroxide and calcium hydroxide are equally satisfactory, and that the addition of some base is necessary for extraction of the alkaloids from the crude drug.

Table III. Effectiveness of various alkaline reagents in freeing alkaloids from crude drug.

Treatment	Total alkaloids, per cent	
	Assay No. 1	Assay No. 2
Ammonium hydroxide	1.42	1.39
Sodium hydroxide	1.47	1.22
Calcium hydroxide	1.36	1.47
None	0.92	0.94

Since the use of calcium hydroxide was effective and it had the added advantages of keeping the drug in a granular condition and of tending to prevent the formation of emulsions in the acetic acid extractions, the assay procedure was modified by substitution of the lime suspension for ammonium hydroxide.

This new procedure was then made use of in an extended series of assays whose purpose was to investigate the influence of solvents in the final extraction. Since the final extract obtained when chloroform-ether was used was distinctly colored and hence contained impurities, it seemed probable that ether or chloroform used separately or in succession would produce a more satisfactory extraction. The results of these tests are given in Tables IV, V, and VI. These data show that chloroform alone extracts as great an amount of material as does the chloroform-ether mixture, about 1.55 per cent. The amounts of alkaloids

Table IV. Percentages of alkaloids extracted using chloroform-ether mixture in the final extraction.

Subsample No.	Alkaloids, per cent
7	1.39
9	1.53
13	1.59
14	1.71
19	1.41
26	1.62
27	1.60
28	1.56
Mean	1.55

Table V. Percentages of alkaloids extracted with ether and then chloroform in the second extraction.

Subsample No.	Percentage alkaloids extracted by		
	Ether	Chloroform	Total
3	0.83	0.43	1.26
4	0.73	0.55	1.28
5	0.87	0.39	1.26
6	0.93	0.37	1.30
8	0.74	0.54	1.28
10	0.74	0.54	1.28
15	0.60	0.79	1.39
16	0.63	0.80	1.43
Mean	0.76	0.55	1.31

Table VI. Percentages of alkaloids extracted with chloroform and then ether in the second extraction.

Subsample No.	Percentage alkaloids extracted by		
	Chloroform	Ether	Total
17	1.56	0.02	1.58
18	1.57	0.02	1.59
Mean	1.56	0.02	1.58

extracted by the respective solvents from the different subsamples vary considerably, but the total yields are generally quite consistent. It must be noted that the total yields, about 1.31 per cent, are uniformly less than the yields obtained by use of the solvent mixture.

DISCUSSION OF ASSAYS

The chemical assay method for estimating total crude alkaloid content of Veratrum viride given by Viehoever and Clevenger (89) appeared to be rather unsatisfactory for general use. The difficulties encountered include the great care that must be taken to prevent evaporation of solvent, the difficulty in filtering the crude drug due to its glutinous nature when treated with ammonium hydroxide or sodium hydroxide, and the formation of emulsions during the acetic acid extraction.

The first difficulty was removed by taking a smaller sample of drug, extracting with the same amount (150 cc.) of solvent and filtering and washing the drug after extraction. The sliminess characteristic of the alkali-treated drug was overcome by use of a suspension of calcium hydroxide in place of ammonium hydroxide or sodium hydroxide. A series of tests indicated that all three basic reagents were equally satisfactory for releasing the alkaloids from the crude drug. In addition, the substitution of calcium hydroxide was effective in preventing emulsion formation.

The procedure thus modified was then applied to a further phase of the investigation. It had been constantly noted that the final chloroform-ether extract was distinctly

colored, a fact pointing to the presence of nonalkaloidal impurities in this extract. A series of assays was made, in which the modified procedure was followed with chloroform-ether as final extractant. The mean of 8 assays by this method showed a total alkaloid content of 1.55 per cent. Two tests were then made in which chloroform was used alone, followed by ether. The total alkaloids extracted by this method averaged 1.58 per cent, thus equal in amount to the yield with chloroform-ether as solvent. Eight more tests were made, but with ether alone until alkaloids were no longer extracted, and then chloroform until complete extraction was attained. Whereas the individual solvents gave quite erratic results, the total of the alkaloids extracted was very consistent, with a mean of 1.31 per cent. The residues thus obtained from the ether extractions were practically colorless. Thus it is evident that the procedure involving successive extraction with ether and then chloroform, which showed a mean alkaloid content of 1.31 per cent, was equally as effective in the <u>extraction of alkaloids</u> as the other two procedures which separated 1.55 and 1.58 per cent of the plant material. The conclusion to be drawn from this fact is that the other 0.2 to 0.3 per cent extracted by the chloroform-ether mixture and chloroform followed by ether was impurities nonalkaloidal in nature.

This conclusion is in part borne out by the insecticidal work of Fisher (25). He applied the modified assay procedure to two commercial samples of Veratrum viride, using chloroform-ether as final solvent. The alkaloid content was nearly the same for the two samples. He used these residues for his experiments, terming them "total alkaloids." He then assayed the samples using ether alone as solvent, followed by chloroform. These fractions he called "ether-soluble alkaloids" and "ether-insoluble alkaloids," respectively. The six fractions, when tested in poison baits on the American cockroach (Periplaneta americana), gave results which led to the conclusions that the "ether-soluble alkaloid" fraction contained most, if not all, of the toxic alkaloids, and that the "ether-insoluble alkaloids" fraction, as evident from its color, contained nonalkaloidal material. The fact that the ratio of the amount of "ether-soluble" to "total alkaloids" in both samples was similar to the ratio of the median lethal dosages of these same materials led to the tentative conclusion that the standardization of the drug for insecticidal purposes should be based on the "ether-soluble" rather than on the "total alkaloid" content.

A further conclusion may be drawn from the work of Fisher. Whereas the total alkaloid contents of the two samples he used were nearly equal, the toxicities of the

total alkaloid fractions were statistically different, thus corroborating on insects much previous work on vertebrate animals which indicated that biological and chemical assays could not be directly correlated.

SEPARATION OF ALKALOID MIXTURE

Methods

The first two experiments on separation of the crude alkaloid mixture from Veratrum viride to be described herein were made on a sample of "Hellebore Root, American" obtained from the S. B. Penick Co. This sample of drug assayed 2.25 per cent total alkaloids by the modified procedure outlined in a preceding section, in which chloroform-ether was employed as final extraction solvent. The third experiment was made on a combination of several lots of the crude drug. The samples were prepared for extraction by grinding them in a Wiley mill to pass a 20-mesh screen.

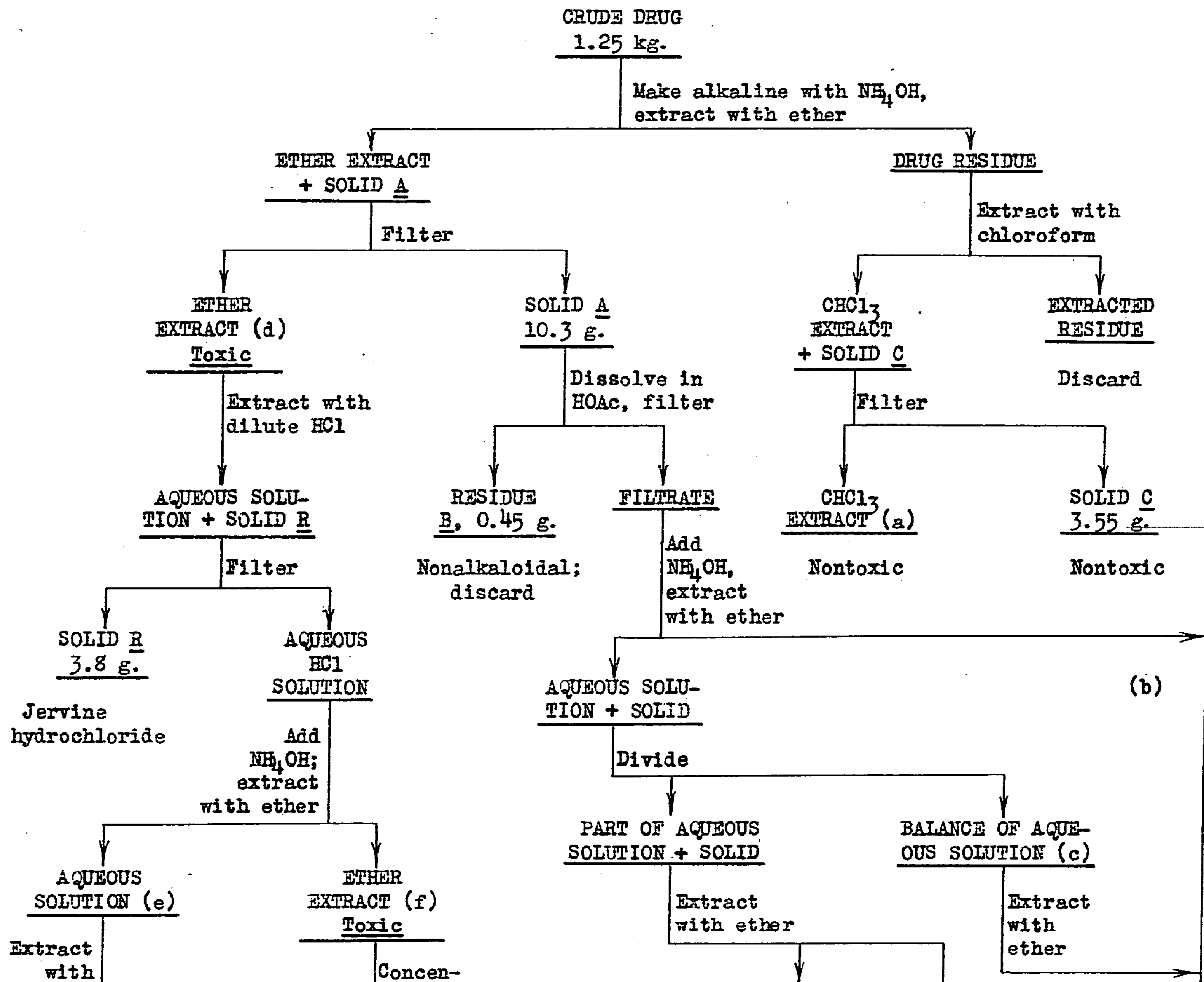
Tests to determine the presence of alkaloids in solutions were made with Mayer's reagent, which is a solution of 10 g. of potassium iodide and 15 g. of mercuric iodide in 100 cc. of water. This solution forms a precipitate with all but the simplest alkaloids in aqueous acid solution. To test organic solvents immiscible with water, such as chloroform and ether, for the presence of alkaloids a small sample of the liquid was extracted with an equal volume of 10 per cent acetic acid solution and the determination was made with Mayer's reagent on this extract.

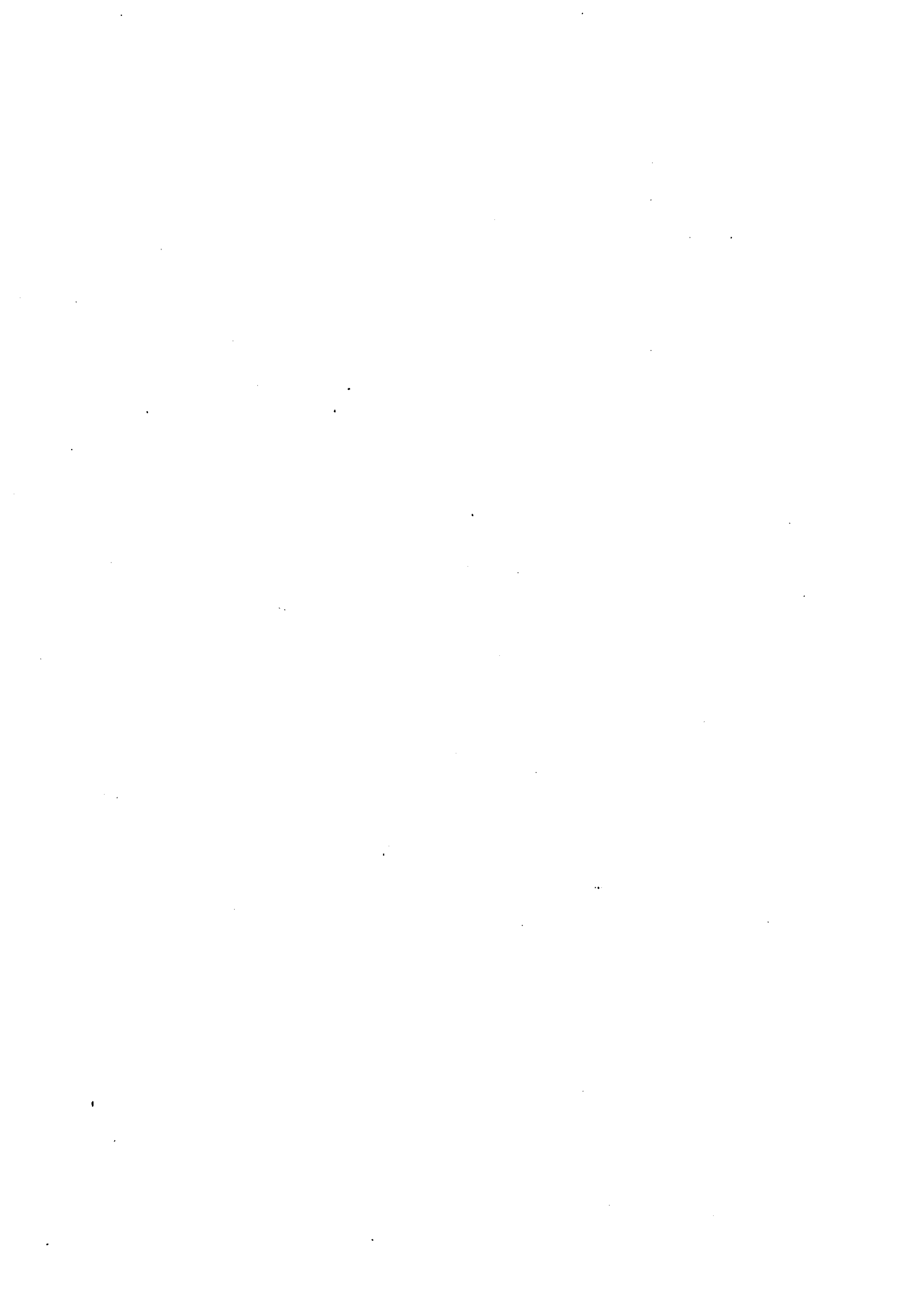
Experiment A.

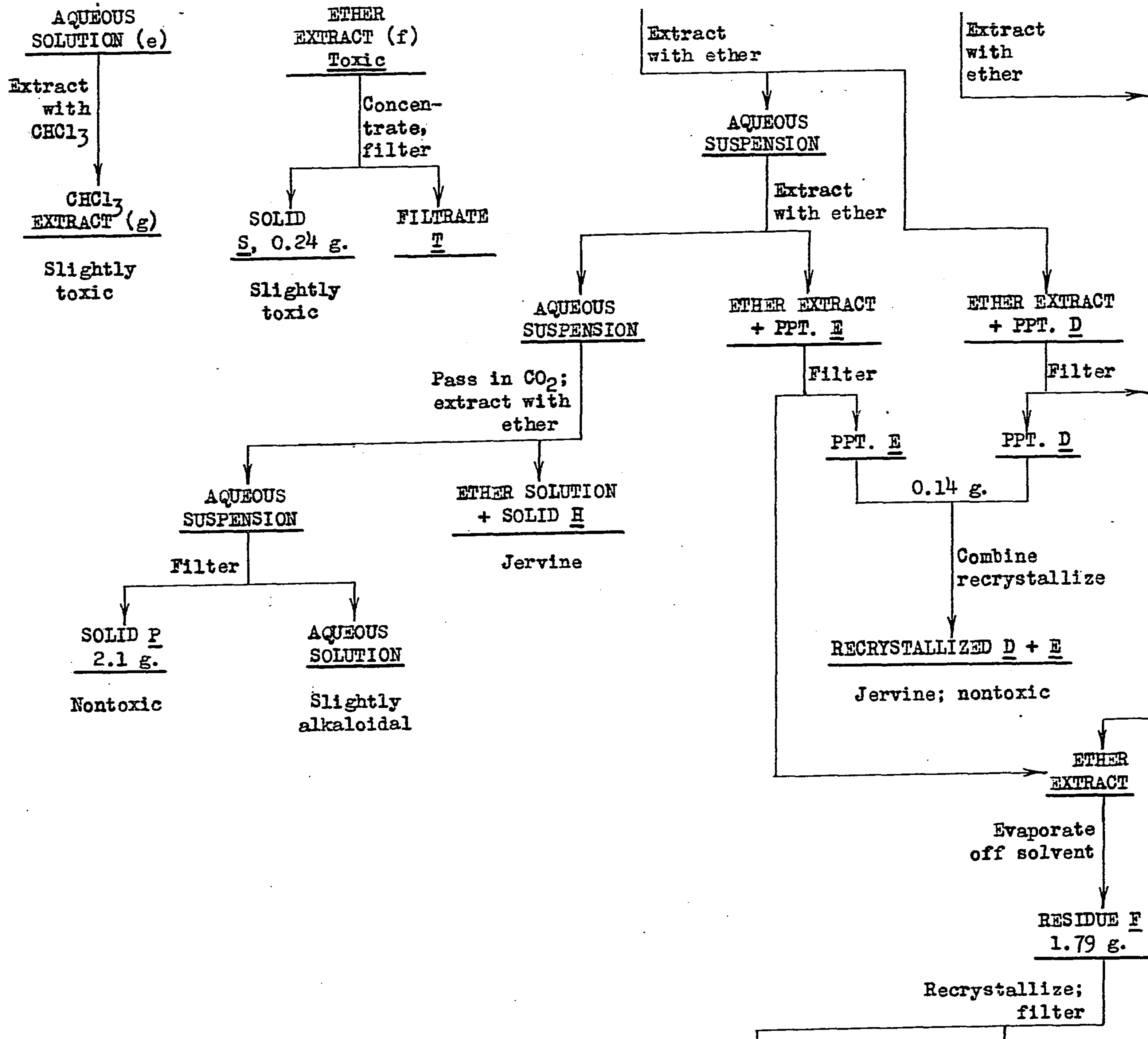
For this experiment, outlined diagrammatically in Fig. 1, 1250 g. of the crude drug was mixed with 500 cc. of 10 per cent ammonium hydroxide solution and packed into a continuous extractor. Here the drug was extracted with ether for about 65 hours. The drug residue was then dried by drawing air through the extractor for 8 hours and the extraction was continued for 72 hours with chloroform as solvent. The extracted drug residue was discarded.

During the chloroform extraction there had separated in the solvent reservoir a light-colored solid C, which was filtered off and washed with chloroform, and, after drying in air, weighed 3.55 g. Both solid C and the chloroform extract (a) were nontoxic when tested on the American cockroach by a procedure to be outlined in a later section.

There had also separated in the reservoir during the ether extraction of the drug 10.3 g. of a tan-colored solid A, which was filtered off, washed with ether and air-dried. The solid A was treated with 160 cc. of 2.5 per cent acetic acid solution, leaving a slight residue B, weighing when air-dried 0.45 g., which was nonalkaloidal and was therefore discarded. The acid filtrate was made alkaline with concentrated ammonium hydroxide, a flocculent, yellow precipitate forming. This suspension was first extracted with a 200-cc. portion of ether, and then with 10 successive


CRUDE DRUG
1.25 kg.
Make alkaline with NH4OH,
extract with ether
ETHER EXTRACT
+ SOLID A
DRUG RESIDUE
Filter
Extract with
chloroform
ETHER
EXTRACT (d)
Toxic
SOLID A
10.3 g.
CHCl3
EXTRACT
+ SOLID C
EXTRACTED
RESIDUE
Discard
Extract with
dilute HCl
Dissolve in
HOAc, filter
Filter
AQUEOUS SOLU-
TION + SOLID R
RESIDUE
B, 0.45 g.
FILTRATE
CHCl3
EXTRACT (a)
SOLID C
3.55 g.
Filter
Nonalkaloidal;
discard
Add
NH4OH,
extract
with ether
Nontoxic
Nontoxic
SOLID R
3.8 g.
AQUEOUS
HCl
SOLUTION
Jervine
hydrochloride
AQUEOUS SOLU-
TION + SOLID
(b)
Add
NH4OH;
extract
with ether
Divide
PART OF AQUEOUS
SOLUTION + SOLID
BALANCE OF AQUE-
OUS SOLUTION (c)
AQUEOUS
SOLUTION (e)
ETHER
EXTRACT (f)
Toxic
Extract
with ether
Extract
with
ether
Extract
with
Concen-





AQUEOUS SOLUTION (e)
Extract with $CHCl_3$
$CHCl_3$ EXTRACT (g)
Slightly toxic
ETHER EXTRACT (f) Toxic
Concentrate, filter
SOLID S, 0.24 g.
Slightly toxic
FILTRATE T
Extract with ether
AQUEOUS SUSPENSION
Extract with ether
Extract with ether
AQUEOUS SUSPENSION
Pass in CO_2; extract with ether
AQUEOUS SUSPENSION
Filter
SOLID P 2.1 g.
Nontoxic
AQUEOUS SOLUTION
Slightly alkaloidal
ETHER SOLUTION + SOLID H
Jervine
ETHER EXTRACT + PPT. E
Filter
PPT. E
ETHER EXTRACT + PPT. D
Filter
PPT. D
0.14 g.
Combine recrystallize
RECRYSTALLIZED D + E
Jervine; nontoxic
ETHER EXTRACT
Evaporate off solvent
RESIDUE F 1.79 g.
Recrystallize; filter

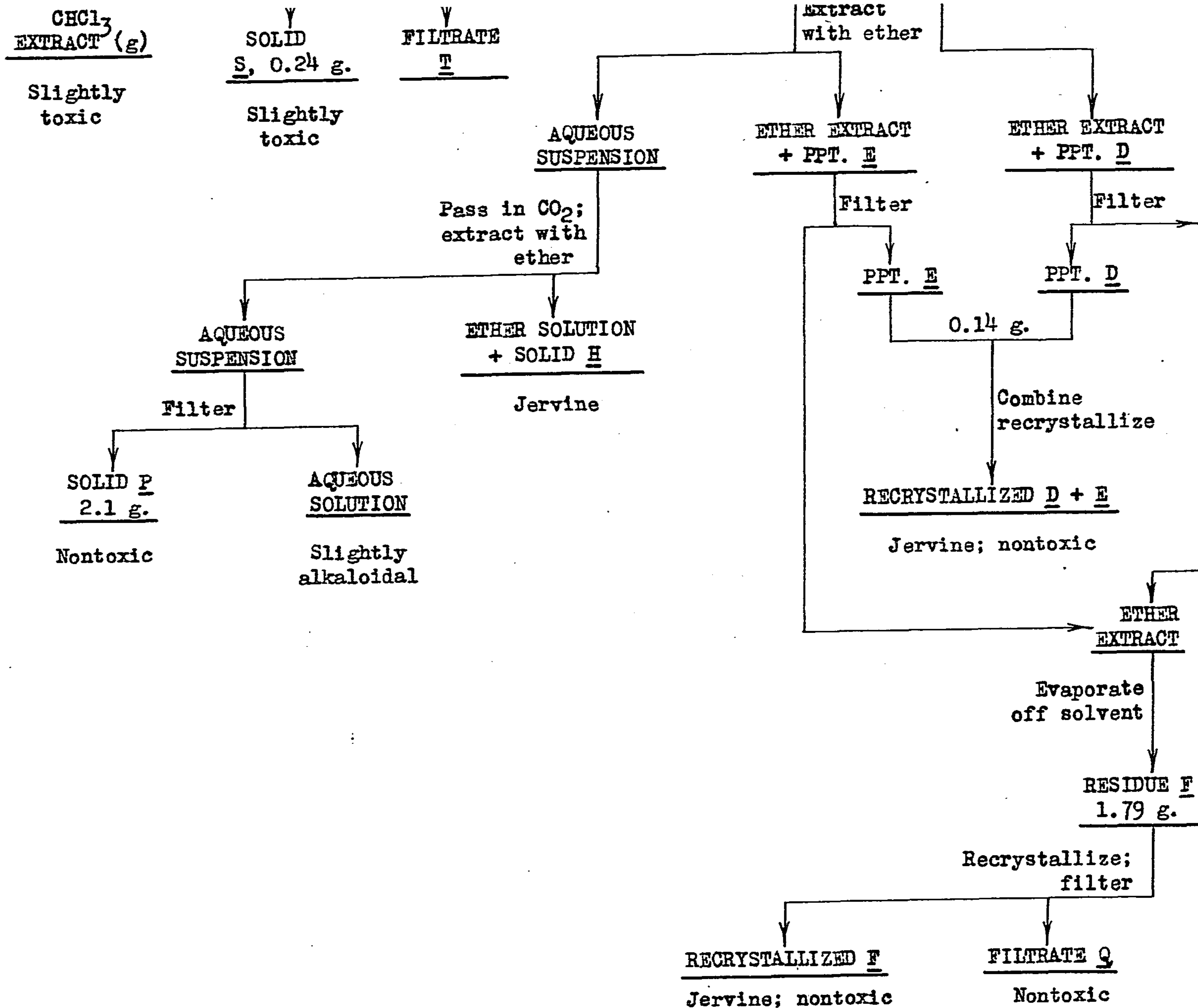


Fig. 1. Schematic outline of procedure followed in Experiment A.

100-cc. portions, the ether extracts being combined (b). The aqueous suspension, which was still strongly alkaloid-containing, was separated into two portions, one containing the solid and the other most of the aqueous solution (c). The suspension was extracted with ether in a continuous extractor; two fractions were collected, the first after 28 hours' extraction, and the second after 17 hours more. From these two extracts there had separated yellow solids along the sides of the reservoir, D and E, respectively. D and E were filtered off, combined, and, after being dried in air, weighed 0.14 g. This fraction was non-toxic.

The two continuous ether extractions had still not removed all the alkaloidal material from the aqueous suspension, so the latter was saturated with carbon dioxide to make the solution more nearly neutral, and the ether extraction was continued for 43 hours longer, a solid H separating in the reservoir. This solid resembled D and E very much. The residual suspension was filtered, yielding a solid P which when air-dried weighed 2.1 g. It was non-toxic. The filtrate gave only a faint test for presence of alkaloids with Mayer's reagent.

The aqueous solution (c) was extracted with ether until all alkaloids had been removed. This ether extract was combined with (b) and the filtrates from D and E, dried

over anhydrous sodium sulfate, and the solvent removed completely by evaporation. The air-dried residue F weighed 1.79 g.

The original ether extract (d), from which A had been removed, was quite toxic on injection into cockroaches. It was extracted with dilute hydrochloric acid (one volume of concentrated acid to nine volumes of water) in 100-cc. portions until all the alkaloids were removed. In the first three acid extractions there separated a light-colored solid R which was filtered off, washed with water, air-dried and found to weigh 3.8 g.

The aqueous acid extract was made alkaline with ammonium hydroxide and extracted with twenty 100-cc. portions of ether, the last of which contained scarcely any alkaloids, whereas the alkaline solution (e) was strongly alkaloidal. The ether extract (f), which was toxic, was concentrated to about 10 cc. and on standing in the icebox a solid S weighing 0.24 g. separated which was slightly toxic. The filtrate T was examined further but yielded no significant information.

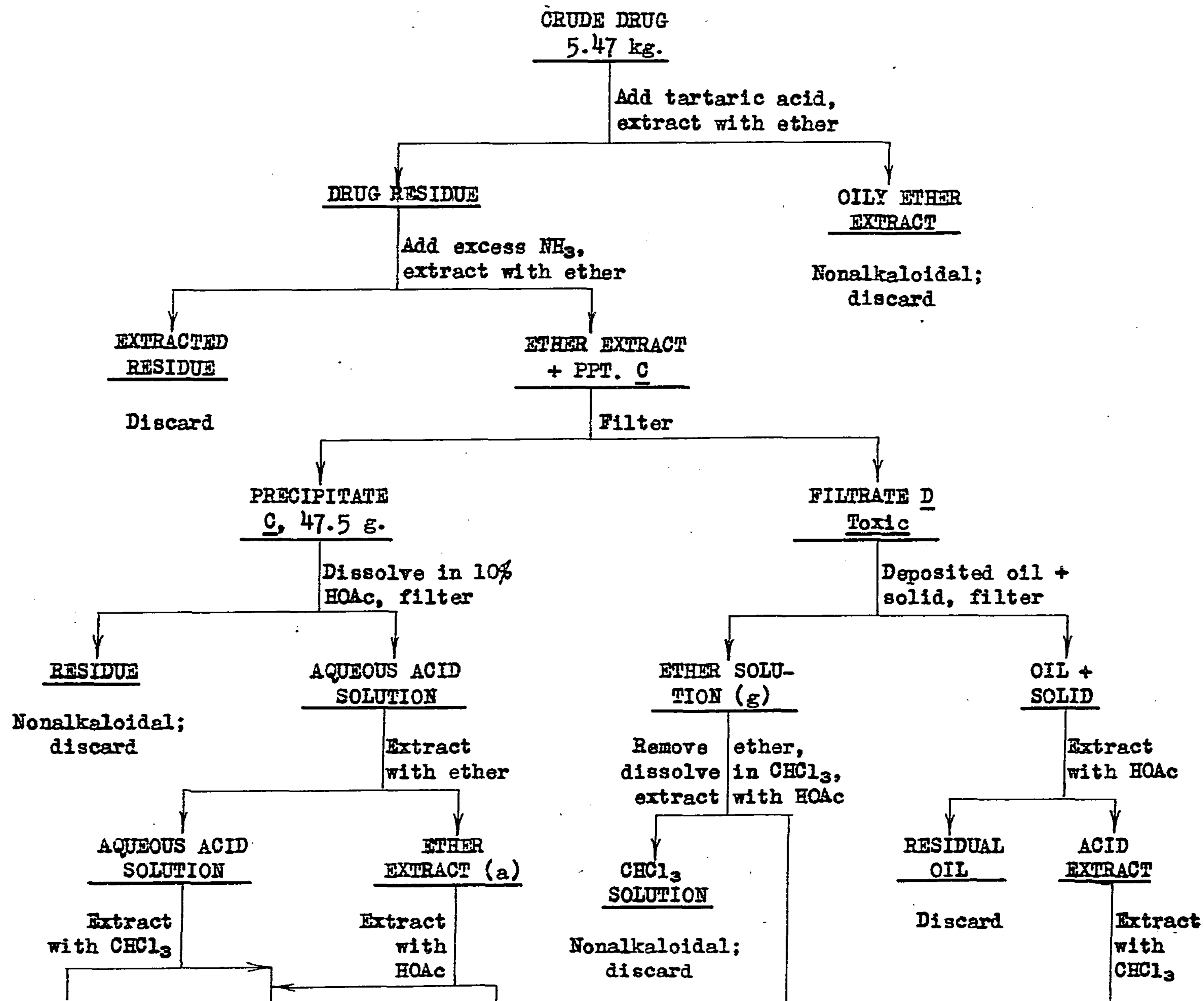
The alkaline solution (e) was extracted with ten 100-cc. portions of chloroform, thus removing all the alkaloids. The chloroform extract (g) was found to be only slightly toxic.

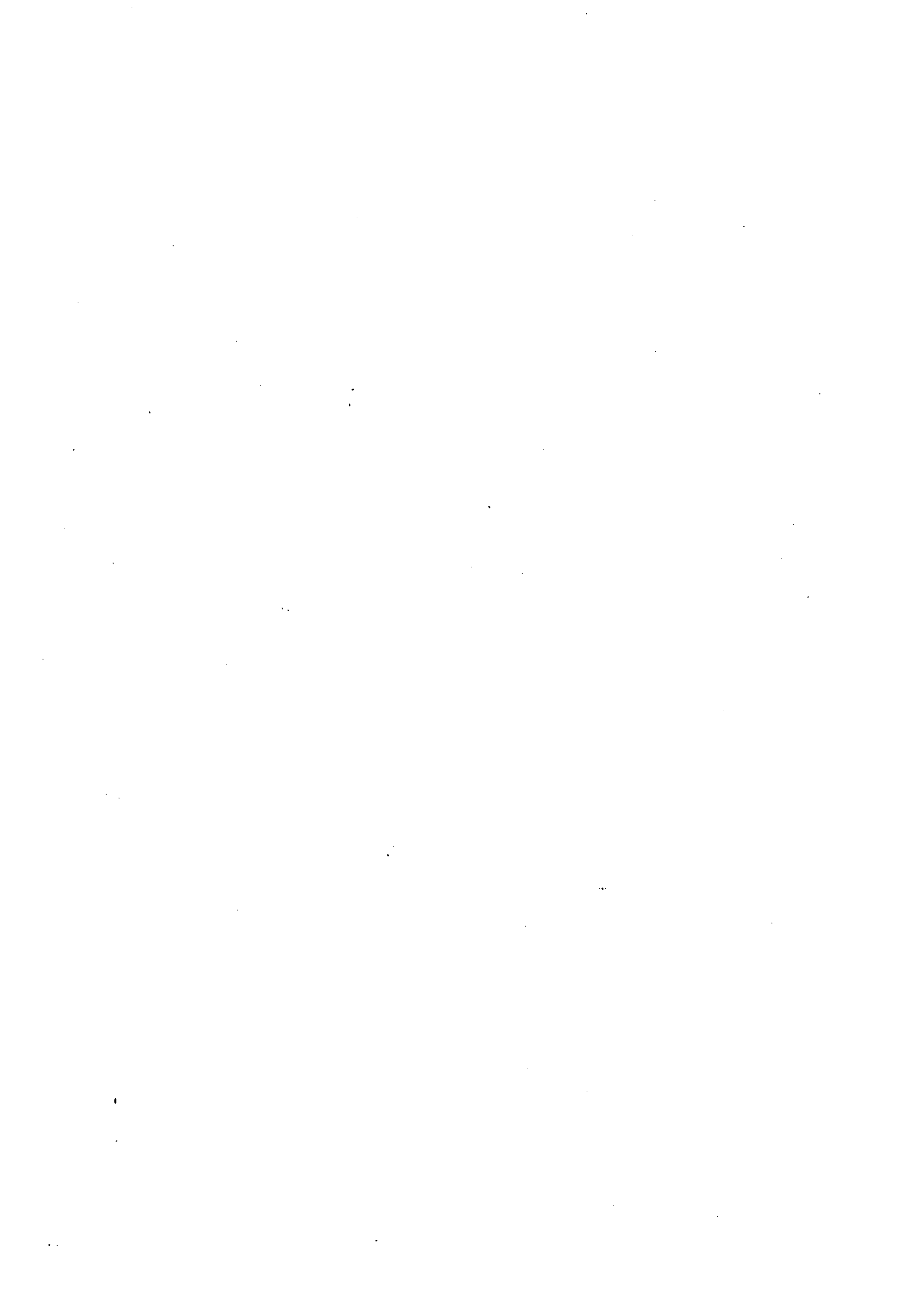
Experiment B.

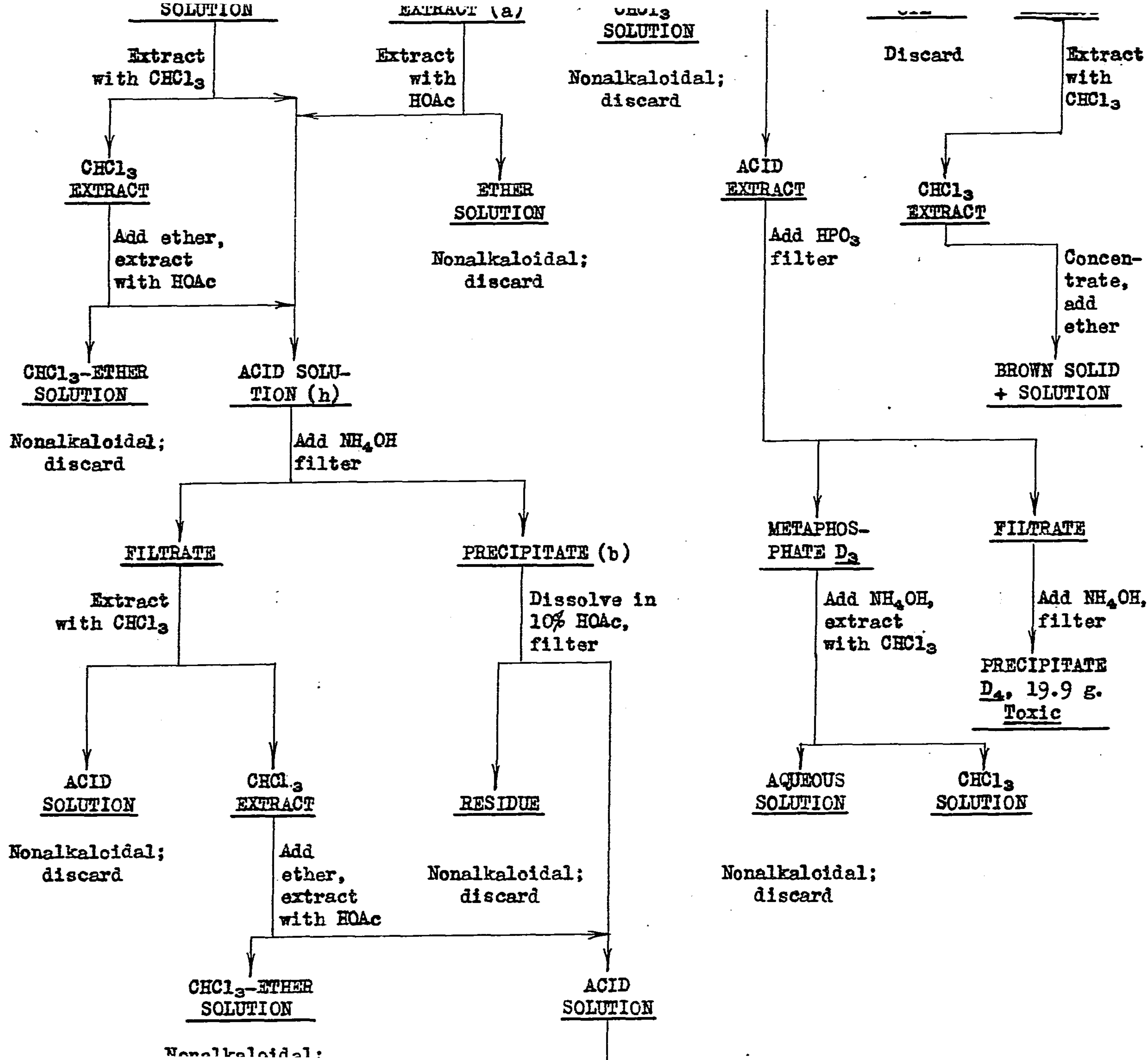
The procedure followed in Experiment B is shown in Figs. 2 and 3. The essential difference from the method of Experiment A was the use of a procedure developed by Salzberger (81) in which jervine was separated from the other alkaloids by precipitation from aqueous acetic acid solutions as the metaphosphate.

The crude drug was from the same lot used in the first experiment. A solution of 175 g. of tartaric acid in 5.15 kg. of water was mixed with 5.47 kg. of the ground drug. The wet drug was placed in a continuous extractor and extracted with ether for 4 days. The resultant ether extract was red-brown in color and contained much oily material but no alkaloids. An excess of ammonia was passed into the extractor, making the drug strongly alkaline, and the ether extraction was continued for 8 days, during which time there separated on the sides of the reservoir a gray solid C. This oily solid was filtered off, washed with a small amount of ether, and dried in air. It weighed 47.5 g.

The solid C was treated with 250 cc. of 10 per cent acetic acid solution, leaving a slight amount of nonalkaloidal residue which was discarded. The acid solution was extracted with six 50-cc. portions of ether which removed some of the coloring matter and a small amount of alkaloids (a). It was then extracted with two 100-cc. portions of


CRUDE DRUG
5.47 kg.
Add tartaric acid,
extract with ether
DRUG RESIDUE
OILY ETHER
EXTRACT
Nonalkaloidal;
discard
Add excess NH_3,
extract with ether
EXTRACTED
RESIDUE
Discard
ETHER EXTRACT
+ PPT. C
Filter
PRECIPITATE
C, 47.5 g.
FILTRATE D
Toxic
Dissolve in 10%
HOAc, filter
Deposited oil +
solid, filter
RESIDUE
Nonalkaloidal;
discard
AQUEOUS ACID
SOLUTION
Extract
with ether
ETHER SOLU-
TION (g)
Remove ether,
dissolve in $CHCl_3$,
extract with HOAc
OIL +
SOLID
Extract
with HOAc
AQUEOUS ACID
SOLUTION
Extract
with $CHCl_3$
ETHER
EXTRACT (a)
Extract
with
HOAc
$CHCl_3$
SOLUTION
Nonalkaloidal;
discard
RESIDUAL
OIL
Discard
ACID
EXTRACT
Extract
with
$CHCl_3$





SOLUTION
EXTRACT (a)
SOLUTION
Extract with $CHCl_3$
Extract with HOAc
Nonalkaloidal; discard
Discard
Extract with $CHCl_3$
$CHCl_3$ EXTRACT
ETHER SOLUTION
ACID EXTRACT
$CHCl_3$ EXTRACT
Add ether, extract with HOAc
Nonalkaloidal; discard
Add HPO_3 filter
Concentrate, add ether
$CHCl_3$-ETHER SOLUTION
ACID SOLUTION (h)
BROWN SOLID + SOLUTION
Nonalkaloidal; discard
Add NH_4OH filter
FILTRATE
PRECIPITATE (b)
METAPHOSPHATE D_3
FILTRATE
Extract with $CHCl_3$
Dissolve in 10% HOAc, filter
Add NH_4OH, extract with $CHCl_3$
Add NH_4OH, filter
PRECIPITATE D_4, 19.9 g. Toxic
ACID SOLUTION
$CHCl_3$ EXTRACT
RESIDUE
AQUEOUS SOLUTION
$CHCl_3$ SOLUTION
Nonalkaloidal; discard
Add ether, extract with HOAc
Nonalkaloidal; discard
Nonalkaloidal; discard
$CHCl_3$-ETHER SOLUTION
ACID SOLUTION

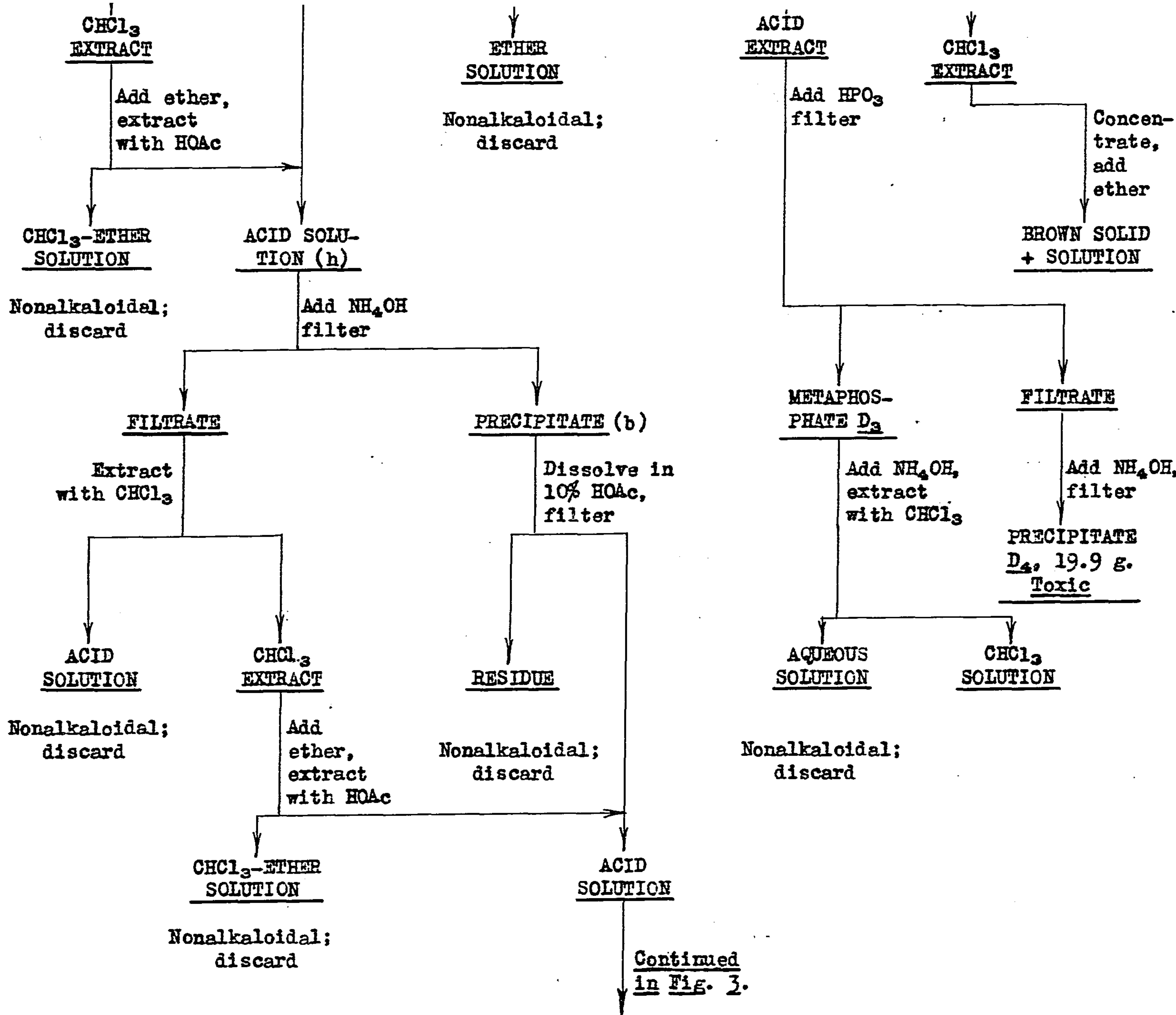
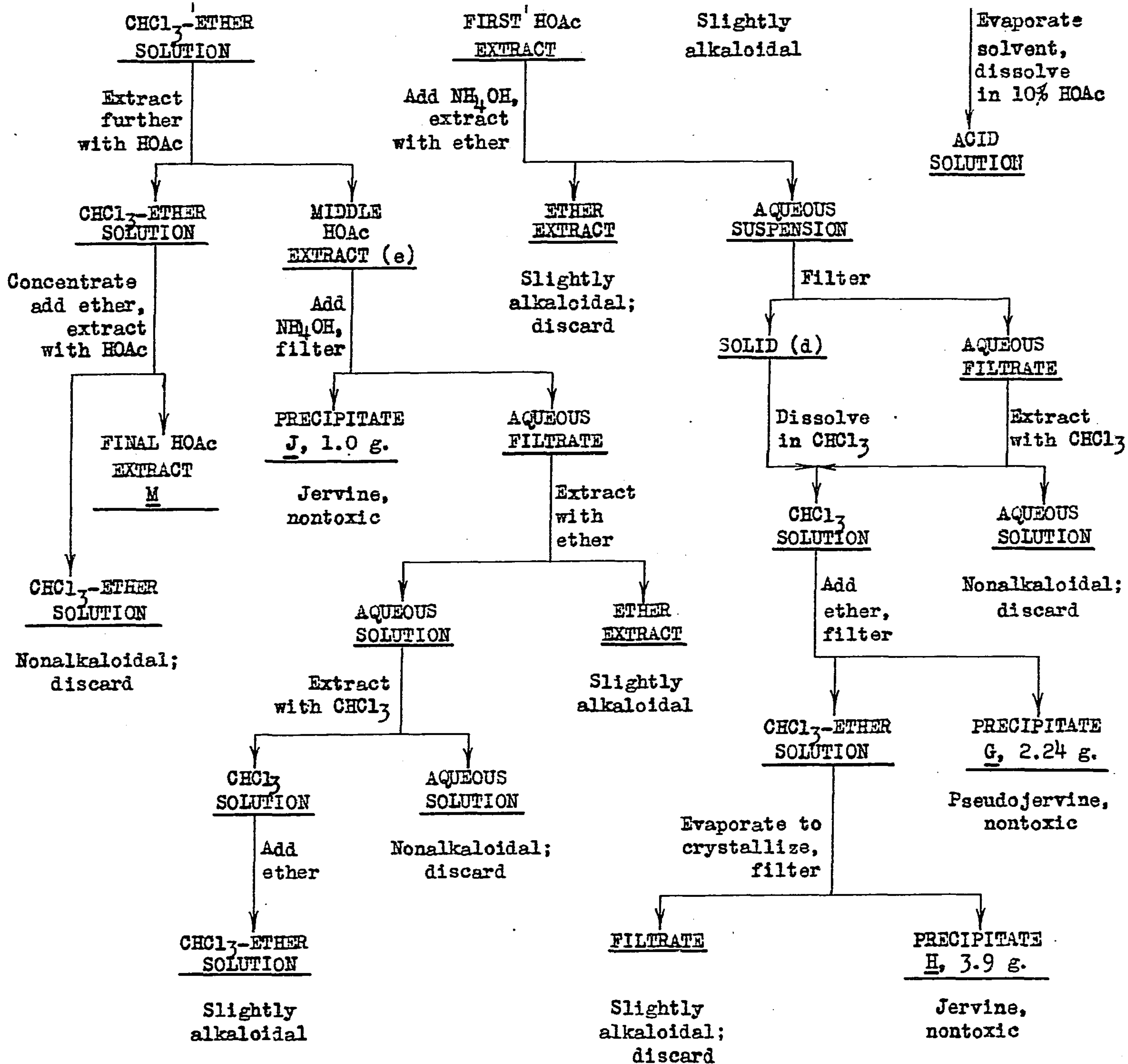


Fig. 2. Schematic outline of procedure followed in Experiment B.

Continued from Fig. 2.

ACID SOLUTION

- Add HPO_3, filter
  - **PRECIPITATE E**
    - Add NH_4OH, extract with $CHCl_3$
      - **AQUEOUS SOLUTION + RESIDUE**
        - Nonalkaloidal; discard
      - **$CHCl_3$ SOLUTION**
        - Concentrate to 1/3, add ether, filter
          - **PRECIPITATE F, 6.8 g.**
            - Jervine, nontoxic
          - **$CHCl_3$-ETHER SOLUTION**
            - Extract partially with 10% HOAc
              - **$CHCl_3$-ETHER SOLUTION**
                - Extract
              - **FIRST HOAc EXTRACT**
                - Add NH_4OH,
  - **FILTRATE**
    - Add NH_4OH, filter
      - **AQUEOUS SOLUTION**
        - Add excess NH_4OH, filter
          - **AQUEOUS FILTRATE (f) Nontoxic**
            - Extract with ether
              - **AQUEOUS SOLUTION**
                - Slightly alkaloidal
              - **ETHER EXTRACT**
                - Evaporate solvent, dissolve in 10% HOAc
          - **PRECIPITATE L, 0.158 g. Toxic**
      - **PRECIPITATE K, 1.47 g.**
        - Slightly toxic



[Fig. 3. Schematic outline] of procedure followed in Experiment B. (Continued from Fig. 2.)

chloroform, the chloroform solution was diluted with an equal volume of ether and was extracted with 10 per cent acetic acid to recover the alkaloids. The residual, colored chloroform solution was discarded. The acid extract was combined with the main body of acid solution. The ether solution (a) was also extracted with 10 per cent acetic acid to recover the alkaloids, and the colored ether solution was discarded. The acid extract was likewise returned to the main body of acid solution.

Ammonium hydroxide was added to precipitate the alkaloids from this acetic acid solution. The precipitate (b) was filtered off, washed with dilute ammonium hydroxide, and dried in air. It weighed 34.4 g. It was treated with 10 per cent acetic acid and filtered from the nonalkaloidal residue. The filtrate from (b) was extracted with chloroform until the alkaloids were all removed, the chloroform extract was diluted with an equal volume of ether, and the solution was extracted with 10 per cent acetic acid. The colored, nonalkaloidal chloroform-ether solution was discarded.

This acetic acid solution was combined with the clarified acid solution of (b), and to it was added a freshly prepared solution of 15 g. of metaphosphoric acid in 50 cc. of water. There formed immediately a flocculent precipitate E which was filtered off and washed with dilute

metaphosphoric acid solution. The precipitate E was suspended in dilute ammonium hydroxide to release the alkaloids from their compounds and the suspension was extracted with chloroform. A small amount of insoluble residue was found to be nonalkaloidal so was discarded. The chloroform solution of alkaloids was concentrated to one-third its volume and was diluted with an equal volume of ether, whereupon a white, crystalline precipitate F formed, which weighed 6.8 g. on being filtered off and dried in air. It was nontoxic.

The chloroform-ether filtrate (c) was then extracted with two 25-cc. portions of 10 per cent acetic acid which removed part of the alkaloids. This acid solution was made alkaline with ammonium hydroxide, precipitating a large amount of material. This suspension was extracted with 50 cc. of ether; the resulting ether extract, being only slightly alkaloid-containing, was discarded. The aqueous suspension was filtered and the precipitate (d) was dissolved in chloroform. The alkaloid-containing filtrate was extracted with chloroform to remove the alkaloids, and the chloroform extract was combined with the solution of (d). This solution was diluted with two volumes of ether, a crystalline precipitate G forming. The latter, when filtered off, washed, and air-dried, weighed 2.24 g. and was nontoxic. On further standing more crystalline solid H

separated from the chloroform-ether solution, was filtered off, and was dried in air. It weighed 3.9 g. and was also nontoxic. The filtrate contained only a small amount of alkaloids so was discarded.

The chloroform-ether filtrate (c), after partial extraction with acetic acid, was extracted with a further four 25-cc. portions of 10 per cent acetic acid, and these extracts were combined as (e). The chloroform-ether solution still contained alkaloids, so was concentrated and diluted with an equal volume of ether, and the acetic acid extraction continued until all the alkaloids had been removed. This final acid extract was combined as M and was not examined further.

The middle portion of acetic acid extract (e) was made alkaline with ammonium hydroxide and filtered from the resulting precipitate J. The latter when air-dried weighed 1.0 g. and was nontoxic. The filtrate from J contained a small amount of alkaloid so was extracted with five 40-cc. portions of ether, yielding on concentration an extract which was slightly alkaloidal and was not worked up further. One 40-cc. chloroform extraction removed the balance of the alkaloids from the aqueous solution, the chloroform extract, after concentration to 10 cc. and dilution with 20 cc. of ether, also being only slightly alkaloid-containing. It was similarly discarded.

The filtrate from the precipitate of metaphosphate E (Fig. 3) was made faintly alkaline with ammonium hydroxide, a tan, amorphous precipitate K forming. This precipitate was filtered off, washed with dilute ammonium hydroxide, and air-dried. It weighed 1.47 g. and was slightly toxic.

The filtrate from K was made strongly alkaline with ammonium hydroxide, a further precipitate L forming. This was also filtered off, washed and dried in air. It weighed 0.158 g. and was toxic. The filtrate from L, (f), was non-toxic, though it still contained alkaloids. It was extracted with ether; the aqueous solution remaining still contained a small amount of alkaloids but was not worked further. The ether extract was evaporated to dryness and the residue was taken up in dilute acetic acid. On further working it yielded no useful information.

The ether filtrate D (Fig. 2) was found to be toxic. On standing for some time in the laboratory there separated an oily layer and a solid material. The ether solution (g) was filtered off, and the residual oil and solid were extracted with dilute acetic acid to remove the alkaloids. The acid solution was extracted with chloroform which removed some of the coloring matter and a small amount of alkaloids. On concentration of the chloroform extract and dilution with ether a brown solid separated out. These fractions were not worked further.

The ether solution (g) was evaporated in a vacuum, and the residual dark oil was dissolved in chloroform and extracted with acetic acid until all the alkaloids had been removed. The acid extract was treated with an excess of freshly prepared metaphosphoric acid solution, a voluminous precipitate $\underline{D_3}$ separating. This was filtered off, suspended in ammonium hydroxide and shaken out with chloroform. The resulting chloroform solution was not worked up since similarly obtained solutions always yielded nontoxic alkaloids, and the residual aqueous solution, being nonalkaloidal, was discarded.

The filtrate from the metaphosphate $\underline{D_3}$ was treated with an excess of ammonium hydroxide and a large amount of precipitate $\underline{D_4}$ was formed. This was filtered off and dried in air. It weighed 19.9 g. and was toxic.

Experiment C.

A third experiment was made in which several lots of crude *Veratrum viride* were combined. The procedure followed was based closely on that outlined for Experiment B, Figs. 2 and 3, but attention was concentrated on fractions found in earlier work to be toxic to the American cockroach and hence of especial interest.

A total of 7.56 kg. of crude drug was wet with a water solution of 200 g. of tartaric acid, allowed to dry in air,

and then extracted continuously with ether for 3 days. The resulting ether solution, containing no alkaloids, was discarded. The crude drug was then dried in air, mixed with an aqueous suspension of calcium hydroxide to make alkaline, replaced in the extractor and extracted with ether for 6 days. As in Experiment B, there separated in the reservoir of the extractor during extraction a gray solid A. This was filtered off from the ether solution B.

The solid A, similar to C of Experiment B, was dissolved in 300 cc. of 10 per cent acetic acid and was extracted with ether for 24 hours to remove extraneous material. The residual acid solution corresponded to solution (h) of Experiment B, from which had eventually been isolated the toxic fraction L, small in quantity. This solution, therefore, was made alkaline with ammonium hydroxide and was filtered. The precipitate H thus obtained, weighing 9.6 g., was not investigated further, since the fractions similarly obtained in Experiment B had consisted predominantly of nontoxic alkaloids. The filtrate J, from precipitate H, was extracted with 25-cc. portions of chloroform, the combined extract was concentrated to 40 cc. and was allowed to stand for two weeks. During this time there separated out slowly a small amount of crystalline material, P, which was filtered off, washed and air-dried. It weighed 0.25 g.

The ether solution E, corresponding to D of Experiment B, was concentrated to remove almost all of the ether, was taken up in chloroform and was extracted with 10 per cent acetic acid in 50-cc. portions, a total of 3000 cc. of extract being collected. This acid solution was made alkaline with ammonium hydroxide and the resulting precipitate D was filtered off and air-dried. It weighed about 45 g.

Forty grams of precipitate D was dissolved in 10 per cent acetic acid and was diluted with water to 250 cc. This solution was cooled in an ice bath and was treated with a freshly prepared solution of metaphosphoric acid, a curdy precipitate forming. This precipitate K weighed, when air-dried, 18.5 g. Since precipitates thus obtained in the previous experiments had yielded only nontoxic alkaloids this fraction was not examined further.

The filtrate from K was made alkaline with ammonium hydroxide and the resulting precipitate L was filtered off. This fraction, which was toxic and corresponded to the toxic fraction D_4 of Experiment B, weighed 15.9 g. The filtrate was slightly alkaloidal and was not investigated further.

Fractions Obtained and Alkaloids Isolated

Some of the fractions separated in the experiments just detailed were investigated further and yielded certain valuable information.

Experiment A.

The combined solids, D and E, weighed 0.14 g. A solution was made of 55 mg. of this fraction in 5 cc. of hot 95 per cent ethyl alcohol, the solution was filtered, and the filtrate was placed in the icebox to crystallize. The recrystallized material was filtered off, washed with alcohol and dried in air. The yield of recrystallized solid was 37 mg. It became colored in the melting point apparatus at about 230°C. and melted with decomposition at 237-239°C.* This recrystallization was repeated, and then dilute methyl alcohol was used as solvent for a third recrystallization. The resulting needles darkened at 230°C.; m.p. 237-241°C. decomposing. This product was jervine, which melts according to Poethke (70) at 243-244°C. (corr.) and according to Saito and coworkers (80) at 243.5-244.5°C. This fraction was nontoxic.

The crude residue F weighed 1.79 g. Fifty mg. were heated with 1 cc. of 95 per cent ethyl alcohol, most of the solid dissolving to form a dark brown solution. From this on standing in the icebox there separated 5 mg. of a needle-like crystalline precipitate which melted at 233-239°C. with decomposition, after darkening about 230°C. The fraction

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*All melting points were taken in the Fisher-Johns micromelting point apparatus equipped with a microscope for observation. The temperature was raised rapidly to about 30° below the expected melting point, after which the rate of heating was lowered to about 3° per minute. The alkaloids and their derivatives with but few exceptions melt with decomposition or decompose before melting.

that did not dissolve in the first 1 cc. of alcohol was dissolved in 2 cc. more of alcohol. From this straw-colored solution there were recovered 3 mg. of needle-like crystals which darkened at 230° and melted with decomposition at 238-243°C. Thus residue F yielded jervine also. Once-recrystallized material from this fraction was non-toxic.

The solid H was in crystalline form so was scraped from the sides of the flask, filtered off, and washed with ether. It darkened above 190° and melted with decomposition at 237-241°C. Its needle-like crystalline form, its melting point, and the fact that it was obtained in a manner similar to that used in collecting D and E indicate that H was also jervine.

The precipitate R which was obtained on hydrochloric acid extraction of the ether extract (d) weighed 3.8 g. when air-dried. A small portion of R was boiled with absolute ethyl alcohol, part going into solution. The filtrate was placed in the icebox to crystallize. The resulting fine, granular crystals were filtered off, washed with more solvent and air-dried. In the melting point apparatus they were observed to darken about 230°C., and a sublimate of clear, colorless rosettes of prism-like crystals began to form. The blackening portions started to melt about 280° and the sublimate melted about 300°C. with decomposition.

The undissolved portion of R began to darken about 200°C., a sublimate of clear rosettes of needles and prisms began to form about 250° and continued to 285°. The brown portion melted about 285-295° and the sublimed crystals melted with decomposition at 300-302°C. The precipitate R thus appeared to be jervine hydrochloride, which according to Saito et al. (79) melts about 308°C.

Experiment B.

The precipitate F, recovered from the metaphosphate precipitate E, was obtained in the form of radiating needle-like crystals. Its melting point was 240-242°C., decomposing. It appeared to be jervine, and was found to be nontoxic.

The precipitate G, obtained in the form of impure crystals, weighed 2.24 g. On heating it melted sluggishly to a dark brown liquid from 242-247°C. This product was very difficult to purify, owing to its pronounced tendency to adsorb impurities whenever precipitated or crystallized. To effect the purification 1.5 g. of the crude fraction was treated with 10 cc. of 10 per cent acetic acid solution, filtered from the insoluble residue, the filtrate diluted with 40 cc. water and 40 cc. methyl alcohol, heated to boiling, neutralized with dilute (1:3) ammonium hydroxide solution till a turbidity formed, and cooled slowly. The

resulting brown, crystalline precipitate, after filtration and air-drying, sintered at 245°, darkened above 250° and melted at 280-295°C. with decomposition. It weighed 0.92 g. Part of this fraction, 0.75 g., was boiled with 25 cc. of methyl alcohol, a dark brown solution forming, and a light tan residue remaining. The latter, weighing 0.43 g., darkened above 275° and melted at 295-305°C. with decomposition. Of this fraction 0.4 g. was dissolved in the least amount of 10 per cent acetic acid necessary, the solution was diluted to 19 cc. with water, 20 cc. ethyl alcohol was added, the solution was heated to boiling and neutralized with 1:2 ammonium hydroxide solution. On cooling slow crystallization took place. The resulting precipitate appeared in the form of colorless platelets, weighing 0.23 g. It darkened above 280° and melted at 298-300°C. with decomposition. It was pseudojervine, which melts with decomposition at 304-305.5°C. according to Poethke (70) and at 300-307°C. according to Salzberger (81). It was non-toxic.

The crystalline precipitate H, weighing 3.9 g., melted at 235-242°C. with decomposition. Three grams of this fraction was dissolved in 15 cc. of 10 per cent acetic acid solution, filtered, diluted with water to 50 cc. and to 90 cc. with methyl alcohol, heated to boiling, and neutralized with 1:3 ammonium hydroxide until a turbidity

appeared in the solution. On cooling 0.11 g. of crystalline solid was obtained. The filtrate from this was again boiled and treated with ammonium hydroxide until a rather large amount of precipitate was formed. This was filtered off after cooling, washed and dried in air. The process was repeated, and a third fraction was obtained. The second and third fractions combined weighed 2.35 g. Of these combined fractions 2.0 g. was dissolved in 40 cc. of boiling methyl alcohol, and 6 cc. of water was added, a turbidity appearing in the solution. On cooling a pure white, needle-like precipitate was obtained, weighing 1.56 g. It darkened above 200° and melted with decomposition at 241-243°C. It was jervine and was nontoxic.

The precipitate J weighed 1.0 g. On recrystallization from dilute ethyl alcohol and a trace of ammonium hydroxide it formed needle-like crystals, melting at 236-241°C. with decomposition. It also was jervine and was nontoxic.

Of the impure, amorphous precipitate K, which was slightly toxic, 3 mg. was dissolved in dilute acetic acid, and a saturated solution of picric acid was added, a yellow precipitate forming slowly. This was filtered off and air-dried. It was then dissolved in a very small quantity of acetone, and ether was added. On standing there separated a dark brown solid which was filtered off, and the filtrate was allowed to evaporate. There formed a small amount of

yellow, crystalline precipitate which was washed with ether and dried. It melted at 230-233°C. This picrate is unidentified.

The amorphous precipitate L, which was toxic, weighed 0.158 g. Numerous experiments were made on this fraction, including attempts at fractional crystallization, sublimation and salt formation, none of which gave positive information.

The crude, brown, amorphous precipitate D_4, weighing 19.9 g., was toxic. Many experiments were made on this fraction, mostly without success. Chromatographic adsorption was tried with unfavorable results. One procedure, however, gave some valuable information. As an example of this procedure, 1 g. of the fraction was dissolved in 1 cc. of 10 per cent acetic acid, diluted with water to 4 cc. and treated with a solution of potassium nitrite to separate out through their insoluble nitroso derivatives the secondary amine alkaloids, such as jervine, probably present in this fraction. A dark brown, sticky precipitate was formed, from which the supernatant liquid was poured. The sticky precipitate after being dried in air amounted on an average to 0.6-0.7 g. Repeated solution in ethyl alcohol and reprecipitation by dilution purified this nitroso fraction to such an extent that crystallization was attained from dilute ethyl alcohol. The crystalline

solid thus obtained melted at best at 228-240°C. with decomposition. This nitroso derivative was probably that of jervine. Nitrosojervine was prepared from pure jervine for comparison and was found to melt after three recrystallizations from dilute ethyl alcohol at 250-254°C. with decomposition. According to Poethke (70) nitrosojervine melts at 246-247°C. with decomposition, and according to Saito et al. (80) at 251-252°C.

The filtrate from the nitroso fraction was made alkaline with ammonium hydroxide and was extracted with ether. Evaporation of the solvent left a slightly colored residue (j) amounting in different experiments to 0.06-0.12 g. The residue was extracted with benzene, the greater part going into solution and a little remaining undissolved. This undissolved fraction was washed with alcohol which took out most of the remaining colored impurities. The residue was then crystallized by solution in dilute acetic acid and reprecipitation with ammonium hydroxide. By recrystallization from a dilute alcoholic acetic acid solution on addition of ammonium hydroxide dendritic crystals were obtained which darkened above 250°C. and charred at 265-270°C. This appeared to be protoveratridine, which melts according to Poethke (69) at 266-267°C. with decomposition and according to Salzberger (81) at 265 °C.

This crystalline product was dissolved in dilute acetic acid, heated and treated with a saturated solution of picric

acid. On cooling crystalline platelets separated out. The air-dried crystals weighed 2.2 mg. They recrystallized to large rhombs above 220°C. and melted and boiled away at 245-252°C. with some decomposition. This picrate appeared to be protoveratridine picrate, which according to Poethke (69) decomposes at 244-246°C. without melting completely.

In one of these experiments involving separation through the nitroso derivatives, the benzene extract from the ether residue was evaporated to dryness. The residue was dissolved in dilute acetic acid and was treated with a saturated picric acid solution. On slow evaporation in a desiccator a crystalline picrate was obtained which darkened above 230°C. and decomposed and sublimed away at 245-251°C. A mixed melting point determination with the previously isolated protoveratridine picrate showed no depression.

Experiment C.

In this experiment extensive work was done on two products, P and L, which were obtained from fractions similar to the toxic fractions L and D_4 of Experiment B.

Fraction P which weighed 0.25 g. when air-dried was crystalline and melted over a range from 180 to 220°C. to an almost colorless oil. It was recrystallized from a concentrated solution in methyl alcohol, forming large

rhombs and cubes which melted partially at 170-175°C., started to decompose and resolidify above 190° and finally melted with decomposition at 215-227°C. From its first isolation from chloroform, its considerable solubility in water, methyl and ethyl alcohols and chloroform, its failure to be precipitated from an aqueous solution by alkalies and its peculiar behavior in the melting point apparatus, this product was thought to be germine, discovered recently by Poethke (69) to be the basic hydrolysis product from the toxic alkaloid germerine. Poethke found that germine had solubilities similar to those just mentioned and observed that it sintered between 160 and 170° and melted about 220°C. Further examination of this fraction will be reported in the next section.

The crude fraction L was found to be toxic to the American cockroach. A one-gram portion of this was treated with dilute hydrochloric acid solution and filtered from the slight residue remaining. The filtrate was diluted with water to 30 cc., 3 cc. of alcohol was added, the solution was heated to boiling and several drops of 70 per cent perchloric acid were added. There was formed a brown precipitate which was filtered off and air-dried. It weighed 0.37 g. The filtrate was investigated extensively but gave no useful information. The precipitated alkaloid perchlorate was suspended in dilute ammonium hydroxide and was extracted

with ether. The ether solution was evaporated to dryness, the residue again taken up in dilute acetic acid, the solution made alkaline with ammonium hydroxide and extracted with ether. The ether solution on evaporation left a residue weighing 0.29 g. This residue was taken up in dilute acetic acid, the solution made alkaline with ammonium hydroxide and the resulting precipitate filtered off. The filtrate from it was investigated further but gave no information. The precipitate was dissolved in alcohol and dilute acetic acid, and the alcohol was evaporated off under vacuum. On evaporation of the alcohol much of the coloring matter separated out and the supernatant acid liquid was poured off. This solution was made alkaline with ammonium hydroxide and was extracted with ether. Evaporation of the ether left a residue weighing 0.18 g. This was recrystallized from ethyl alcohol by diluting the hot solution and formed needle-like crystals which melted at 235-237°C. with decomposition. On recrystallization from dilute ethyl alcohol the product melted at 239-243°C. with slight decomposition. This fraction appeared to be rubijervine, which melts according to Poethke (70) at 239-240°C. with decomposition. Further examination of this fraction was made and will be reported in the next section.

Two grams of fraction L was dissolved in 1:1 hydrochloric acid and the solution was diluted to 250 cc. This

diluted solution was placed in a continuous extractor and was extracted with chloroform. Nine successive portions of extract were collected, all containing small amounts of alkaloidal material. Then 10 cc. of concentrated ammonium hydroxide was added to the aqueous solution and the extraction was continued for 24 hours, giving extract number 10. A further 24-hour extraction gave fraction number 11. After addition of 10 cc. more of ammonium hydroxide extracts 12 and 13 were collected. The process was repeated for collection of extracts 14 and 15, 16 and 17, 18 and 19. Addition of the next 10 cc. of ammonium hydroxide made the aqueous solution slightly alkaline. Continued chloroform extraction gave extract number 20. A further addition of 10 cc. of ammonium hydroxide made the solution strongly alkaline and the extraction was continued till no further alkaloidal material was removed, the final extract being number 21.

All of the first 19 extracts were evaporated to small volumes, giving small amounts of amorphous product. On standing extracts 20 and 21 yielded crystalline material which had the same characteristics as did fraction P and proved to resemble germine.

STUDY OF PURE ALKALOIDS

Jervine

Free base.

Pure jervine melted at 241-243°C. with decomposition, after previously darkening above 200°C. Poethke (70) found that jervine crystallized from methyl alcohol contained one molecule of solvent of crystallization, and that obtained from dilute ethyl alcohol contained two molecules of water of crystallization. The data obtained here show that the alkaloid crystallized from dilute methyl alcohol contains one molecule of water of crystallization.

Analysis

Calculated for $C_{26}H_{37}O_3N \cdot CH_3OH$: 7.22% loss.
Calculated for $C_{26}H_{37}O_3N \cdot 2H_2O$: 8.05% loss.
Calculated for $C_{26}H_{37}O_3N \cdot H_2O$: 4.19% loss.
Found on drying at 110°C. in vacuum over P_2O_5:
4.36%, 4.21%.

Determination of rotatory power

α_D^{23} -1.57° (ethanol, 7.584 mg. air-dried sample in 0.7335 cc.) $[\alpha]_D^{23}$ -158.5°, calculated as anhydrous alkaloid.

α_D^{20} -1.20° (10% acetic acid, 5.740 mg. air-dried sample in 0.7335 cc.) $[\alpha]_D^{20}$ -160.1°, calculated as anhydrous alkaloid.
Poethke (70) $[\alpha]_D^{20}$ -154.5° (ethanol), -167.6° (chloroform); Saito et al. (80) $[\alpha]_D^{20}$ -150° (ethanol).

Hydrochloride.

Jervine formed a hydrochloride which melted at 300-302°C. with decomposition. Saito et al. (78) gave "about 308°C." as its melting point.

Hydroiodide.

The hydroiodide of jervine was prepared by adding a potassium iodide solution to a solution of jervine in dilute acetic acid. The amorphous precipitate thus obtained was filtered off, washed with water and dried. An attempt was made to recrystallize this product from acetone, but it was only slightly soluble in this solvent. It was successfully recrystallized by solution in a large volume of methyl alcohol, addition of water and standing overnight. The recrystallized jervine hydroiodide darkened above 260°C. and melted at 288-290°C. with strong decomposition. Salzberger (81) in an attempted preparation of jervine methiodide obtained a compound melting at 275°C. which he said was jervine hydroiodide.

Picrate.

Jervine picrate, which had not been previously reported, was prepared by dissolving 200 mg. of jervine in dilute acetic acid, diluting with water to 50 cc. and precipitating with an excess of picric acid solution. The resulting amorphous precipitate was dried, dissolved in the least amount of acetone necessary for solution, and the acetone solution was diluted with peroxide-free, dry ether. There separated yellow crystals, weight when dry 187 mg., which darkened above 210°C. and charred completely without melting at 274-284°C.

Analysis

Calculated for $C_{26}H_{37}O_3N \cdot C_6H_3O_7N_3$: N, 8.74.

Found: N, 8.60, 8.60.

Nitrosojervine.

Treatment of an acetic acid solution of jervine with potassium nitrite solution yielded a white precipitate which when recrystallized three times from dilute ethyl alcohol melted at 250-254°C. with decomposition. According to Poethke (70) nitrosojervine melted at 246-247°C. (corr.) with decomposition, and according to Saito et al. (78) it melted at 251-252°C. with decomposition.

Pseudojervine

The melting point of pseudojervine was 298-300°C. with decomposition, after darkening above 280°C. The melting point of the pure alkaloid given by Poethke (70) was 304-305.5°C. (corr.) with decomposition. Pseudojervine, recrystallized by solution in dilute acetic acid, addition of ethyl alcohol and treatment with ammonium hydroxide, contained no solvent of crystallization. The pure alkaloid was only slightly soluble in chloroform or absolute ethyl alcohol but was much more soluble in a mixture of the two solvents.

Analysis

Loss in weight on drying at 105°C. in vacuum over P_2O_5: 0.32%.

Determination of rotatory power

α_D^{23} -0.64° (1:3 ethanol-chloroform, 3.518 mg. air-dried sample in 0.7335 cc.) $[\alpha]_D^{23}$ -133.4°.

α_D^{18} -0.77° (1:6 ethanol-chloroform, 4.270 mg. dried sample in 0.7335 cc.) $[\alpha]_D^{18}$ -132.5°.

α_D^{22} -0.99° (10% acetic acid, 5.454 mg. air-dried sample in 0.7335 cc.) $[\alpha]_D^{22}$ -133.1°.

Poethke (70) $[\alpha]_D^{20}$ -139° (7:43 ethanol-chloroform).

Rubijervine

Free base.

Rubijervine on recrystallization from dilute ethyl alcohol formed needles melting at 239-243°C. with slight decomposition. Previous reports of the melting point of rubijervine are: Poethke (70), 239-240°C. with decomposition; Salzberger (81), 240-246°C.; Wright and Luff (106), 236°C. A total of about 10 mg. of this alkaloid was obtained, and since its melting point was close to that of jervine it was desired to distinguish between them.

Hydroiodide.

The only crystalline salt of rubijervine reported in the literature is the hydroiodide prepared by Poethke (70). Therefore this salt was made by addition of a few drops of potassium iodide solution to a solution of the alkaloid in dilute acetic acid. The resulting precipitate was filtered off, dried and recrystallized by solution in acetone (in which it was quite soluble) and reprecipitation with ether. Rubijervine hydroiodide was thus obtained in rosettes of colorless crystals melting at 269-273°C. with decomposition. Poethke stated that this compound melted at 261-262°C. Rubijervine is thus distinguished from jervine, the hydroiodide of which was only slightly soluble in acetone and

melted at 288-290°C. with much decomposition.

Protoveratridine

Free base.

Protoveratridine was obtained in very small amount in these experiments. It darkened above 250°C. and charred at 265-270°C. Poethke (69) reported its melting point to be 266-267°C. with decomposition, and Salzberger (81) gave it as 265°C.

Picrate.

Protoveratridine picrate was prepared by addition of saturated picric acid solution to a hot solution of a few milligrams of the alkaloid in dilute acetic acid. On cooling 2.2 mg. of crystalline platelets separated out which were filtered off and air-dried. These platelets recrystallized to large rhombs above 220°C., darkened above 230°C., and melted and sublimed at 245-252°C. with some decomposition. Protoveratridine picrate according to Poethke (69) decomposes at 244-246°C. without complete fusion.

Germine

Free base.

Germine on recrystallization from a concentrated

solution in methyl alcohol formed rhombs and cubes which melted partially at 170-175°C., darkened and resolidified in part above 190°C. and melted with decomposition at 215-227°C. Poethke (69) observed that germine sintered between 160 and 170°C. and melted about 220°C. Germine was soluble in chloroform, methyl and ethyl alcohols, acetone and water, and somewhat in ether.

Germine crystallized from methyl alcohol contained varying amounts of solvent of crystallization. The loss in weight on drying in a vacuum over P_2O_5 at 110-120°C. or in a Pregl block at 110°C. in a current of dry air varied in 13 tests from 10.06 to 12.76 per cent.

Calculated for $C_{26}H_{41}O_8N \cdot 2CH_3OH$: 11.45% loss.

Calculated for $C_{26}H_{41}O_8N \cdot 4H_2O$: 12.70% loss.

Calculated for $C_{26}H_{41}O_8N \cdot 3H_2O$: 9.84% loss.

Poethke (69) found that germine crystallized from methyl alcohol lost 13.34 and 12.65% of its weight on drying.

Determination of rotatory power

α_D^{16} +0.26° (10% acetic acid, 9.192 mg. dried germine in 0.8162 cc.) $[\alpha]_D^{16}$ +23.1°.

α_D^{24} +0.22° (10% acetic acid, 8.974 mg. dried sample in 0.8162 cc.) $[\alpha]_D^{24}$ +20.0°.

Poethke (69) $[\alpha]_D^{20}$ +21.1° (dilute acetic acid).

From the observations of melting characteristics, solubilities, solvent of crystallization and optical

rotatory power, the product here obtained appeared to resemble the germine of Poethke. However, microanalytical data did not give as close checks of the theoretical formula established by him as were desired. These results are shown and are compared with the actual data of Poethke (69).

Analysis

	C	H	N
Calculated for $C_{26}H_{41}O_8N$:	C, 62.99;	H, 8.34;	N, 2.83
Found:	C, 63.53;	H, 8.78;	N, 3.16
	63.74	8.76	3.09
	63.89	8.63	3.11
	63.39	8.60	3.18
	63.40	8.63	3.08
	63.60	8.90	3.17
Mean:	C, 63.59;	H, 8.72;	N, 3.13
Average deviation from calculated:	C, +0.60;	H,+0.38;	N,+0.30
Found by Poethke:	C, 63.08;	H, 8.55;	N, 3.07
	62.96	8.52	

It was felt that these analytical data were reasonably reliable since parallel carbon-hydrogen and Dumas nitrogen determinations made on a known alkaloid, cevine, $C_{27}H_{43}O_8N$, and on hippuric acid, $C_9H_9O_3N$, gave the following satisfactory results:

	C	H	N
Calculated for $C_{27}H_{43}O_8N$:	C, 63.61;	H, 8.51;	N, 2.75
Found:	C, 63.45;	H, 8.50;	N, 2.79
	63.55	8.68	

Calculated for $C_9H_9O_3N$:	C, 60.25; H, 5.06; N, 7.82
Found:	C, 60.20; H, 5.14; N, 7.87
	60.40 5.26 7.80
	60.30 5.23 7.70
	60.50 5.30
Mean:	C, 60.35; H, 5.23; N, 7.79
Average deviation from calculated:	C, +0.10; H,+0.17; N,-0.03

Conclusions might be drawn from these analyses that the formula for germine, $C_{26}H_{41}O_8N$, given by Poethke (69) is erroneous, or that the compound isolated from Veratrum viride in these experiments is different from Poethke's germine obtained from V. album, despite their similarities in physical properties. A careful study of Poethke's paper (69) on germine, its methylethylacetate, protoveratridine, and the latter's methylethylglycolate, germerine, shows that that author has ample support for his suggested empirical formulas for these alkaloids. His formulas are based on the analyses of the alkaloids and numerous salts, on titrimetric determinations of their equivalent weights and on estimation of the molecular weights of the ester alkaloids by titration of the acid residues formed on saponification. On the other hand, there is considerable likelihood of the same alkaloidal unit being present in two such closely related species as Veratrum viride and V. album, in which

case the compound here isolated would truly be Poethke's germine and the failure of the analyses to check his formula would remain unexplained. There is equal likelihood of two distinct, though similar, alkaloidal units being present in these species, the physical properties of the two being indistinguishable but the chemical formulas being somewhat different.

For lack of final proof on this point, the assumption has been made that the alkaloid under discussion is identical with Poethke's germine.

Comparison with cevine.

The formula of germine, $C_{26}H_{41}O_8N$, is very similar to that of cevine, $C_{27}H_{43}O_8N$, the difference being one carbon and two hydrogen atoms. Cevine is the basic hydrolysis product of cevadine and veratridine from commercial veratrine which are prepared from sabadilla seeds (Schoenocaulon officinale). Poethke (69) first called attention to this similarity and pointed out further the agreements and disagreements in other essential properties of the two alkaloids. In Table VII are listed many of these properties of cevine and germine, from which it is evident that these alkaloids while being very similar, are yet not identical. An obvious possibility from the formulas that cevine might be the methyl ether of germine is weak, for Macbeth and Robinson (49) found no methoxyl in cevine.

A further supposition of homology is possible. Should homology exist the problem of the structure of the germine molecule, and hence of the highly toxic alkaloid germerine, would be greatly simplified for the structure of cevine is being elucidated by Jacobs and Craig (39, 40, 41, 42) and Craig and Jacobs (19, 20). These authors have thus far published information which indicates the disposition of the nitrogen atom, eleven carbon atoms and two oxygen atoms in the cevine molecule.

The supposition of homology is partially discounted by the fact that cevine on treatment with alcoholic potassium hydroxide forms crystalline potassium cevine while germine on similar treatment forms no precipitate.

It was felt that further comparisons might be made between the chemical reactivities of cevine and germine in order to shed light on the possible relationship between these alkaloids. To this end hydrogenation experiments were carried out on the two alkaloids.*

The hydrogenation experiments were made on 16- to 51-mg. samples in an apparatus with a total volume of about 35 cc., a 5.5-cc. buret capable of being read to ±0.01 cc., and a device for holding the sample out of contact with the catalyst in the closed apparatus. The catalysts used were

*The author wishes to express his thanks to Dr. L. C. Craig of the Rockefeller Institute for Medical Research who very kindly sent the sample of cevine on which these experiments were made.

Table VII. Comparative physical and chemical properties of cevine and germine.

Property	Cevine	Germine
Formula	$C_{27}H_{43}O_8N$	$C_{26}H_{41}O_8N$
Melting point	softens 155-160°; melts 195-200° (27)	softens 160-170°; melts 210-220° (69) softens 170-175°; melts 215-227° (obs.)
Soluble in	H_2O, CH_3OH, C_2H_5OH $CHCl_3$, acetone	H_2O, CH_3OH, C_2H_5OH, $CHCl_3$, acetone
Specific rotation:		
in C_2H_5OH	-17.52° (49); -25.1°, -24.0° (obs.)	+4.8° (69)
in CH_3OH	-15.36° (49); -23.3° (obs.)	-----
in CH_3COOH (10%)	-7.5° (obs.)	+21.1° (69); +23.1°, +20.0° (obs.)
Crystallized from:		
CH_3OH	Yes (obs.)	Yes (69, obs.)
H_2O	with 3.5 H_2O (27, obs.)	with 3 H_2O (69)
Methiodide	m. p. 253-257° (42)	no good product (69)
Oxide	m. p. 275-278°	m. p. 249° (69)
Methoxyl content	0 (49)	0 (69)

Continued on next page.

Table VII. (Concluded)

Property	Cevine	Germine
Active H (Zerewitinoff)	6 (27)	6 (69)
Active H (acetylation)	2 (27)	5 (69)

Note: The data on cevine were taken partly from the literature and partly from direct observations on the compound. Those for germine were taken from the work of Poethke (69) on germine from Veratrum album and from the work here reported on germine isolated from V. viride, thus serving as a comparison of the germine from the two sources.

Raney's nickel catalyst and the platinum oxide catalyst of Adams and Shriner, both prepared in the usual manner. In hydrogenations with the nickel catalyst, the nickel and solvent were saturated with hydrogen first since varying amounts of hydrogen were taken up, and then the compound was added. When platinum oxide was used, the catalyst and compound were ordinarily hydrogenated together since correction could be made for the amount of hydrogen equivalent to the weighed catalyst.

Preliminary experiments were made on hydrogenation of several compounds with known hydrogenation products in order to study the characteristics of the apparatus and catalysts, and to determine the correspondence between actual and calculated volumes of hydrogen absorbed. The compounds studied were fumaric acid, strychnine, jervine, cevine and germine. The results of these experiments are presented in Table VIII.

Hydrogenation of fumaric acid to succinic acid took place rapidly in methyl alcoholic solution with Raney's nickel catalyst. The product isolated by evaporation of the solvent melted at 185-186.5°C. The melting point for succinic acid is 185°C.

Strychnine had previously been hydrogenated by Robinson (76) to dihydrostrychnine in acetic acid with palladium chloride suspended on norite. Dihydrostrychnine on

Table VIII. Results of hydrogenation experiments.

Compound hydrogenated	Catalyst	Solvent	Atoms of H absorbed	Time for absorption, hours
Fumaric acid	Ni	CH_3OH	2	2
Strychnine	Ni	CH_3OH	2	18
"	Pt	CH_3COOH	2	2
"	Pt	CH_3COOH	2	2
"	Pt[a]	CH_3COOH	2	2
Jervine	Ni	CH_3OH	0	24
"	Ni	CH_3COOH	0	16
"	Pt	CH_3COOH	4	--[b]
"	Pt	CH_3COOH	4	--[c]
Cevine	Pt	CH_3COOH	2	24
"	Pt	CH_3OH	2	48
"	Pt	CH_3OH	2	24
Germine	Ni	CH_3OH	0	20
"	Ni	CH_3OH	0	12
"	Ni	CH_3OH	0	2
"	Pt[a]	C_2H_5OH	0	18
"	Pt	CH_3COOH	0	6

(a) Catalyst hydrogenated first, then compound added.
(b) Two in 2 hrs., 2 more in 20 hrs.
(c) Two in 2 hrs., 2 more in 48 hrs.

recrystallization from 50 per cent aqueous methyl alcohol was obtained as $C_{21}H_{24}O_2N_2 \cdot 2H_2O$, melting at 220-222°C. Catalytic hydrogenation of strychnine takes place at the carbon-to-carbon double bond. The experiments here reported showed that strychnine could be hydrogenated to dihydrostrychnine with Raney's nickel catalyst in methyl alcohol in 18 hours, and was similarly hydrogenated with the platinum catalyst in glacial acetic acid in two hours. The product melted at 220-226°C.

Jervine was not successfully hydrogenated with Raney's nickel catalyst in methyl alcohol or glacial acetic acid. However, it was hydrogenated in glacial acetic acid with the platinum catalyst, four atoms of hydrogen being absorbed, the first two in two hours and the second two in 20 to 48 hours. Similar results were obtained by Saito et al. (80) in the hydrogenation of jervine with platinum oxide catalyst.

Cevine was hydrogenated in methyl alcohol or glacial acetic acid solution with the platinum oxide catalyst of Adams and Shriner, two atoms of hydrogen being absorbed in 24 hours. The product was isolated and was recrystallized from a concentrated solution in methyl alcohol. The resulting crystals melted between 173 and 185°C., crystallized partly between 195 and 205°C., started to decompose and effervesce at 223°C., crystallized in part again and finally

melted at 260-262°C. Jacobs and Craig (42) had been unable to hydrogenate cevine with the platinum oxide catalyst but they did succeed in obtaining the absorption of one mole of hydrogen with Raney's nickel catalyst. They isolated the product, dihydrocevine, $C_{27}H_{45}O_8N$, which softened with effervescence at 220°C. due to loss of solvent, but resolidified and then melted at 263-265°C. It had the rotation $[\alpha]_D^{25}$ -8° in methyl alcohol.

Determination of rotatory power

α_D^{23} -0.10° (methanol, 5.420 mg. dried dihydrocevine in 0.8162 cc.) $[\alpha]_D^{23}$ -15.1°.

α_D^{25} -0.40° (methanol, 19.145 mg. dried sample in 0.8162 cc.) $[\alpha]_D^{25}$ -17.0°.

Analysis

Calculated for $C_{27}H_{45}O_8N$: C, 63.36; H, 8.87.

Found: C, 63.10; H, 8.94.

Numerous attempts were made to hydrogenate germine, all without success. The trials were made with Raney's nickel catalyst in methyl alcohol and with the platinum oxide catalyst of Adams and Shriner in ethyl alcohol and in glacial acetic acid. It thus appears that cevine and germine are distinctly different in their reactivities toward hydrogenation.

TOXICOLOGICAL EXPERIMENTS

Methods

The method of injecting solutions into the American cockroach (Periplaneta americana) used in these experiments was somewhat modified from that described by Campbell (11) in 1932 and modified by Yeager, Wooley and Brown (107) in the same year.

The injection needle was made by drawing out a piece of 2-mm. glass tubing to a fine point about 0.1 to 0.2 mm. in diameter. The tip was beveled on a fine Carborundum stone to facilitate penetration of the insect cuticula. The needle was attached with a short piece of rubber tubing to a horizontally clamped 0.1-cc. glass pipette calibrated in 0.01-cc. divisions. The volume expelled was measured to the nearest 0.001 cc. by dividing the pipette divisions into ten equal parts. The calibrated pipette was attached by a long piece of rubber tubing to a glass mouthpiece, and injection was made by oral pressure.

The injections were made through one of two locations, either through the coxa-femur conjunctiva of the left hind leg, or into the conjunctiva at the proximal end of the left hind coxa, the former method being preferred. For the

coxa-femur injection the insects were held in the left hand, between the thumb and first two fingers, ventral side uppermost, the two hind legs being held between the edge of the thumb and the third finger, and the needle was inserted cephalad along the coxa. For the proximal coxal injection the insects were held between the thumb and third finger, ventral side up, the left hind leg being distended between the first and second fingers, and the needle was inserted cephalad into the body cavity.

After the injection each insect was retained for observation in an individual, small, wire-screen cage and was supplied with food and water continually until death or termination of the experiment. The criterion of death was failure to respond to pinching of the tarsi, antennae, and cerci.

Before injection the insect was weighed to the nearest 0.01 g. and the volume of solution to be injected was calculated at the rate of 0.05 cc. per 0.9 g. of body weight of the roach. Knowledge of the concentration of the solutions injected permitted calculation of the weight of fraction injected in milligrams per gram of body weight.

The solutions of the alkaloid fractions were made up by weighing the samples to the nearest 0.002 mg., dissolving them in small volumes of 10 per cent acetic acid solution, nearly neutralizing with sodium hydroxide or ammonium

hydroxide solution with methyl red as indicator, and diluting to volume with distilled water. The solutions of fractions obtained from Experiment A were neutralized with sodium hydroxide and injected through the proximal coxal conjunctiva; those from Experiments B and C were neutralized with ammonium hydroxide and injected through the coxa-femur conjunctiva.

Control insects injected with a solution of acetic acid nearly neutralized with sodium hydroxide or ammonium hydroxide showed no injurious effects beyond a temporary lameness in the injected leg. The control insect tests were distributed throughout the course of the injection experiments. Equal numbers of males and females were used throughout to eliminate possible sex differences in reaction.

It should be noted that some of the cruder fractions did not dissolve completely when prepared for injection, but it was felt that the alkaloid portion of these fractions was dissolved since the acetates of these alkaloids are water-soluble.

Results

In Tables IX and X is presented a compilation of the injection tests on the American cockroach with the alkaloid fractions obtained by the procedures of Experiments A and B.

Table IX. Summary of toxicological tests on the American cockroach (Periplaneta americana) of alkaloidal fractions obtained in Experiments A and B.

Fraction	Dosage, mg./g.	Number of insects injected	Number of insects killed	Other effects
Experiment A:				
Total alkaloids from assay	0.044	6	6	All knocked down; death in less than 96 hours.
Solid C	0.044	6	1	No knockdown; death in 18 hrs.
Chloroform extract (a)[a]	0.044	6	0	No effect.
Recrystallized D and E, jervine	0.044	6	0	3 knocked down; up in 1 hour.
Solid P	0.044	6	0	No effect.
Recrystallized F, jervine	0.044	6	0	No effect.
Filtrate Q[a]	0.044	6	0	All knocked down.
Ether extract (d)[a]	0.044	10	7	All knocked down; death in less than 96 hours.
Chloroform extract (g)[a]	0.044	6	2	All knocked down; convulsive action; death in less than 96 hours.

Continued on next page.

Table IX. (Continued)

Fraction	Dosage, mg./g.	Number of insects injected	Number of insects killed	Other effects
Ether extract (f)[a]	0.044	10	10	All knocked down; 8 dead in less than 96 hours, 1 in 101 and 1 in 153 hours.
Solid S	0.044	10	2	All knocked down; severe convulsive action; death in less than 96 hrs.
Experiment B:				
Total alkaloids from assay	0.044	6	6	All knocked down; death in less than 96 hours.
Precipitate F, crude jervine	0.044	6	0	No effect.
Precipitate G, crude pseudo-jervine	0.044	6	0	No effect.
Precipitate H, jervine	0.044	6	0	No effect.
Precipitate J, jervine	0.044	6	0	No effect.
Precipitate K	0.044	10	1	7 knocked down, 3 partially paralyzed; death in less than 96 hrs.

Continued on next page.

Table IX. (Concluded)

Fraction	Dosage, mg./g.	Number of insects injected	Number of insects killed	Other effects
Precipitate L	0.044	6	5	All knocked down; death in 24 hrs.
Aqueous filtrate (f)	--[b]	6	0	1 knocked down.
Ether filtrate D	--[c]	6	6	All knocked down; 4 dead in less than 72 hours, 2 in 149 hours.

(a) The solvent was evaporated from a portion of these solutions and the residue dissolved in the indicated concentration.
(b) This filtrate was injected without further treatment.
(c) Solvent evaporated from 1 cc. of filtrate, residue allowed to stand with dilute acetic acid, and decanted acid solution injected after partial neutralization.

Table X. Effect on the American cockroach of varying doses of certain toxic fractions obtained in Experiment B.

Fraction	Dosage, mg./g.	Number of insects injected	Number of insects killed	Other effects
D_4	0.5	3	3	All knocked down; death in less than 19 hours.
"	0.044	4	4	All knocked down; death in less than 48 hours.
"	0.022	4	1	All knocked down; 1 dead in 5 days.
"	0.0044	4	0	All knocked down; up in 2 hours.
Residue (j)	0.044	4	3	All knocked down; deaths in 72 hours.
"	0.022	4	3	All knocked down; deaths in 48 hours.
"	0.0044	4	0	All knocked down; 2 up in 2 hours.

Reference to Table IX and Figs. 1 to 3 (pages 45, 49 and 50) indicates that the fractions isolated in the procedures followed in the chemical and physical separations were tested for the presence of toxic constituents by the injection method. It is further evident that the toxicity can be traced through a series of separations and found to reside chiefly in one or two individual fractions at the end of the series.

In Experiment B the procedure in many parts was similar to that of Experiment A, so the corresponding fractions obtained in the two experiments were often tested only on their first isolation. This fact is noted to account in part for the relative scarcity of toxicological tests in the first part of the work on precipitate C in Experiment B (Fig. 2).

Table X presents some interesting data. Fraction D_4 of Experiment B was quite toxic. All the insects injected at the dosage of 0.044 mg. per g. were killed rapidly, while of those injected at 0.022 and 0.0044 mg. per g. all were knocked down but only one died at the greater concentration. This indicates that the median lethal dose for fraction D_4 is between 0.044 and 0.022 mg. per g. In the chemical examination of this fraction, treatment with potassium nitrite separated out a sticky precipitate from which nitrosojervine was crystallized. The filtrate from

this nitroso fraction was extracted with ether and the solvent removed, leaving residue (j). This procedure brought about a slight concentration of the toxic components for the median lethal dose of residue (j) appears to be between 0.022 and 0.0044 mg. per g. From residue (j), by a procedure previously outlined, a small amount of the alkaloid, protoveratridine, was crystallized. This alkaloid was shown by Poethke (69) to be a hydrolysis product of germerine which Haas (31) found to be highly toxic to the frog, cat and rabbit.

The fraction L of Experiment C was obtained in a manner similar to that by which D_4 was prepared in Experiment B. Fraction L was tested for toxicity to the roach and found to be considerably more toxic than D_4, the median lethal dose, as apparent from the data in Table XI, being less than 0.011 mg. per g. From this fraction, as well as from fraction P, the pure alkaloid, germine, was crystallized. Germine was proved by Poethke (69) to be a hydrolysis product of protoveratridine and hence of germerine. It was therefore of interest to test this pure alkaloid for toxicity to the cockroach. This was done and the resultant data are also presented in Table XI. It appears that germine is much less toxic than the fraction L from which it was isolated. The experiments of Haas (31) on frogs, cats and rabbits showed that germine was much less toxic than its parent alkaloid, germerine.

Table XI. Effect on the American cockroach of varying doses of fraction L from Experiment C, of germine and of cevine.

Material tested	Dosage, mg./g.	Number of insects injected	Number of insects killed	Other effects
Fraction L	0.044	4	4	All knocked down; deaths in 48 hours.
"	0.022	4	4	All knocked down; deaths in 72 hours.
"	0.016	8	7	All knocked down; deaths in 72 hours.
"	0.011	12	7	All knocked down; deaths in 96 hours.
Germine	0.44	8	3	6 knocked down; deaths in 96 hours.
"	0.264	12	5	10 knocked down; deaths in 72 hours.
"	0.176	8	2	2 knocked down; deaths in 48 hours.
"	0.044	4	0	No knockdown.
Cevine	0.088	12	6	9 knocked down; 5 of dead were males; deaths in 72 hours.
"	0.044	4	1	2 knocked down; death in 7 days.

Since a chemical comparison was made between germine and cevine, the hydrolysis product of cevadine from commercial veratrine, it seemed of interest to compare also the toxicities of the two alkaloids. A few tests were made on roaches with cevine, the data for which are in Table XI. It appears that the median lethal dose for cevine is about 0.088 mg. per g., while that for germine is about 0.264 mg. per g., the former therefore being more toxic.

Observations and Discussion

The toxicological tests made in conjunction with the chemical studies on *Veratrum viride* appeared to be of much value in determining which procedures effected concentration of the toxic components, and which eliminated nontoxic constituents. They showed further that jervine and pseudojervine, alkaloids present in *V. viride*, were nontoxic to roaches, and finally that germine, prepared from highly toxic crude fractions, and isolated for the first time from *V. viride*, was also relatively nontoxic. The toxicological tests of the pure alkaloids on cockroaches are of special interest for they constitute the first experiments on insects with pure alkaloids from the Veratrums, despite the fact that these plants have been known and used as insecticides (hellebore) for a great length of time.

It would have been highly desirable to have used larger numbers of insects in the toxicological tests, especially in those in which the effects of various doses were studied. This was not feasible in many cases because of the small amounts of some of the fractions and alkaloids available from the fractionation procedures. Despite this difficulty it is felt that in general quite definite conclusions may be drawn because of the clear-cut distinctions among the toxic effects of closely related doses, as evident from these data. The microinjection technique itself is a factor that probably contributes much to this differentiation, because of the accuracy with which dosage may be controlled and the rapidity with which the component introduced may reach the vital centers of the insect without the necessity for penetration of the cuticular membrane or the gut epithelium.

A short summary of the more prominent physiological actions observed during the course of these experiments on cockroaches is given. The toxic, crude fractions, such as $\underline{D_4}$ and $\underline{L}$ of Experiment B and $\underline{L}$ of Experiment C, generally produced immediate knockdown of the roaches, even at sublethal doses. This knockdown progressed rapidly to almost complete paralysis which continued for about two to four days and ended in death or was terminated in less than 24 hours with eventual complete recovery. It is noteworthy

that in but few cases did death take place in less than 24 hours. These observations are in accord with those of Haas (31) on frogs who noted that when lethal doses of germerine were injected death occurred during the period of complete paralysis which lasted about 24 to 48 hours after injection. The experiments with germine gave evidence of a different reaction. Most of the insects injected with germine solutions were affected shortly after injection by an apparently irritant action, characterized by rapid bodily vibration and motor incoordination. This was followed after several hours by paralysis in those insects which eventually died. Here again the experiments of Haas on frogs and rats showed similar effects for germine, a preliminary period of great excitability and irritability being prominent, leading in the case of toxic doses to eventual paralysis.

DISCUSSION OF CHEMICAL AND TOXICOLOGICAL STUDIES

One of the questions that has been discussed and experimented upon by many investigators in the past is that of the total amount of alkaloids present in the two important species of *Veratrum*, *V. album* in Europe and *V. viride* in America. A second question closely related to the first and of more importance from the standpoint of pharmacology and economic entomology is the relative toxicities of the total alkaloid fractions from these two *Veratrum* species. From the numerous total alkaloid determinations reported in the literature for the two species, it seems apparent that the ranges of concentrations overlap considerably but that on an average the total is slightly higher in *V. album*. The total alkaloid content is generally somewhat over one per cent in both species but occasionally is very low, 0.17 per cent, as was observed in one sample of *V. viride* purchased during these investigations.

Physiological and pharmacological studies and bioassays reported by many investigators are almost unanimously in favor of the claim for greater potency in *V. album*. This claim is supported to a considerable extent by the work of Poethke (68, 69, 70, 71) on *V. album* and the investigations

here reported on V. viride. Poethke showed that by one procedure he was able to isolate from the total crude alkaloid fraction, which amounted to 0.45 per cent of the crude drug, 14 per cent germerine, 1.4 per cent protoveratridine, 0.5 per cent jervine and 0.4 per cent rubijervine, leaving 50 per cent amorphous alkaloids. By another procedure he separated from the drug about 0.6 per cent of total alkaloids which yielded about 16 per cent protoveratrine, 10 per cent germerine, 2 per cent rubijervine, 4 per cent jervine and 68 per cent amorphous alkaloids. The two alkaloids, germerine and protoveratrine, were shown by the work of Haas (31) and Eden (23) to be highly toxic and physiologically active on vertebrates. The alkaloids obtained in small quantities by Poethke, namely, jervine, pseudojervine and rubijervine, had previously been shown by Lissauer (48) to be almost inactive on vertebrates.

In contrast with this the present work on Veratrum viride showed that the predominant alkaloids of American Veratrum were jervine and pseudojervine, which appeared to be almost nontoxic to the roach. Jervine was obtained as about 17 per cent of the total crude alkaloid fraction and pseudojervine as about 3.3 per cent of the crude. Rubijervine was also obtained, but in extremely small quantities. Similar results have previously been obtained even on V. album. Wright and Luff (104, 106) were

able to crystallize only these inactive alkaloids, jervine pseudojervine and rubijervine, from both Veratrum species and Saito et al. (78, 79) obtained only jervine in 23 per cent yield from the total alkaloids occurring in V. album grown in Japan.

The reasons for the difference in the alkaloids predominant in the two species of Veratrum and even in different samples of the same species are obscure. Some of the more prominent suppositions to explain this difference are that geographical source and season of collection play an important role.

During this investigation there were isolated from V. viride, besides jervine, pseudojervine and rubijervine, the two alkaloids, protoveratridine and germine, which had not previously been found in the American species. Protoveratridine was obtained in quite small amounts, in the order of a few milligrams, and germine was crystallized in amounts equal to about one per cent of the total alkaloids.

Protoveratridine had first been discovered by Salzberger (81) in V. album and it and germine were separated by Poethke (69) in his work on the same species. Poethke studied the interrelationships of the alkaloids and found that partial hydrolysis of germerine yielded 1-methylethylglycolic acid and protoveratridine and that the latter on hydrolysis produced 1-methylethylacetic acid and germine.

Haas (31) tested Poethke's germerine, protoveratridine and germine on vertebrate animals including the frog, cat, rat and rabbit. He showed that germerine was highly toxic, the median lethal dose for the frog being 0.009 mg. per g. and for the rat 0.0037 mg. per g. Germine was lethal to the frog only at 0.5 mg. per g. and to the rat at 2.0 mg. per g. The germine here isolated from V. viride was tested against the American cockroach by injection and its median lethal dose was about 0.3 mg. per g. The alkaloidal fraction from which germine was the only crystalline product obtained was highly toxic, its median lethal dose for the roach being less than 0.01 mg. per g.

Since germine is a hydrolysis product of the toxic alkaloid, germerine, and the fraction from which germine was crystallized was highly toxic, it seemed at least possible that germerine was present in this fraction. However, despite numerous experiments based partly on the methods of Poethke and partly on other procedures, no germerine could be obtained. A somewhat similar experience was reported by Poethke (71). In his investigation of the amorphous alkaloid fraction from V. album, which amounted to fifty per cent of the total alkaloids, he was unable to isolate a crystalline product, but on hydrolysis he obtained from ten grams of amorphous alkaloids four grams of germine, a very small amount of veratric acid and larger amounts of acetic

and l-methylethylacetic acids. Poethke ruled out the obvious assumption that this germine was formed by hydrolysis of germerine or protoveratridine whose crystallization had been prevented by amorphous impurities. In such a case the amorphous alkaloid fraction must have consisted of approximately fifty per cent germerine or protoveratridine. He had observed that germerine had readily been crystallized from amorphous alkaloid mixtures with benzene and could not understand why so considerable a part of germerine would not have crystallized here had it been present. Furthermore, saponification would have yielded also l-methylethylglycolic acid and this was not observed. The great insolubility of protoveratridine in the solvents used argued against the presence of large amounts of this alkaloid, for the amorphous alkaloids were readily soluble in these solvents.

Another possible reason for the failure in these experiments to isolate germerine (assuming its presence in the plant) is that the procedures used were sufficiently drastic to degrade the compound to a slight extent or to effect some sort of isomeric change resulting in alteration of certain properties such as crystallizability, though the latter is somewhat unlikely. The effect of optical isomerization has been observed in another plant insecticide, derris, wherein racemization of certain of the constituents was found to take place during the extraction procedures,

and the resultant racemes were less toxic than the optically active isomers originally present.

The suggestion of Poethke that germine and cevine might be closely related from a structural standpoint, e.g., by homology, because of the similarity of their molecular formulas and many of their physical properties, may probably be rejected. Poethke himself pointed out several of the differences in physical properties between them, notably the difference in their optical rotatory powers. He found a different number of active hydrogen atoms in germine by acetylation than had previously been found in cevine. The experiments here reported comprising attempts at hydrogenation of the two alkaloids showed that cevine could be hydrogenated with the platinum oxide catalyst of Adams and Shriner. Jacobs and Craig (42) had hydrogenated cevine with Raney nickel as catalyst. In the present investigation germine was not successfully hydrogenated with either of these catalysts. These differences in chemical reactivity point to a more deep-seated structural difference between the two alkaloids than would be afforded by homology.

Although no germerine or protoveratrine were obtained in the present experiments the presence in Veratrum viride of some toxic alkaloid such as these is definitely indicated, especially by the separation of highly toxic crude alkaloid fractions. It is quite probable that this

toxicity might be due originally to germerine or protoveratrine or both and that these alkaloids, since they are rather unstable to hydrolytic agents, may have been eventually partly degraded even by the mild treatments employed in these investigations. In the case of protoveratrine this would be a quite plausible explanation for this alkaloid is readily hydrolyzed and Poethke was unable to crystallize its basic hydrolysis product. However, were germerine also present in considerable amount the inability to crystallize it or its partial hydrolysis product, protoveratridine, in appreciable amounts, as pointed out by Poethke, is not understood.

CONCLUSIONS

1. The procedure for chemical determination of total alkaloid content of *Veratrum viride* has been modified to facilitate mechanical manipulation, to decrease the amount of nonalkaloidal impurities carried through the extraction procedure and to increase the precision of the determination.

2. From the work of Fisher (25) in testing the alkaloid fractions separated by the chemical assay method, total alkaloid content is not a measure of the toxic effect of this insecticide on the American cockroach.

3. The presence in *Veratrum viride* of jervine, pseudojervine and rubijervine has been confirmed. Rubijervine was separated in very small amounts, but jervine and pseudojervine were the predominant alkaloids isolated from the plant.

4. These latter alkaloids were found to be almost nontoxic to the American cockroach.

5. Germerine and protoveratrine, alkaloids highly toxic to vertebrates, which have been isolated from the European species, *Veratrum album*, could not be isolated from *Veratrum viride*, the American species.

6. Protoveratridine in small amounts and germine in larger quantities, both hydrolysis products of germerine,

were isolated for the first time from <u>Veratrum viride</u>.

7. Germine was somewhat toxic to the American cockroach, its median lethal dose being about 0.3 mg. per g.

8. The crude alkaloid fractions from which germine and protoveratridine were separated were highly toxic, indicating the possible existence in <u>Veratrum viride</u> of some toxic alkaloid such as germerine.

9. Germine resembled cevine, the hydrolysis product of cevadine and veratridine from sabadilla seeds, in chemical formula and many physical properties, but did not resemble it in chemical reactivity toward catalytic hydrogenation.

10. The microinjection procedure for testing crude alkaloidal fractions and pure alkaloids for toxicity to the American cockroach was very satisfactory. It was especially valuable in determining the efficacy of fractionation procedures in concentrating the toxic components.

SUMMARY

The purposes of this investigation were to improve the procedures for chemical determination of the total alkaloid content of *Veratrum viride*, to compare the chemical with the biological assay on insects, to separate the crude, physiologically active mixture into its constituents, and to test them for toxicity to insects.

An extensive review of the literature relating to *Veratrum viride* was prepared. While no bibliographic summary was attempted, the subject was covered from the standpoints of botany, chemistry, physiology and pharmacology, economic entomology, and assays, and it is felt that practically all the important references were presented.

An investigation of the procedure for chemical assay of the alkaloid content of *Veratrum viride* was made and several modifications in method were made which resulted in increased ease of manipulation and accuracy of results.

The crude alkaloid mixture was investigated with a view to separation into its components, and the somewhat involved methods were described in detail and illustrated by charts. The several fractions of special interest thus obtained were investigated further and five alkaloids were isolated in pure form and identified. These alkaloids were

jervine, pseudojervine, rubijervine, protoveratridine and germine.

Jervine, pseudojervine and rubijervine had been isolated from Veratrum viride by earlier investigators and their presence in the plant was thus confirmed. Jervine and pseudojervine were found to be the predominant alkaloids separated from Veratrum viride, while rubijervine was obtained in small amounts.

Protoveratridine and germine were separated from Veratrum viride for the first time, the former in small amounts and the latter in somewhat larger quantities. These two alkaloids had been shown by another investigator to be hydrolysis products of the alkaloid, germerine, which he had obtained from Veratrum album.

Neither germerine nor protoveratrine, the latter also previously isolated from Veratrum album, was obtained in these investigations on Veratrum viride, but their presence in this plant is not, of course, thereby excluded. In fact, the existence in the plant of some such alkaloid as germerine is to some extent indicated by the very fact of the isolation of germine and protoveratridine, and also by the considerable toxicity of the alkaloidal fractions from which these compounds were isolated.

Because of suggested homology between germine and another alkaloid, cevine, the reactivities of these two

alkaloids to catalytic hydrogenation were studied. It was concluded that they were probably not structurally related to the extent suggested.

The toxic properties of the fractions separated during this investigation, including those from which protoveratridine and germine were obtained, as well as of some of the pure alkaloids isolated, were tested by microinjection of their solutions into the American cockroach, *Periplaneta americana* (L.). In this way the progress of the fractionation procedures was followed biologically and the concentration of the toxic components by these procedures was observed. Further, the alkaloids, jervine and pseudojervine, were found to be almost nontoxic to the cockroach and the median lethal dose of germine was determined to be about 0.3 mg. per g. for the American cockroach. These toxicological experiments with pure alkaloids were the first in which pure alkaloids from *Veratrum viride* were tested on insects.

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ACKNOWLEDGMENTS

The writer expresses his sincere appreciation of the suggestions and assistance given by Dr. I. B. Johns during this investigation and of the advice given on the toxicological experiments and the interest in the problem shown by Dr. C. H. Richardson.

The writer is also indebted to Dr. R. A. Fisher, formerly of the Department of Zoology and Entomology, Iowa State College, for his assistance in reviewing the entomological literature and in making some of the assay determinations on the drug, and to the Industrial Science Research Institute for financial support of this project.