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The Separation, Micro-Estimation and Distribution of the Alkaloids of Hemlock (*Conium maculatum* L.)

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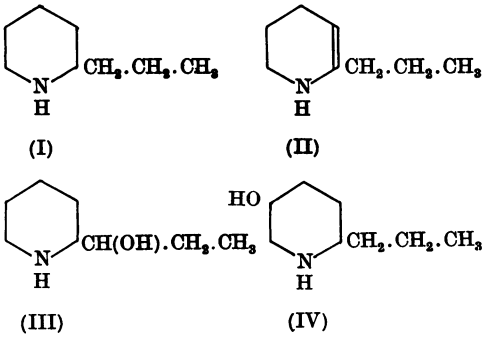
From the tissues of *Conium maculatum* the alkaloids coniine (I), *N*-methylconiine, γ-coniceine (II), conhydrine (III) and pseudoconhydrine (IV) have been isolated (Henry, 1949; Marion, 1950; Cromwell, 1955).

The alkaloids (III) and (IV) are present in very small amount and were first isolated from the residues from the separation of the major alkaloids of the group.

Methods for large-scale isolation and separation of the alkaloids have been described by Wolffenstein (1895) and Braun (1905). Farr & Wright (1904) have described titrimetric and gravimetric methods for the estimation of the total alkaloids (expressed as coniine) of hemlock tissues, and Madaus & Schindler (1938) have estimated the total alkaloid content of tissues during the growth and reproductive phases of the plant. Methods for the estimation of the individual alkaloids of the group do not appear to have been described; the object of the present work was to develop methods for the separation and micro-estimation of each alkaloid of the group, and to use these methods for a study of the distribution of the alkaloids in the plant during the phases of growth and reproduction.



MATERIALS AND METHODS

Plant material. C. maculatum is a biennial growing wild in many parts of Britain. Material from both first- and second-year plants growing locally was collected as required. Seed was obtained from wild plants and seedlings were raised in boxes in the greenhouse.

Preparations. Synthetic (±)-coniine was prepared from 2-methylpyridine by the method of Koller (1926) and converted into the hydrochloride. After repeated recrystallization from warm acetone, a product melting sharply at 212° was obtained. *N*-Methylconiine was prepared from (±)-coniine by methylation with potassium methyl sulphate (Passon, 1891), and converted into the hydrochloride which was purified by recrystallization from warm acetone. The purified *N*-methyl-(±)-coniine melted at 168°. (±)-Conhydrine was prepared from pyridine-2-aldehyde by the method of Lautenschläger & Onsager (1918). After repeated recrystallization from light petroleum the base melted at 99–100°. The method of Hofmann (1885) was used for the preparation of γ-coniceine from bromoconiine. The base was converted into the hydrochloride and purified by band chromatography on Whatman no. 3MM paper as described below. The purified product (92 mg.) melted at 142°.

The method of Lipp (1892) was used for the preparation of 2-methylpiperidein from 6-bromohexan-2-one prepared by the method of Anderson, Crawford & Sherrill (1946). The base was converted into the aurichloride, which was purified by recrystallization from hot water (m.p. 143–144°). Pipecolinic acid hydrochloride was prepared from 2-methylpyridine by the method of Clemo & Ramage (1931). The product, after recrystallization from ethanol containing 40% (v/v) of benzene, melted at 257°. It was not found possible to prepare pseudoconhydrine synthetically and, as a supply of the natural alkaloid was not available, a study of this minor alkaloid could not be undertaken in the present work.

Extraction of alkaloids from the tissues of Conium maculatum

The alkaloids are readily volatile in steam and can be isolated from the macerated tissues after release of the free bases with alkali. For bulk isolation it has been found advantageous to extract the alkaloids from fresh material with ethanol before steam distillation rather than to distil directly. By the adoption of this preliminary extraction, difficulties likely to be encountered in the handling of large quantities of macerated material are avoided and the distillation can be carried out smoothly and without frothing. Fully developed green fruits were used for the extraction. Alternatively, leaves from plants in the first year of growth were used. A total of 3 kg. of material was extracted in batches of 300 g. with 95% ethanol for 5 min. in a Waring Blendor. The ethanolic extract was filtered through muslin, the residue squeezed out and washed once with 95% ethanol. The washings were added to the filtrate, and a further filtration (Whatman no. 1 paper) was carried out to remove cell debris which passed through the muslin. The filtrate was acidified to pH 2 by the addition of 10N-HCl and transferred to a large evaporating basin, and the ethanol removed on a boiling-water bath. The dark-brown residue was extracted with warm water (100 ml.), the solution filtered and transferred to the flask of a steam-distillation apparatus. A saturated solution of NaCl (50 ml.) was added and the solution made alkaline (pH 10) by the addition of a solution of Na_2CO_3 (20%, w/v). Steam was passed in until the distillate no longer reacted alkaline. The mixture of alkaloids was shaken out of the distillate with chloroform and finally extracted from the chloroform with 0·5N-HCl. On evaporation of the acid solution on the water bath, the hydrochlorides of the alkaloids crystallized out. A portion of the mixture of hydrochlorides was benzoylated in alkaline solution (Braun, 1905), and unchanged *N*-methylconiine extracted from the ether extract with HCl (10%, v/v). On evaporation of the acid solution, the residue of *N*-methylconiine was recrystallized from warm acetone. A mass of white crystals melting at 188° was obtained. The m.p. of pure *N*-methyl-(+)-coniine is given as 188–189° (Marion, 1950). A picrate was prepared which melted at 119°. The remaining portion of the alkaloids obtained from the extract of fruits was set aside for the isolation of (+)-coniine by the method of band chromatography. The mixture of alkaloid hydrochlorides obtained from leaf material was made strongly alkaline with NaOH solution and steam-distilled, and the alkaloids were extracted from the distillate with ether. After removal of the ether, the oily residue was fractionally distilled, and the fraction distilling at 170–173° was collected and used for the isolation of γ-coniceine by band chromatography. The higher-boiling fraction which remained in the distillation flask was similarly used for the isolation of conhydrine.

For the determination of the distribution of the alkaloids in tissues by the method of paper chromatography, 1–2 g. (wet wt.) of tissue (leaves, flowers or fruits) was ground with sand in a mortar and the mash (15 ml.) transferred to the flask of an all-glass semi-micro steam-distillation apparatus. A saturated solution of NaCl (10 ml.) was added, followed by 1 g. of MgO. The receiving flask contained 5 ml. of 0·1N-HCl. Steam was passed into the apparatus for 20 min., during which time approx. 35 ml. of distillate was collected. The distillate was transferred to a flat-bottomed evaporating basin and allowed to evaporate slowly overnight on a water bath maintained at 55°. The residue of alkaloid hydrochlorides was dissolved in ethanol and spotted on the papers. Root tissues contain low concentrations of alkaloids, and it was necessary to use larger quantities of material (up to 50 g. wet wt.) in order to obtain the minimum amount of alkaloids detectable on paper. For the quantitative determination of the alkaloids in roots, leaves, flowers and fruits the material was dried in an oven with forced draught at a temperature not exceeding 60°, finely powdered, and stored in a desiccator until required for use. Drying at low temperature did not appear to result in any appreciable loss of alkaloids from leaves, flowers or fruits, but results obtained with young roots gave some indication that a proportion of the small content of alkaloids may be lost in the drying process.

For the micro-estimation of the alkaloids, 0·2–1 g. of powdered material was placed in the flask of the distillation apparatus. The powder was thoroughly wetted with ethanol, and water (15 ml.) and a saturated solution of NaCl (10 ml.) were added, followed by 1 g. of MgO. The distillation was carried out as described above.

Colour reactions of the hemlock alkaloids

Colour tests have been described by Dilling (1909). These tests are based on the earlier work of Melzer (1898), who showed that coniine, a secondary base, reacts with CS_2 to form the coniine salt of coniylthiocarbamic acid, which

with various metallic salts, including salts of copper, gives coloured complexes soluble in ether or toluene. Other secondary bases, e.g. conhydrine, γ-coniceine and piperidine, give a positive reaction with thiocarbamate, but coniine appears to be the only hemlock alkaloid that gives a coloured complex with uranyl nitrate (or acetate) which is soluble in toluene (Dilling, 1909). Gabutti (1906) has described a test for coniine in which a red colour develops on the addition of a few drops of a dilute solution of sodium nitroprusside to a solution of coniine (or one of its salts) to which one or two drops of a dilute solution of Na_2CO_3 have been added. On subsequent addition of a drop of acetaldehyde, the red colour slowly changes to violet.

For the colorimetric determination of quinine, Marshall & Rogers (1945) described a method based on the reaction of the alkaloid with bromothymol blue to form a yellow compound soluble in benzene and other organic solvents. It was found that the hemlock alkaloids also reacted with bromothymol blue to give yellow compounds soluble in benzene or toluene. A summary of the colour tests applied to solutions of the pure synthetic alkaloids is given in Table 1.

Separation of the alkaloids by paper chromatography

For the separation of the individual alkaloids from mixtures the ascending one-dimensional method was used with Whatman no. 1 or no. 3MM paper. A single-phase mixture of *tert.*-pentanol–*tert.*-butanol–N-HCl in water (9:3:2, by vol.) was the most satisfactory solvent system. The papers were cut into strips 35 cm. × 19 cm., washed for 5 min. in N-HCl and subsequently for 5 min. in water and dried in air. Preliminary washing was necessary to remove from the paper impurities that produced a yellow band with the solvent system, which interfered with the movement of the faster-moving alkaloids. The alkaloids were spotted on the paper as the hydrochlorides (in ethanolic solution) and the chromatograms run for 18 hr. The papers were dried in air at room temperature until most of the solvent was removed, and finally in an oven with forced draught at a temperature of 60° to remove all traces of HCl. The reagents used for the detection of the spots were I_2 (0·2%) in light petroleum and ninhydrin (0·1%) in acetic acid. The I_2 reagent was poured on the paper, and as the solvent evaporated the alkaloids became visible as brown spots which gradually faded. The ninhydrin reagent was used as a spray and the papers were heated at 105° to develop the colours. Should any HCl remain on the paper after drying, charring and disintegration of the paper will take place at this temperature. Violet colours are given with coniine, conhydrine, piperidine and 2-methylpiperidine, and yellowish brown colours with γ-coniceine and other unsaturated secondary bases. No colour is given by *N*-methylconiine. In addition, for the detection of γ-coniceine and other piperideines, a solution of sodium nitroprusside (1%, w/v) made alkaline with 10% (w/v) Na_2CO_3 solution was used as a spray. This solution gives red spots with γ-coniceine and other piperideines.

As much as 100 mg. of each of the alkaloids can be separated from mixtures by use of a series of 3MM papers (35 cm. × 45 cm.) on which the alkaloid solution is applied as a band 0·5 cm. wide and 42 cm. in length. A mixture of the hydrochlorides of the alkaloids (in ethanol) which contained the equivalent of 5 mg. of each alkaloid was applied as a band to each paper, and the chromatograms were run for 18–24 hr. with the solvent system. The papers were dried as described above, and the separated bands detected with the iodine reagent and marked with a pencil. After fading of the colours the bands were cut out and eluted with ethanol, and the eluates from all the papers used were combined. On removal of the ethanol, the hydrochlorides of coniine and *N*-methylconiine crystallized out. The hydrochlorides of conhydrine and γ-coniceine are deliquescent, but crystallize slowly on drying in a desiccator. On drying, the crystals of γ-coniceine hydrochloride became green and finally rose-pink, colour changes characteristic of pure γ-coniceine hydrochloride. When the filter paper is heavily loaded the separation of coniine from *N*-methylconiine presents some difficulty, as the band of *N*-methylconiine tends to merge with that of coniine. However, the line of demarcation of the coniine zone can be made visible by application of the ninhydrin reagent in the form of a streak across the bands.

By the use of band chromatography the alkaloids extracted from fruits of *C. maculatum* were isolated in amounts sufficient for characterization. Thus *N*-methyl-(+)-coniine was separated as the hydrochloride (75 mg.), m.p. 186–187° (mixed m.p. with a pure specimen of *N*-methyl-(+)-coniine hydrochloride showed no depression). Similarly, (+)-coniine was obtained as the hydrochloride (52 mg.) which melted at 219–220° and showed no depression of m.p. when mixed with (+)-coniine hydrochloride obtained from pure synthetic (±)-coniine hydrochloride by resolution through the (+)-tartrate. From leaf material of young plants in the first year of growth, γ-coniceine hydrochloride (95 mg.), m.p. 140–142°, and conhydrine (30 mg.), m.p. 119°, were isolated. The specimen of γ-coniceine hydrochloride showed no depression of m.p. when mixed with the pure synthetic compound. The m.p. of natural conhydrine ((+)-conhydrine) is given as 120·6° (Marion, 1950). The aurichloride prepared from the isolated conhydrine melted at 132° (pure conhydrine aurichloride melts at 133°).

Conhydrine (as hydrochloride) was eluted from the papers with ethanol. The ethanol was removed, the residue dissolved in water (5 ml.) and the solution made strongly alkaline with NaOH and extracted with chloroform. On removal of the chloroform, a residue of conhydrine remained which was dissolved in light petroleum and allowed to crystallize slowly. The crystals appeared in stellate masses. The pure natural alkaloids isolated in the manner described above gave the same colour reactions and, when run on chromatograms, the same R_F values as the synthetic alkaloids.

Micro-estimation of the alkaloids. (*a*) γ-Coniceine. This alkaloid can be estimated in the presence of the other alkaloids by means of the nitroprusside colour reaction. After steam distillation, as described above, the distillate (approx. 35 ml.) was transferred to a volumetric flask and the volume made up to 50 ml. with water. A portion (10 ml.) of this solution was pipetted into a tube, and 0·2 ml. of Na_2CO_3 solution (10%, w/v) added, followed by 0·3 ml. of a freshly prepared solution of sodium nitroprusside (1%, w/v). A period of 5 min. was allowed for maximum colour development and the intensity of the colour read in a Spekker absorptiometer (A. Hilger and Co.) with the Ilford spectrum blue filter and 2 cm. cells. Fading of the red colour takes place after a period of 15 min. Over the range from 20 to 200 μg. the extinction was proportional to the concentration of γ-coniceine ($E = 0{\cdot}45$ for 100 μg.).

(*b*) The micro-estimation of coniine, *N*-methylconiine, conhydrine and γ-coniceine after separation on paper (Whatman no. 3MM). An ethanolic solution containing

Table 1. *Colour reactions of hemlock alkaloids and related substances*

Alkaloid	Colour test: $CS_2 + CuSO_4$	CS_2 + uranyl nitrate	Sodium nitroprusside	Sodium nitroprusside + acetaldehyde	Bromothymol blue
Coniine	Brown colour soluble in ether and amyl alcohol	Orange-red colour soluble in toluene	Negative	Slow violet	Yellow, soluble in benzene
Conhydrine	As for coniine	Orange-red colour insoluble in toluene	Negative	Slow purple	As for coniine
N-Methylconiine	Negative	Negative	Negative	Negative	As for coniine
γ-Coniceine	As for coniine	Orange colour insoluble in toluene	Red	Slow violet	As for coniine
Methylpiperidein	As for coniine	Orange colour insoluble in toluene	Red	Slow violet	As for coniine
Piperidine	As for coniine	Orange colour slightly soluble in toluene	Negative	Violet	As for coniine

500 μg./ml. of each of the alkaloids was prepared and 0·1 ml. applied as a spot to the paper by means of an Agla (Burroughs Wellcome and Co.) micrometer syringe. The chromatogram was run for 18 hr. and the separated spots were detected with the iodine reagent and marked with pencil. After the brown colour had faded each spot was cut out, cut into small pieces into a test tube and eluted with five successive portions of 2 ml. of boiling ethanol, which were combined in a centrifuge tube of 15 ml. capacity. The centrifuge tube was placed in a water bath, a small porcelain chip added, and the ethanol carefully evaporated off. The ethanol-free residue was dissolved in 3 ml. of water, and 1 ml. of phosphate buffer (0·1 M-KH_2PO_4, adjusted to pH 7·0 with 0·1 M-KOH) and 3 ml. of a saturated solution of bromothymol blue in water, were added.

The tubes were stoppered and gently shaken for 5 min. Benzene (5 ml.) was added and the tubes were well shaken for 1 min. The emulsion was broken by centrifuging, if necessary, and the clear benzene layer pipetted off and dried with anhydrous Na_2SO_4. The colour intensity was read with the Ilford spectrum violet filter and 0·5 cm. cells. A water extract of filter paper cut out from a region adjoining the spots, and treated as above, served as a blank. The intensity of colour given by the eluates of the spots, when referred to calibration curves of each of the alkaloids (10–100 μg.), showed that recovery from the paper at a concentration of 50 μg. was 95%. After chromatography on paper a range of concentrations (10–100 μg.) of each of the alkaloids showed a variation in recovery of 93–102%. It was found that the calibration curves for each alkaloid were linear and almost identical ($E = 0{\cdot}62$ for 100 μg.). The above method was used for the determination of the alkaloids in the fruits and other tissues of *C. maculatum*. The dried and powdered plant material (0·5–1 g.) was steam-distilled and the distillate evaporated as described for the estimation of γ-coniceine. The residue was dissolved in ethanol (5 ml.) and transferred to a 10 ml. graduated stoppered cylinder. The basin was washed with successive small portions (1 ml.) of ethanol and the washings were added to the cylinder to make a final volume of 10 ml. A total of 0·2 ml. of the solution was applied to the paper as a spot with the aid of an Agla syringe. The procedure adopted for tissue extracts in which the concentration of any particular alkaloid was very low was as follows. The residue of mixed alkaloid hydrochlorides was dissolved in ethanol and transferred quantitatively to a 15 ml. conical centrifuge tube. The ethanol was carefully evaporated off and exactly 1 ml. of ethanol added to dissolve the residue. Of this solution 0·2 ml. was applied to the paper. Separation and estimation of the alkaloids were carried out as described above.

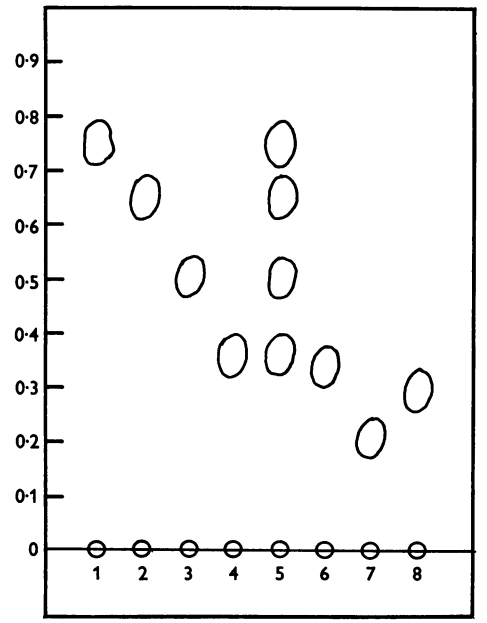


Fig. 1. Chromatograms of the hemlock alkaloids and related compounds. (1) Coniine (R_F 0·74); (2) *N*-methylconiine (R_F 0·64); (3) conhydrine (R_F 0·50); (4) γ-coniceine (R_F 0·36); (5) mixture of (1), (2), (3) and (4); (6) piperidine (R_F 0·34); (7) 2-methylpiperidein (R_F 0·20); (8) pipecolinic acid (R_F 0·29). Whatman no. 3 MM paper. Solvent system: *tert.*-pentanol–*tert.*-butanol–N-HCl (9:3:2, by vol.).

As an alternative to the bromothymol blue method, estimation of the secondary bases coniine, conhydrine, and γ-coniceine can be carried out by application of the thiocarbamate reaction of Melzer (1898). To 3 ml. of the solution (neutral) containing 10–100 μg. of alkaloid, 0·5 ml. of copper acetate soln. (1%, w/v) and 0·1 ml. of NH_3 soln. (10%, v/v) were added, followed by 5 ml. of a 5% (v/v) solution of CS_2 in *n*-pentanol. The tubes were stoppered, shaken for 1 min. and allowed to stand until the pentanol layer separated out. The pentanol was pipetted off, dried with anhydrous Na_2SO_4, and the intensity of the brown colour measured in the Spekker absorptiometer with the Ilford spectrum violet filter and 0·5 cm. cells. A blank was run as described for the bromothymol blue method. The calibration curve is linear ($E = 0{\cdot}58$ for 80 μg. of coniine).

RESULTS

Chromatography. By use of the solvent system *tert.*-pentanol–*tert.*-butanol–N-HCl (9:3:2, by vol.) with Whatman no. 3MM paper, separation of four of the five alkaloids of *C. maculatum* can be achieved. The R_F values of the pure synthetic alkaloids and some related compounds are shown in Fig. 1. In Figs. 2 and 3 the results of the chromatographic separation of the natural alkaloids present in the tissues in various stages of growth and reproduction of the plant are given. It will be observed that the relative amounts of alkaloids present (as judged by the area of the spots) vary with the stage of development and reproduction of the plant. An unknown steam-volatile base (R_F 0·33) which gave a positive ninhydrin reaction was present in small amount in leaves and flowers, and traces were found in the root tissues of young plants. It was thought that this base might be piperidine (R_F 0·34), but tests on eluates from the spots were negative for this compound. However, the frequent appearance of the spot on chromatograms suggested that it may represent pseudoconhydrine, the fifth alkaloid of the group. Examination of root tissues of young plants revealed a very low alkaloid content consisting largely of γ-coniceine. Sap exuding from the stumps of decapitated plants appeared to be virtually devoid of alkaloids, but a compound having an R_F value of 0·21 and giving a red colour with the nitroprusside reagent was present in very small amount. The presence of amino acids in the exuding sap was established by spraying chromatograms of the sap with the ninhydrin reagent.

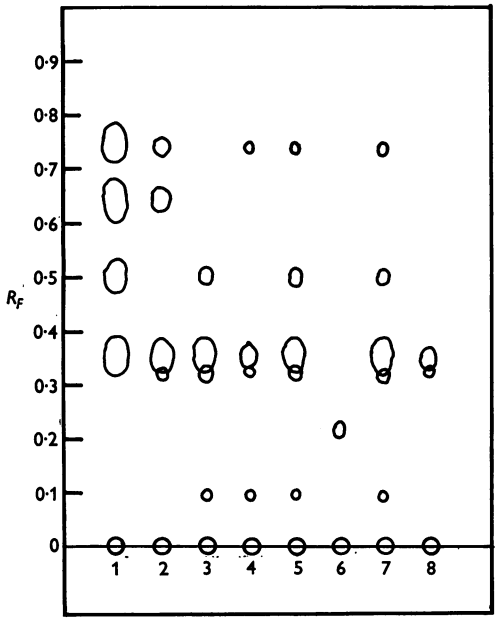


Fig. 2. Chromatograms of steam distillates of tissues of *C. maculatum* in various phases of vegetative growth (first year). (1) Mixture as Fig. 1 (5); (2) young seedlings (cotyledon stage); (3) seedlings; (4) roots of young plants; (5) leaves of young plants; (6) sap exuded from root of decapitated plants; (7) leaves of mature plant; (8) fibrous roots of mature plant. Sap exuding from the stumps of decapitated plants was collected, made slightly acid with HCl (1%, v/v), evaporated slowly to dryness at 50°, the residue dissolved in ethanol (0·5 ml.) and the solution applied directly to the paper.

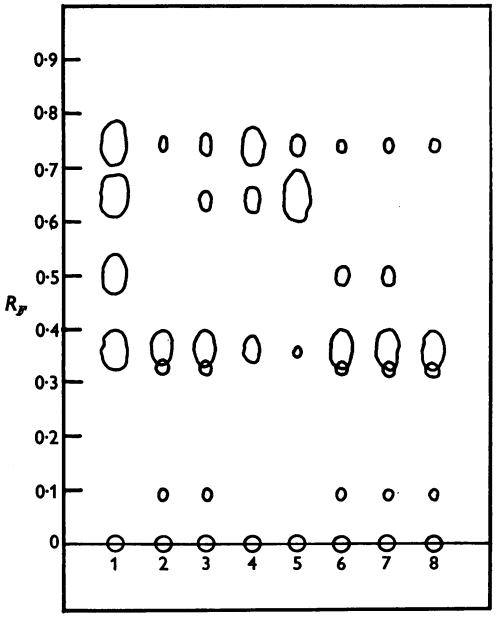


Fig. 3. Chromatograms of steam distillates of tissues of *C. maculatum* in second year of growth. (1) Mixture as Fig. 1 (5); (2) flowers (bud stage); (3) flowers; (4) young developing fruits; (5) mature green fruits; (6) young leaves developed from crown; (7) leaves of mature plant; (8) petioles of leaves of mature plant

Micro-estimation of the alkaloids. The accuracy of the methods developed for the micro-estimation of the alkaloids was tested by adding known amounts of each alkaloid in admixture to tissues and determining the percentage recovery at different concentrations (Tables 2 and 3). The concentration of alkaloids run on the chromatograms was adjusted to fall within the range of the calibration curves, either by varying the concentration or the volume of the solution applied to the paper. In the estimation of γ-coniceine the dilution of the steam distillate was varied where necessary. For tissues containing

Table 2. *Recovery (μg.) of added γ-coniceine from tissue (mature green fruits) of* Conium maculatum *after distillation*

Nitroprusside method. Percentage recovery values of duplicate analyses are given in parentheses.

γ-Coniceine (μg.) added to 0·2 g. of tissue	0	100		200		400		600		800		900	
γ-Coniceine recovered (μg.) (*a*)	26	118	(90)	210	(94)	408	(95)	580	(94)	794	(96)	875	(95)
(*b*)	24	112		212		398		594		790		882	

Table 3. *Recovery (μg.) of added conhydrine, coniine and* N*-methylconiine (in admixture) from tissue (leaves of young plants) of* Conium maculatum *after distillation and chromatographic separation*

Bromothymol blue method. Percentage recovery values of duplicate analyses are given in parentheses.

Alkaloid (μg.) added to 0·2 g. of tissue		0	100		200		300		400		500	
Alkaloid recovered (μg.)												
Conhydrine	(*a*)	46	118	(84)	211	(88)	306	(89)	398	(90)	484	(88)
	(*b*)	50	130		225		312		408		480	
Coniine	(*a*)	Trace	92	(93)	185	(94)	275	(94·5)	380	(94)	470	(95·5)
	(*b*)	Trace	94		192		292		375		485	
N-Methylconiine	(*a*)	—	94	(97·5)	194	(96)	275	(93)	372	(93)	466	(94·6)
	(*b*)	—	101		190		284		370		480	

Table 4. *Variation in alkaloid content of tissues of* Conium maculatum *during phases of growth and reproduction (cf. Figs. 2 and 3)*

For the estimation of γ-coniceine duplicate samples of tissue were steam-distilled and samples were taken in duplicate from each distillate; for the estimation of conhydrine, coniine and *N*-methylconiine duplicate samples of tissue were steam-distilled and chromatograms from each distillate run in duplicate. The figures therefore represent the mean of four values. Mean deviations are given in parentheses.

	Alkaloid content (mg./100 mg. dry wt.)				
	γ-Coniceine	Conhydrine	*N*-Methylconiine	Coniine	Total alkaloid
(1) Plants in first year of growth					
Seedlings	0·15 (0·007)	0·02 (0·002)	—	—	0·17
Leaves of young plants	0·29 (0·008)	0·03 (0·002)	—	Trace	0·32
Roots of young plants	<0·01	—	—	Trace	<0·01
Leaves developed from crowns of resting plants (October)	0·42 (0·01)	—	—	0·21 (0·03)	0·63
Fibrous roots of resting plants grown in sand culture (October)	0·08 (0·003)	—	0·04 (0·002)	0·03 (0·003)	0·15
Fleshy roots of resting plants (October)	0·08 (0·003)	—	—	0·04 (0·002)	0·12
(2) Plants in second year of growth					
Leaves	0·86 (0·02)	0·02 (0·002)	—	0·06 (0·003)	0·94
Flowers	0·75 (0·015)	—	0·09 (0·004)	0·20 (0·03)	1·04
Young developing fruits	0·36 (0·007)	—	0·25 (0·02)	0·42 (0·03)	1·03
Mature green fruits	0·01 (0·0015)	—	0·85 (0·025)	0·21 (0·025)	1·07

low concentrations of alkaloid the weight of dry tissue taken for extraction was increased. Recovery of added alkaloids was generally good, and with the exception of conhydrine (recovery 84–90%) the percentage recoveries varied between 90 and 97%.

Distribution of alkaloids in tissues

For the determination of conhydrine, coniine and *N*-methylconiine, the bromothymol blue method was used after separation of the alkaloids by chromatography. The incomplete separation of γ-coniceine from the unknown secondary base (R_F 0·33) precluded the use of the chromatographic method for the estimation of this alkaloid. The more specific nitroprusside method was therefore used for the determination of γ-coniceine. The variations in alkaloid content of tissues of the plant in various stages of growth and reproduction are shown in Table 4. Chromatographic examination of very young seedlings in the cotyledon stage showed the presence of γ-coniceine, coniine and *N*-methylconiine. In older seedlings, coniine and *N*-methylconiine were absent, and γ-coniceine (accompanied by small amounts of conhydrine) was the predominant alkaloid. In leaves of young plants in active growth γ-coniceine was the major alkaloid, but as the plants reached maturity towards the end of the first year of growth the content of coniine increased. The alkaloid content of root tissues of actively growing plants was very low, and no accumulation of alkaloids was observed in the roots of plants from which the stems and leaves had been removed. Roots of plants in the resting stage contained a much higher proportion of γ-coniceine and coniine than roots of young plants in active growth. Fibrous roots of plants in the resting stage which were grown in sand contained *N*-methylconiine in addition to coniine and γ-coniceine. With renewed vegetative growth in the spring the developing leaves contained much γ-coniceine, small amounts of coniine, and no *N*-methylconiine. Conhydrine and the unidentified base (R_F 0·33) were present in small amount. The onset of the reproductive phase was marked by a complete change in the distribution of the alkaloids. In flowers and developing fruits the content of coniine increased rapidly, and that of γ-coniceine showed a progressive decrease. When the fruits reached maturity *N*-methylconiine was the predominant alkaloid.

DISCUSSION

Nitroprusside test. This test for coniine, as described by Gabutti (1906), was found to be negative when applied to solutions of pure synthetic coniine hydrochloride. On the other hand, solutions of γ-coniceine hydrochloride and other unsaturated piperideines gave a strong red colour with the nitroprusside reagent, and it is concluded that the formation of the red colour depends on the presence of a double bond in the molecule. It is evident that the samples of coniine used by Gabutti (1906) for the test contained traces of γ-coniceine.

Micro-estimation of the alkaloids. The accuracy of the methods described depends largely on complete distillation of the steam-volatile alkaloids and on good separation of the alkaloids on paper. Overloading of the chromatograms results in poor separation of coniine from *N*-methylconiine and of conhydrine from γ-coniceine. Leaf tissues contain much γ-coniceine and little conhydrine, and care is required in determining the exact area of the conhydrine spot on chromatograms of these tissues. Thus the lower recoveries of conhydrine recorded in this work may have been due either to loss on the chromatogram or to incomplete distillation, especially as the volatility of conhydrine is lower than that of the other alkaloids.

Distribution of the alkaloids. The results recorded show that the principal alkaloid of leaves and young tissues is γ-coniceine. The presence of conhydrine in association with γ-coniceine in seedlings suggests that these alkaloids may be the first to be formed in the plant. The occurrence of coniine and *N*-methylconiine in very young seedlings could be attributed to translocation from the seed, which contains both alkaloids in small amount. The concentration of conhydrine never reaches a high level in the tissues, and it is possible that this alkaloid is the immediate precursor of γ-coniceine, into which it can be converted by dehydration (Löffler & Tschunke, 1909). Alternatively, γ-coniceine (or a related piperideine) formed by cyclization of an amino-aldehyde or aldimine in the manner suggested by Mann & Smithies (1955) may be the precursor of the other alkaloids. The observations that root tissues of young plants contain relatively low amounts of alkaloid, and that alkaloids are virtually absent from sap exuding from roots of decapitated plants, provide an interesting comparison with results obtained from similar experiments on *Atropa belladonna* (Cromwell, 1943), *Datura stramonium* (James, 1949) and *Nicotiana tabacum* (Dawson, 1941). The root tissues of these solanaceous plants are rich in alkaloids, and sap exuding from the roots of decapitated plants contains substantial amounts of alkaloid. These facts, in conjunction with the results of grafting experiments, have favoured the view that the root is the principal site of synthesis of alkaloid in members of the Solanaceae (Mothes, 1955). It would be unwise to draw conclusions from the present work regarding the site of synthesis of the hemlock alkaloids, as little information has yet been gained on the movement of the alkaloids in the plant. It might be expected that if alkaloids are synthesized in the root

and move upwards to the shoot system some accumulation might take place in the roots of decapitated plants, provided that precursors were present in sufficient amount. However, no accumulation was observed in the roots of decapitated plants, at least during the period of one week after decapitation. The higher concentration of alkaloids in the roots of plants in the resting stage could be attributed either to a downward movement from the shoot system at the end of the growing season or, if synthesis takes place to any extent in the roots, to a restriction in upward movement to the leaves. The possibility that the root is an active site of synthesis cannot be ruled out, for small amounts of γ-coniceine and coniine are present in the roots of young plants, and traces of an unknown compound (R_F 0·21) which gave a red colour with nitroprusside were detected in bleeding sap. If upward transport of alkaloid from roots to shoot were vigorous the concentration of alkaloids in the root system may remain very low even if active synthesis is taking place in the root. Nevertheless, the conclusion is reached that in *C. maculatum* alkaloid synthesis appears to take place more readily in the tissues of the shoot than in the root.

SUMMARY

1. The alkaloids of *Conium maculatum* have been separated by the method of paper chromatography.

2. Methods for the micro-estimation of the individual alkaloids have been described.

3. These methods have been used for a study of the distribution of the alkaloids in the tissues of the plant during the phases of growth and reproduction.

4. The major alkaloid of *C. maculatum* in the vegetative state is γ-coniceine. In flowers and immature fruits the content of coniine and *N*-methylconiine progressively increases and the content of γ-coniceine decreases during development. In mature fruits the major alkaloid is *N*-methylconiine.

5. The significance of these results is discussed in relation to the synthesis of the alkaloids in the plant.

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Investigations on Rat Serum Albumin Marked with Tritium-Labelled Leucine

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Limitations that have been found in the use of isotopes for the investigation of protein metabolism include the low sensitivity of the techniques of measurement of stable isotopes and, with radioactive isotopes, the difficulty and expense of preparing labelled protein of sufficiently high specific activity.

Although ^{3}H-containing hydrogen of high specific activity has been available for some time, at comparatively low cost, its use as a label has not been extensive, owing to the lack of a convenient method of assay. However, routine assay is now possible by the combustion-bomb method of production of gas for counting (Payne & Done, 1954), followed by