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THE ALKALOIDS OF HEMLOCK (*CONIUM MACULATUM* L.)—III.

THE PRESENCE OF BOUND FORMS IN THE PLANT

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Abstract—Evidence is produced to indicate that the major alkaloids of hemlock, coniine and γ-coniceine, occur as bound forms as well as the normal free salts. These bound forms vary from ethanol-soluble substances which readily break down into coniine or γ-coniceine, to larger, more resistant, water-soluble complexes which on vigorous treatment break down to alkaloid-like compounds. The quantities vary during fruit development and it is suggested that the alkaloids are built up into a series of compounds of increasing complexity which may play an important part in the metabolism of the fruit.

INTRODUCTION

A PREVIOUS paper[1] demonstrated that rapid changes in the alkaloidal pattern in developing fruits of hemlock (*Conium maculatum* L.) occurred at two-or four-hourly intervals. In particular when the coniine content was high that of the γ-coniceine (differing only by the presence of a double bond in the piperidine ring) was low, and vice versa. This indicated that one alkaloid may be readily converted into the other by simple oxidation-reduction processes. Recently Leete and Adityachaudhury[2] have unambiguously demonstrated this for one direction by feeding specifically labelled γ-coniceine to hemlock plants and showing that radioactive coniine, labelled after the same pattern, could be extracted a few days later. In our next paper* we report work with generally labelled alkaloids which indicates that this process goes on in both directions.

We also reported earlier that coniine was the major alkaloid of the fruit, but it seemed to "disappear" in significant quantities at intervals. Similar studies on *Papaver somniferum* also demonstrated rapid fluctuations in the alkaloidal pattern and the periodical "disappearance" of morphine, one of the major alkaloids.[3,4] Subsequent work on the metabolism of morphine in the plant indicates that it is converted into several bound forms which are translocated to the developing ovules and seeds and which seem to play a part in seed viability.[5] We therefore decided to investigate whether the hemlock alkaloids also occurred in bound forms. Both Cromwell[6] and Fairbairn and Suwal[1] reported the presence of unknown Dragendorff-positive substances in hemlock whose low R_f values might indicate they are larger molecules and more polar than the normal free alkaloids. Preliminary work which we

* Part IV—*Phytochem.* **7**, 1599 (1968).

[1] J. W. FAIRBAIRN and P. N. SUWAL, *Phytochem.* **1**, 38 (1961).
[2] E. LEETE and N. ADITYATHAUDHURY, *Phytochem.* **6**, 219 (1966).
[3] J. W. FAIRBAIRN and G. WASSEL, *Phytochem.* **3**, 253 (1964).
[4] J. W. FAIRBAIRN, A. PATERSON and G. WASSEL, *Phytochem.* **3**, 577 (1964).
[5] J. W. FAIRBAIRN and S. EL-MASRY, *Phytochem.* **6**, 499 (1967), **7**, 181 (1968).
[6] B. T. CROMWELL, *Biochem. J.* **64**, 259 (1956).

carried out with thin-layer chromatograms indicated that several of these compounds did exist in hemlock and that with some of them the Dragendorff colour developed only slowly after spraying, indicating a breadown from a parent Dragendorff-negative substance.

This present paper reports the results of investigations on these substances using special extraction and counter-current methods. For convenience these new compounds will be referred to as "low R_f (LRF) compounds" in contrast to the normal alkaloidal salts whose R_f values were nearly always significantly higher in the chromatographic systems used.

RESULTS

General Phytochemical Investigation

In view of the fact that some of the presumed bound forms may be unstable in acid or alkali, we decided to avoid marked changes in pH in all extraction and chromatographic procedures. These unconventional methods meant that the "free" alkaloidal salts would be extracted in their native form and not as hydrochlorides, sulphates or acetates as is usually done. It was therefore necessary to determine the properties of these native (ionic) salts of the alkaloids so as not to confuse them with the (presumed non-ionic) LRF compounds. Fractionation of plant material with water and ethanol led to the separation of three groups of these compounds.

(*a*) *Water-soluble LRF compound.* This compound (or compounds) was insoluble in ethanol or even 50% ethanol; on spraying with Dragendorff's reagent it only slowly developed an orange colour. In contrast to the normal alkaloidal salts, it would not diffuse out of intact killed fruits. It was stable in water and dilute acids or alkalies: however, on heating in 6 N HCl for half an hour an ethanol-soluble substance was formed. This gave an immediate red colour with Dragendorff's reagent and had an R_f value identical with that of coniine in one solvent system but it differed, however, from coniine in other systems. This LRF compound therefore appeared to be a stable, fairly large, water-soluble substance which on vigorous treatment broke down into an alkaloid-like compound. It was present in the vegetative parts and in young fruits during the first 2 or 3 weeks after fertilization but was absent at later stages. As these stages were of particular interest to us, we decided to postpone a detailed study of this compound until later.

(*b*) 70% *ethanol-soluble LRF compounds.* Although soluble in 70% ethanol, these compounds were insoluble in pure ethanol. They were stable to acid and alkali at room temperature but after heating in 6 N HCl for 4 hr yielded coniine, γ-coniceine and a third Dragendorff positive substance similar in R_f values and colour reactions to that derived from Compound *B* (see below). These compounds occurred in the fruits during active stages of development.

(*c*) *Ethanol-soluble LRF compounds.* Although extracted by 70% ethanol, these compounds were also soluble in pure ethanol. They occurred in the fruits during the stages of active growth (3–5 weeks after fertilization) and we therefore attempted to fractionate them by the use of selective solvents, counter-current methods and continuous flow TLC.[7] By these means four compounds were separated and named *A*, *B*, *C* and *E*. Compound *A* was stable in all conditions tried: Compound *B*, on vigorous treatment with acid or alkali, broke down into an alkaloid-like substance of low R_f value. Compound *C* was less stable, and on mild treatment with acid or alkali yielded coniine. Compound *E* also broke down readily on mild treatment and yielded γ-coniceine. A fifth LRF compound (Compound *D*) was found to be an intermediate between Compound *C* and coniine. Compounds *C* and *E* differ

[7] M. BRENNER and A. NIEDERWIESER, *Experimenta, Basle* **17**, 237 (1961).

from their corresponding alkaloids and from *N*-methylconiine in chromatographic behaviour (Table 1). Compound *C* is also freely soluble in ethyl acetate in contrast to coniine salts; it also migrated at a different rate during counter-current fractionation.

TABLE 1. R_f VALUES AND COLOUR REACTIONS OF SOME OF THE FREE AND THE BOUND ALKALOIDS

Substance	Chromatographic system			Colour with Dragendorff's reagent
	Silica Gel G ($CHCl_3$/MeOH (1:1))	Silica Gel G (MeOH/NH_4OH (97:3))	Alumina ($CHCl_3$/MeOH (4:1))	
Coniine hydrochloride	0·60	0·30	0·47	Red–violet
γ-Coniceine hydrochloride	0·28	0·67	0·80	Red
N-Methylconiine hydrochloride	0·16	0·50	0·71	Orange
Compound *C*	0·08	0·30	0·46	Red–violet
Compound *E*	0·00	—	—	Orange

Native Forms of the Alkaloidal Salts

In all chromatographic systems, the "native" forms of coniine and γ-coniceine had R_f values identical to those of their hydrochlorides (Table 1), hydrobromides, oxalates, tartrates and sulphates. Compounds *C* and *E* are therefore not mere "native" forms of the alkaloids, nor could they be the free bases, as the latter, if they did occur naturally in the plant, would have been removed by chloroform during the preliminary fractionation (see Experimental).

Fluctuations in the LRF Compounds during Fruit Development

Samples of the developing fruits, 2, 3 and 4 weeks after fertilization, were collected at 2-hr intervals and the ethanol-soluble compounds extracted and examined by thin-layer chromatography. In the 2-week-old samples LRF compounds were only detectable in the sample collected at 16.00 hr. In the 3- and 4-week samples much larger amounts of the LRF substances were present. The amounts, judging by the size of the spots and intensity of the orange–red colour after spraying with Dragendorff's reagent, varied considerably from sample to sample with the largest quantities tending to occur in the 16.00 hr samples.

DISCUSSION

It is obvious from the work described here that a series of compounds are present in hemlock which can be hydrolysed to form alkaloids or alkaloid-like substances. In this sense, the alkaloids can be considered as intermediates rather than end-products. Two of these compounds are readily broken down into the known alkaloids, coniine and γ-coniceine. In contrast, some of the compounds are resistent to decomposition under mild conditions and yet others are resistant to even more drastic conditions. In practically all cases, however, it was possible to demonstrate the release of substances which behaved like alkaloids in that they could be extracted from alkaline solution into organic solvents and gave a positive Dragendorff colour.

A tentative interpretation of these facts is that the alkaloids in hemlock are built up into a series of compounds of increasing complexity: those in the leaf being the most polar as they

are insoluble in ethanol (even 50 per cent) but readily soluble in water. Examination of the fruits at different stages of development shows that other less polar bound forms appear during critical stages of growth when marked changes in the pericarp tissues and in the alkaloidal pattern take place.[8] Furthermore at this stage the concentration of these bound forms seems to vary rapidly during the day. These observations suggest that the newly discovered compounds may play some part in the metabolism of the fruit.

Unfortunately, the amounts present are small and we found it extremely difficult to separate sufficient quantities to establish the facts we have. Tedious and prolonged phytochemical techniques may be needed to isolate quantities of sufficient purity for structure determinations. In the next paper we describe the application of radioactive techniques to establish in more detail the relationship between these compounds and the alkaloids and the rapid fluctuations in quantity which seem to occur.

EXPERIMENTAL

Preliminary Fractionation

Conium maculatum L. (Variety C)[8] was used throughout the work. To minimize decomposition, freshly collected plant material (usually the fruits) was immediately blended several times with 70% ethanol. The extract was filtered; two volumes of $CHCl_3$ and sufficient water were added to give two layers. The dark-green lower layer was washed with dilute ethanol, the washings returned to the upper layer, and the lower layer discarded. The upper layer was evaporated to a syrup and poured into an excess of ethanol where a gummy precipitate formed. The supernatant layer contained most of the Compounds *A*, *B*, *C*, *D* and *E* as well as the normal alkaloidal salts: the gummy material contained traces of these together with the 70% ethanol-soluble LRF compounds. The marc, from the original extraction, was extracted with water to yield the water-soluble LRF compounds.

Separation of Compound C

The supernatant ethanol layer, referred to above, was evaporated to dryness and subjected to counter-current distribution using both layers from $CHCl_3$:methanol:water (3:1:1). After sixty transfers, the upper layers, containing most of the LRF compounds, were combined, carefully evaporated to dryness and refractionated using the same counter-current system. By this means a fraction containing Compound *C* (and traces of *D*), but no other LRF compounds, together with the alkaloidal salts of coniine, γ-coniceine and *N*-methylconiine, was obtained. Coniine salts were separated by extraction with acetone, in which they are insoluble, and further counter-current fractionation removed the γ-coniceine. *N*-Methylconiine was separated by TLC on neutral alumina and $CHCl_3$:methanol (4:1) (see Table 1). The small quantities of Compound *D* originally present had now been eliminated. Compound *C* was eluted from the alumina layer with methanol: on evaporation to dryness a yellow residue was obtained in too small a quantity to attempt recrystallization.

Compound E

This was separated from the supernatant ethanol layer by continuous TLC using the BN chamber devised by Brenner and Niederwieser[7] and the silica gel/$CHCl_3$/MeOH system of Table 1. After 5 hr continuous running, all the normal alkaloids were at the top of the plate, and Compound *E* (and *C*) was well removed from the starting line on which Compounds *A* and *B* were retained. Unfortunately it was not possible to elute the band containing *C* and *E* with neutral solvents; it was necessary to use 0·5 N HCl in ethanol. The slow elution process (3 hr) in these acid conditions led to the decomposition of both compounds with the production of coniine and γ-coniceine.

Compounds A *and* B

The fraction containing these compounds from previous counter-current treatment were combined, evaporated and re-cycled for sixty transfers in the system $CHCl_3$:methanol:water (2:2:1). The fractions containing Compound *A* or Compound *B* were combined and evaporated to dryness. No other LRF compounds were present.

Decomposition of Compounds C, D *and* E

Compound *C* was treated with N HCl for 30 min at room temperature, the mixture made alkaline and extracted with $CHCl_3$. A little HCl was added to the $CHCl_3$ layer which was then evaporated to

[8] J. W. FAIRBAIRN and S. B. CHALLEN, *Biochem. J.* **72**, 556 (1959).

dryness. The amount of substance formed was too small for recrystallization but it was identified as coniine by co-chromatography with coniine hydrochloride in four systems (Table 1, and Cromwell[6]). Similar results were obtained when Compound *C* was treated with N KOH at room temperature. When Compound *C* had been stored in the refrigerator for 2–3 months and re-chromatographed a new substance had been formed which was called Compound *D*. This was eluted, and on treatment with HCl produced coniine. Although Compound *E* was not separated from Compound *C*, the mixture on treatment with dilute acid at room temperature yielded coniine (from Compound *C*) and γ-coniceine which was identified by co-chromatography with authentic γ-coniceine[6] and by colour reaction with alkaline nitro-prusside.[8]

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