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

## ISOTOPIC STUDIES OF THE BOUND FORMS OF ALKALOIDS IN THE PLANT

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**Abstract**—When radioactive γ-coniceine is fed to stalks subtending actively growing umbels almost all the radioactivity passes into the umbels. Here it is rapidly converted into the bound forms previously described[1] and into coniine. Conversely, when radioactive coniine is fed, radioactive bound forms and γ-coniceine are produced. The specific activities of all these compounds fluctuated fairly rapidly indicating that they are involved in important metabolic processes. The specific activities of the free alkaloids did not fall markedly even after a period of 19 days and this indicates a re-cycling system. The similarity of this situation to that obtaining with the pyridine nucleotides is discussed and it is suggested that the alkaloids may represent moieties of nucleotide-like compounds concerned with oxidation-reduction processes in this plant.

IN OUR previous paper[1] we have shown that the plant contains a number of bound forms of the alkaloids, that is, substances of low $R_f$ value (LRF compounds) which on mild or vigorous treatment with acids and alkalies break down into coniine, γ-coniceine and alkaloid-like compounds. The quantities present in the fruit seemed to fluctuate in the same way as did the alkaloids, especially 2–4 weeks after fertilization. We have therefore attempted to investigate in greater detail these fluctuations and the relationship of the LRF compounds to the alkaloids by feeding radioactive alkaloids to the plant.

## RESULTS

### γ-*Coniceine Feeding*

Earlier work[2] had indicated that γ-coniceine enters the fruits via the schizogenous ducts as well as by the vascular bundles. We therefore examined the stalks (peduncle and pedicels) below the umbels histologically and histochemically and found schizogenous ducts containing piperideines in the cortex and in the outer regions of the phloem; piperideines were also present in the xylem vessels. Radioactive γ-coniceine hydrochloride solution was therefore fed to these tissues from a polythene cup[3] attached to the peduncle below an umbel after having gently scraped away the epidermal tissues from the peduncle. Maximum amounts of ethanol-soluble radioactivity reached the fruits 3–4 days after feeding. In the 1965 experiments this represented 25 per cent of the fed activity (16 per cent in the fruits and 9 per cent in the stalks). Examination of the fruits showed that the coniine and the crude LRF compounds had become radioactive. In the 1966 experiments 60–75 per cent of the fed activity was

[1] J. W. FAIRBAIRN and A. A. E. R. ALI, *Phytochem.* **7**, 1593 (1968).
[2] J. W. FAIRBAIRN and S. B. CHALLEN, *Biochem. J.* **72**, 556 (1959).
[3] J. W. FAIRBAIRN, A. PATERSON and G. WASSEL, *Phytochem.* **3**, 577 (1964).

found in the 70 per cent ethanol extract of the fruits and stalks 3–4 days after feeding; practically all the radioactivity in the cups had entered the plant 2 days after feeding.

Samples were collected at various time intervals after feeding, their 70 per cent ethanol extracts fractionated and the radioactivities of the coniine, γ-coniceine and crude LRF compounds determined. The results are shown in Table 1. The single plant which supplied the umbels collected at 10.00, noon and 14.00 hr, four days after feeding, was dug up at 15.00 hr on the same day and immediately divided into roots, leaves, umbels (other than the fed ones), stems and branches. Each was separately extracted with 70 per cent ethanol and the radioactivities of these extracts determined. The results showed that 67·8 per cent of the fed

TABLE 1. DISTRIBUTION OF RADIOACTIVITY IN 70% ETHANOL-SOLUBLE SUBSTANCES OF THE FRUIT AFTER FEEDING RADIOACTIVE γ-CONICEINE HYDROCHLORIDE

| Interval after feeding | Time of day | Radioactivity (dpm × $10^3$ per umbel) (specific activities, dpm/μmole, in brackets) | | | |
|---|---|---|---|---|---|
| | | Coniine | γ-Coniceine | Compound *A*+*B* | Compound *C*, *D*, *E* |
| 1 day | 6.00 hr | 3·16 (39) | 2·29 (120) | 0·40 | 2·60 |
| | noon | 6·18 (63) | 10·36 (241) | 0·97 | 4·50 |
| | 18.00 hr | 6·01 (76) | 10·18 (496) | 0·96 | 2·30 |
| 2 days | noon | 6·32 (121) | 9·60 (457) | 1·20 | 3·50 |
| | 18.00 hr | 6·22 (152) | 13·77 (467) | 2·38 | 3·10 |
| 3 days | 6.00 hr | 7·31 (127) | 11·43 (313) | 1·85 | 3·80 |
| | noon | 20·77 (216) | 12·64 (665) | 1·85 | 6·07 |
| | 18.00 hr | 26·17 (164) | 6·63 (577) | 2·74 | 2·70 |
| 4 days | 6.00 hr | 32·00 (173) | none — | 1·70 | 8·32 |
| | 8.00 hr | 27·03 (173) | traces — | 2·06 | 3·07 |
| | 10.00 hr | 18·10 (149) | 9·36 (435) | 2·26 | 8·22 |
| | noon | 19·73 (174) | 21·18 (432) | 4·20 | 5·50 |
| | 14.00 hr | 21·00 (170) | 18·42 (405) | 2·90 | 6·17 |
| | 16.00 hr | 19·42 (140) | 7·33 (458) | 2·50 | 12·70 |
| | 18.00 hr | 24·80 (222) | 9·05 (532) | 3·50 | 3·02 |
| 19 days | noon | 15·41 (378) | 4·33 (n.d.) | n.d. | n.d. |

γ-Coniceine hydrochloride fed to the peduncle = 1164 dpm/μmole; 140 × $10^3$ dpm per umbel. Fed to the peduncle, 17 days after fertilization.

radioactivity was in the fed umbels (fruits and stalks), 0·9 per cent in the other umbels, 2·2 per cent in the leaves, 1·6 per cent in the stems and branches and 1·7 per cent in the roots. This shows clearly that the movement of γ-coniceine and its products is mainly in an upward direction towards the developing fruits.

*Hydrolysis of the Radioactive LRF Compounds*

Some of the fractions containing Compounds *C*, *D* and *E* (Table 1) were treated with cold 0·5 N HCl then alkalinized and the liberated alkaloids extracted into chloroform and the coniine and γ-coniceine separated by band chromatography. The quantities and radioactivities of each alkaloid were determined and the specific activities, compared with those for the free alkaloids given in Table 1, are shown in Table 2.

TABLE 2. SPECIFIC ACTIVITIES OF THE FREE AND BOUND ALKALOIDS OCCURRING SIMULTANEOUSLY IN THE FRUIT

| Sample* | Specific activity (dpm/μmole) | | |
|---|---|---|---|
| | Alkaloid | Free | Liberated from *C*, *D* and *E** |
| 1st day, 6.00 hr | γ-coniceine | 120 | 147 |
| 1st day, noon | γ-coniceine | 241 | 277 |
| | coniine | 63 | 100 |
| 4th day, 6.00 hr | coniine | 173 | 337 |

* Same samples as in Table 1.

*Coniine Feeding*

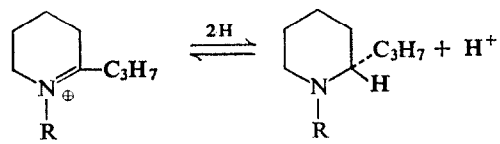
Radioactive coniine (generally labelled) was fed to umbels 2 weeks after fertilization in the same way as for γ-coniceine. Samples were collected at intervals between the first and twenty-first day after feeding and the 70% ethanol extracts of the fruits and stalks examined. Quite surprisingly no γ-coniceine at all was found in any of the fruit samples even though it was present in the stalks. Each umbel, containing approximately 120 fruits, had been fed with 3·3 mg coniine hydrochloride, all traces of which had entered the plant from the cups 2 days after feeding. The γ-coniceine of the stalks was found to be radioactive with a specific activity not much less than that of the fed coniine. In the fruits, radioactive coniine was present as well as radioactivity in the LRF compounds, and in other substances.

## DISCUSSION

This work has established the following facts; (*a*) at the active phase of fruit development the movement of γ-coniceine is almost entirely upward from the stalk into the fruits, (*b*) on reaching the fruit the γ-coniceine is fairly rapidly converted into coniine and into other bound forms of the alkaloids (LRF compounds). The fact that at any one time the γ-coniceine LRF Compound *E* is of the same specific activity as the "free" γ-coniceine (Table 2) suggests that the latter is incorporated intact into Compound *E* and fairly rapidly; (*c*) the specific activities and amounts of the alkaloids (especially γ-coniceine) and the LRF compounds show considerable variation with time (Table 1) and indicate that all are implicated in active metabolism, (*d*) the specific activity of the coniine rises gradually and fluctuates but even after 19 days shows no sign of disappearing. This indicates that the total coniine content of the fruit (free and bound) is fairly constant and that the periodical "disappearance" of the free coniine, previously reported,[4] is due to temporary reversible conversion into non-alkaloidal bound forms. There is normally a significant "pool" of free coniine, about 5–15 times as big as the γ-coniceine "pool",[4] and this may explain why the free coniine in Table 2 is less radioactive than the bound coniine, owing to dilution, in contrast to the free and bound γ-coniceine. At intervals these bound forms evidently return into metabolic circulation so that the coniine ultimately reappears as γ-coniceine. This is confirmed by the fact that after feeding radioactive coniine we were able to recover radioactive γ-coniceine.

[4] J. W. FAIRBAIRN and P. N. SUWAL, *Phytochem.* **1**, 38 (1961).

The result of this and previous work therefore establishes that the concentrations of coniine and $\gamma$-coniceine fluctuate rapidly during active growth in the fruits. An interchange between these two is indicated by the fact that their concentrations are negatively correlated[4] and confirmed by the radioactive tracer work of Leete[5] and our own. The fact that their specific activities do not diminish even after 19 days (Table 1) suggests a re-cycling system and raises the question whether these alkaloids are involved in reversible oxidation-reduction processes. The chromatographic behaviour of the bound forms[1] suggests that the non-alkaloid moiety is attached to the reactive nitrogen atom, as this would become quarternary in $\gamma$-coniceine and tertiary in coniine complexes, and so increase the basicity. Waldi[6] has shown that in slightly acidic silica gel systems with neutral running solvents increasing basicity of a series of compounds leads to decreasing $R_f$ values. The LRF compounds have distinctly lower $R_f$ values than those of the coniine and $\gamma$-coniceine[1] and this may be due to increased basicity due to attachment of a moiety to the nitrogen as indicated above. If so, the bound forms could react as follows:




The close similarity to the $NAD^+ \rightleftharpoons NADH + H^+$ is obvious and it may well be that some of our bound froms represent nucleotide-like compounds with the alkaloids corresponding to the nicotinamide moiety. Kisaki and Tamaki[7] have already referred to such a possibility for nicotine and quote the work of Fukuzumi *et al.*[8] who replaced the nicotinamide moiety of NAD with nicotine and found that the resulting nucleotide was active. Similarly Anderson, Ciotti and Kaplan[9] produced a series of nucleotides of varying activities by replacing the nicotinamide with amine or carbonyl functions.

The co-occurrence of free alkaloids with several bound forms of increasing complexity and the evidence for a re-cycling system is also closely parallel to the position of the pyridine nucleotides. Gholson[10] has postulated that in most organisms, NAD (nicotinamide adenine dinucleotide) is readily broken down into free nicotinamide by the ubiquitous NAD-glycohydrolase. Some of the liberated nicotinamide is converted into nicotinic acid which is then incorporated into nicotinic acid mononucleotide. This, in turn, is used for the production of fresh NAD. Some of the free nicotinamide is "bled off" from the system either as the free compound or as a derivative, e.g. *N*-methyl nicotinamide. The similarity of this situation to that in *Conium maculatum* with its re-cycling system of free alkaloids and bound forms of varying complexity and the production, at times, of *N*-methyl coniine and other derivatives[4] is sufficiently close to warrant further investigation into the possibility that the alkaloids are intimately connected with important oxidation-reduction processes in this plant.

[5] E. LEETE and N. ADITYACHAUDHURY, *Phytochem.* **6**, 219 (1967).
[6] D. WALDI, *Thin Layer Chromatography*, p. 279, Academic Press, New York (1965).
[7] T. KISAKI and E. TAMAKI, *Phytochem.* **5**, 293 (1966).
[8] T. FUKUZUMI, H. TAKAHARA and K. ARAI, *Sci. Papers Central Res. Inst. Japan Monopoly Corp.*, No. **106**, 63 (1964).
[9] B. M. ANDERSON, C. J. CIOTTI and N. O. KAPLAN, *J. Biol. Chem.* **234**, 1219, 1226 (1959).
[10] R. K. GHOLSON, *Nature* **212**, 933 (1966).

## EXPERIMENTAL

*Preparation and Feeding of Radioactive Alkaloids*

Radioactive γ-coniceine was prepared by exposing young hemlock plants (variety *C*)[2] to $^{14}CO_2$ for 30 hr in a perspex chamber.[11] The aerial parts were macerated in 70% methanol for a week and filtered: the filtrate was acidified with tartaric acid and evaporated to small volume. The green sludge was filtered off, the filtrate made alkaline and the alkaloids extracted into $CHCl_3$. This was re-extracted into acid and the process repeated two or three times. The γ-coniceine was separated by band chromatography using Cromwell's system[12] and further purified by band chromatography on silica gel plates.[1] Radioactive coniine was prepared in a similar manner from a Minnesota variety. This variety differs from our variety *C* and from Cromwell's[12] in that coniine is the major alkaloid of the vegetative parts. The alkaloids (as HCl-ides in water) were fed to the plant by gently scraping away 2 cm of the superficial tissue of the peduncle about 10 cm below an umbel and attaching a polythene cup at this position.[3] The solution of radioactive alkaloid was placed in the cup and after absorption the cup was filled with water to wash in the remainder of the radioactivity. After about 2 days all radioactivity fed entered the plant.

*Separation and Radioactivities of the Alkaloids and LRF Compounds*

Each sample consisted of two to four umbels (250 to 500 fruits). The fruits were rapidly separated from the stalks and both separately macerated with 70% ethanol after grinding in a glass mortar with sand. The alkaloids and LRF compounds were isolated by the methods already described[1] and their radioactivities determined in a Packard Tricarb scintillation spectrometer (Model 3003) using suitable corrections for quenching effects. The quantities of alkaloid were determined as previously described.[4]

*Acknowledgement*—We would like to thank Professor E. Leete for supplying us fruits of his "Minnesota" variety and the United Arab Republic for a grant to one of us (A. A. E. R. A.).

[11] A. A. E. R. Ali, The Alkaloids of *Conium maculatum* L. as Possible Intermediates in its Metabolism. Thesis, University of London (1967).
[12] B. T. Cromwell, *Biochem. J.* **64**, 259 (1956).