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STUDIES ON THE BIOGENESIS OF SOME SIMPLE AMINES AND QUATERNARY AMMONIUM COMPOUNDS IN HIGHER PLANTS.

TRIMETHYLAMINE IN *CHENOPODIUM VULVARIA* L.

B. T. CROMWELL and M. RICHARDSON*

Department of Botany, University of Hull, and Department of Biology, Queen Elizabeth College, London, W.8

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Abstract—The content of trimethylamine in the tissues of *Chenopodium vulvaria* L. was studied during the phases of growth and reproduction and in relation to the development of the epidermal glands. Bacteria were shown to be responsible for the production of trimethylamine from choline in non-sterile enzyme preparations. Bacteria were not responsible for the production *in vivo* of trimethylamine by plants of *C. vulvaria*. Choline-Me-^{14}C did not give rise to trimethylamine in cuttings of *C. vulvaria* apart from traces which resulted from the spontaneous radiosensitive decay of the radioactive choline. The major radioactive compound isolated from tissues of *C. vulvaria* which had been fed with choline-Me-^{14}C was identified as betaine; traces of two other radioactive compounds which were tentatively identified as dimethyl glycine and trimethylamine-N-oxide-^{14}C were also isolated. Trimethylamine-N-oxide-^{14}C was isolated from cuttings of *C. vulvaria* which had been fed with trimethylamine-^{14}C. No significant radioactivity was observed in trimethylamine isolated from cuttings which had been fed with trimethylamine-N-oxide-^{14}C, phosphoryl choline-Me-^{14}C, methylamine-^{14}C, betaine-^{14}C, and sodium formate-^{14}C. Feeding of methionine-Me-^{14}C to plants of *C. vulvaria* resulted in a slight incorporation of radioactivity into trimethylamine isolated from the tissues and substantial labelling in a number of other quaternary ammonium bases present in the plant.

INTRODUCTION

SEVERAL complex nitrogen containing compounds, especially choline, have been regarded as the most likely immediate precursors of trimethylamine in biological systems. Choline was suggested as the precursor of trimethylamine by early workers[1,2] but without experimental support. The ability of certain bacteria to produce trimethylamine from choline is well established[3–6] and Hayward and Stadtman[7,8] have prepared cell-free extracts of *Vibrio cholinicus* (= *Desulfovibrio desulfuricans* Beij.) which degrade choline anaerobically with the production of trimethylamine, ethanol and acetic acid.

Cromwell[9] prepared an extract from the leaves of *Chenopodium vulvaria* L. and demonstrated the production of trimethylamine when the extract was incubated with choline at 24° and pH 7·8 for 48 hr in the presence of thymol as an antiseptic. Ethylene glycol was detected

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1 C. WEHMER, *Die Pflanzenstroffe* (2nd Ed.) Fischer, Jena (1929–31).
2 F. R. POWER and V. K. CHESNUT, *J. Am. Chem. Soc.* **47**, 1751 (1925).
3 F. E. DYER and A. J. WOOD, *J. Fisheries Research Board., Canada* **7**, 17 (1947).
4 G. N. COHEN, B. NISMAN and M. RAYNAUD, *Compt. rend.* **225**, 647 (1947).
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6 V. MONSOUR and A. R. COLMER, *J. Bacteriol* **63**, 597 (1952).
7 H. R. HAYWARD and T. C. STADTMAN, *J. Bacteriol* **78**, 557 (1959).
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9 B. T. CROMWELL, *Biochem. J.* **46**, 580 (1950).

in the reaction mixtures and suggested that choline aminase enzyme was present. These findings were in close agreement with a scheme[10] which suggested that the methyl amines in plants might arise from a hydrolytic cleavage of the corresponding methyl ethanolamines which are intermediates in the biosynthesis of choline.

Stein von Kamienski[11] showed that amines were not produced by homogenates of flowers of *Sorbus aucuparia* L. and various species of *Crataegus* in the presence of thymol, chloroform, toluene and sodium fluoride. However he observed strong bacterial development in homogenates to which thymol was added and suggested that in the experiments of Cromwell[9] trimethylamine might have been produced by bacterial breakdown. Thymol was also found to be unsatisfactory as an antiseptic in experiments on the conversion of choline to betaine by homogenates of root tissue of *Beta vulgaris* L.[12]

In the present work the suggested role of choline as a precursor of trimethylamine in the tissues of *Chenopodium vulvaria* has been re-examined and the possibility that a compound other than choline is the immediate precursor of trimethylamine has been investigated.

RESULTS

The production of trimethylamine in plants of *C. vulvaria* does not appear to be restricted to any one phase of the growth of the plant (Fig. 1), nor does the onset of flowering appear to affect the production of this amine. Table 1 shows the variation in content of trimethylamine in the leaves of the plant. The content of trimethylamine is always greatest (per unit weight) in the apical leaves and shows a progressive decline as the leaf expands irrespective of the state of maturity of the plant as a whole.

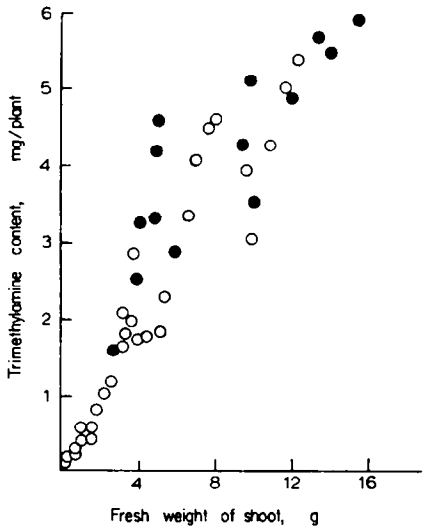

FIG. 1. TRIMETHYLAMINE CONTENT OF SHOOTS OF *C. vulvaria* L.

Plants in flower (●), plants not in flower (○). Trimethylamine isolated by vacuum distillation at 40°, and estimated by the modified periodide method described in the text.

[10] B. T. CROMWELL, *Biochem. J.* **45**, 84 (1949).
[11] E. STEIN VON KAMIENSKI, *Planta*, **50**, 331 (1958).
[12] B. T. CROMWELL and S. D. RENNIE, *Biochem. J.* **58**, 318 (1964).

TABLE 1. TRIMETHYLAMINE CONTENT OF LEAVES OF *C. vulvaria*

	Trimethylamine content (μg/g fresh weight)	
	Young plants	Plants in flower
Apical leaves	904	982
Median leaves	405	370
Basal leaves	197	126

Trimethylamine isolated by vacuum distillation and estimated by the iodine-potassium iodide method described in the text. Each value is the mean of 5 replicate determinations.

Trimethylamine was produced when enzyme extracts prepared[9] from the leaves of *C. vulvaria* were incubated with choline, but was shown to be the result of the action of contaminating micro-organisms whose growth was only slightly suppressed by the use of thymol.

Seedlings of *C. vulvaria* grown from surface sterilized seed under sterile conditions on sand irrigated with a nutrient solution produced trimethylamine in the absence of micro-organisms. The mean content of trimethylamine in sterile seedlings (359 μg/g fresh wt.) was only slightly lower than the content of seedlings grown under non-sterile conditions (417 μg/g).

Metabolism of Choline-Me-^{14}C by Cuttings of C. vulvaria

A low degree of incorporation of radioactivity into trimethylamine isolated from cuttings of *C. vulvaria* to which choline-Me-^{14}C had been administered under sterile conditions was shown to be entirely due to the spontaneous radiosensitive decomposition of the choline-Me-^{14}C which is known to be quite rapid at room temperature.[13,14] The major radioactive compound produced was betaine (Fig. 2), and its i.r. spectrum and electrophoretic behaviour

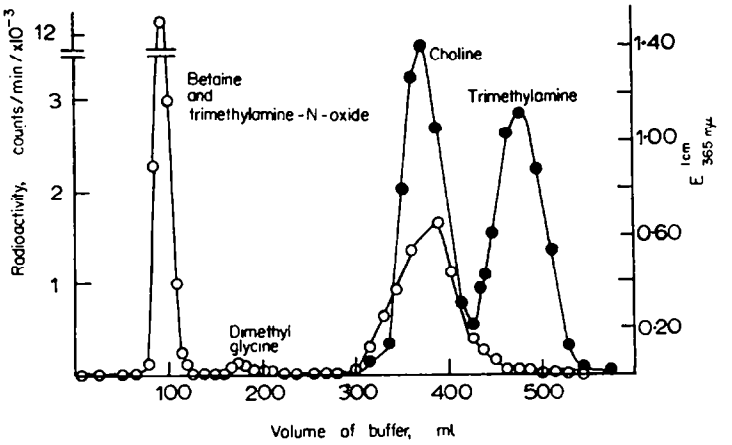

FIG. 2. SEPARATION OF BASES FROM *C. vulvaria* AFTER FEEDING WITH ^{14}C CHOLINE.
Chromatography on a column (1·17 cm × 135 cm.) of Zeo-Karb 226 buffered at pH 7·3 and eluted with buffer pH 5·3 of an extract from a plant of *C. vulvaria* L. which had received 12 μc (0·25 mg) of choline-Me-^{14}C for 72 hr. Radioactivity (○); $E^{1\,cm}_{365\,m\mu}$ of the periodide derivatives of the quaternary ammonium compounds (●). All other experimental details as given in the text.

[13] R. H. LEMMON, M. A. PARSONS and D. M. CHINN, *J. Amer. Chem. Soc.* **77**, 4139 (1955).
[14] I. SERLIN, *Science* **126**, 261 (1957).

TABLE 2. ELECTROPHORETIC MOBILITIES AND ION EXCHANGE PAPER CHROMATOGRAPHIC R_f VALUES OF QUATERNARY AMMONIUM COMPOUNDS

Compound	Electrophoretic mobility. Distance moved in 90 min at 400 V (cm)	R_f values	
		Amberlite SA-2 solvent: 1 N HCl	Amberlite WA-2 solvent: 0·2 M phosphate-citrate buffer, pH 5·2
Phosphoryl choline	5·3	0·95	—
Betaine	5·9	0·90	0·82
Choline	15·8	0·59	0·29
Neurine	16·8	—	—
Trimethylamine-N-oxide	17·2	0·38	0·41
Trimethylamine	20·4	0·58	0·25
Dimethyl glycine	—	0·81	0·76
Tetramethylammonium hydroxide	18·9	—	—

Experimental details as given in text.

(Table 2) was in agreement with data obtained from an authentic sample. Two other compounds were isolated in small amounts: the first was thought to be trimethylamine-N-oxide but its i.r. spectrum did not reveal the absorption band at 940 cm^{-1} characteristic of this compound.[15] The second compound was only recorded as minute traces in extracts of plants which had been fed with choline-Me-^{14}C for more than 48 hr. The behaviour of this compound in a number of solvent systems indicated that it might possibly be dimethyl glycine. It was however not present in sufficient amounts to make a positive identification possible.

Table 3 shows the relative amounts of radioactivity found in the various quaternary ammonium bases extracted from *C. vulvaria* after the administration of choline-Me-^{14}C.

TABLE 3. RADIOACTIVITIES OF QUATERNARY AMMONIUM BASES ISOLATED FROM *C. vulvaria* AFTER THE ADMINISTRATION OF [Me−^{14}C] CHOLINE

Duration of feeding (hr)	Radioactivities (counts/min × 10^{-6})		
	Choline	Betaine	Trimethylamine-N-oxide
12	1·81	9·93	—
24	2·68	11·97	0·064
48	1·99	13·00	0·098
72	1·68	9·38	0·092
15*	7·69	1·15	—

* In atmosphere of nitrogen.

Except for the last experiment, where 0·147 mg (6 μc) was fed, 0·245 mg (10 μc) of [Me−^{14}C] choline was administered in each experiment.

Radioactive compounds separated initially on a column (1·15 × 135 cm) of Zeo-Karb 226 as described in the text, and subsequently purified by band chromatography.

Radioactivity determined by liquid scintillation counting.

[15] P. A. GIGUERE and D. CHIN, *Can. J. Chem.* 39, 1214 (1961).

Similarly choline-Me-^{14}C administered to sterile leaf discs and sterile excised roots of *C. vulvaria* was converted to betaine. The traces of radioactivity which appeared in trimethylamine isolated from these tissues never exceeded the amounts produced in control samples of choline-Me-^{14}C by spontaneous radiosensitive decay.

Metabolism of Trimethylamine-^{14}C by Cuttings of C. vulvaria

When labelled trimethylamine-^{14}C was fed to cuttings of *C. vulvaria* radioactivity was subsequently found in a compound which was identified as trimethylamine-N-oxide. The specific activity (Table 4) of the purified trimethylamine-N-oxide isolated indicated that there was little or none present in the plants under normal conditions.

TABLE 4. RADIOACTIVITY OF TRIMETHYLAMINE-N-OXIDE ISOLATED FROM CUTTINGS OF *C. vulvaria* AFTER THE ADMINISTRATION OF [^{14}C] TRIMETHYLAMINE

Duration of feeding (hr)	[^{14}C] trimethylamine-N-oxide isolated	
	(counts/min $\times 10^{-4}$)	(specific activity $\times 10^{-3}$)
24	1·72	6·83
48	3·19	6·49

Trimethylamine $0{\cdot}78 \times 10^3$ counts/min/μmole (2·17 counts/min total) fed in each case.

Trimethylamine-N-oxide isolated initially on a column of Zeo-Karb 226, as described in the text, followed by fractionation on a column of Dowex 50 (H^+ form, 200–400 mesh) with 1 N HCl as described by Friedman *et al.*[57]

Radioactivity determined by liquid scintillation counting. Trimethylamine and trimethylamine-N-oxide estimated by the I_2/KI method as described in the text.

Utilization of (^{14}C) *Labelled Compounds as Precursors for Trimethylamine Production in* C. vulvaria

Betaine-Me-^{14}C and methylamine-^{14}C did not give rise to any detectable radioactivity in trimethylamine when they were fed to cuttings of *C. vulvaria.* Sodium formate-^{14}C gave rise to slight traces of radioactivity in trimethylamine but this radioactivity was less than 0·006 per cent of the total radioactivity taken up by the plant.

Phosphoryl choline-Me-^{14}C was metabolized to choline and betaine but the traces of radioactivity found in the trimethylamine were due to the radiosensitive decomposition of the choline.

Feeding with L-methionine-Me-^{14}C gave rise to small amounts of radioactivity in trimethylamine in *C. vulvaria* but not in cuttings of *C. rigidum* and *C. botrys* L. (which do not normally produce trimethylamine). The distribution of radioactivity in compounds other than trimethylamine after feeding *C. vulvaria* with L-methionine-Me-^{14}C was investigated by chromatography of the extracts of the plants on columns of Dowex 50 W8. Figure 3 shows the elution curves of one such experiment. The unknown radioactive compound in Peak 1 yielded a spot with the following R_f values on paper chromatography: 0·03 (in 5% concentrated ammonia in 95% ethanol), and 0·20 (butan-1-ol–acetic acid–water (12/3/5, by vol). This spot gave an immediate violet colour when sprayed with Dragendorff's reagent, which faded after

24 hr. With the iodoplatinic acid reagent the spot became slowly bleached, indicating the possible presence of a sulphur containing amino-acid. The compound also gave a blue-grey colour with iodine, and a distinct dark blue spot with phosphomolybdic acid and stannous chloride. Peak 2 showed the same chromatographic behaviour and straining reactions as N-methylethanolamine, and Peak 3 was tentatively identified as N,N-dimethylethanolamine.

Peak 4 contained both radioactive choline and radioactive betaine. These two compounds were separated by column chromatography on Zeo Karb 226. In addition to traces of radioactive trimethylamine, peak 5 also contained another radioactive compound which gave no colour reactions with any of the location reagents used for the detection of the quaternary ammonium bases and tertiary amines.

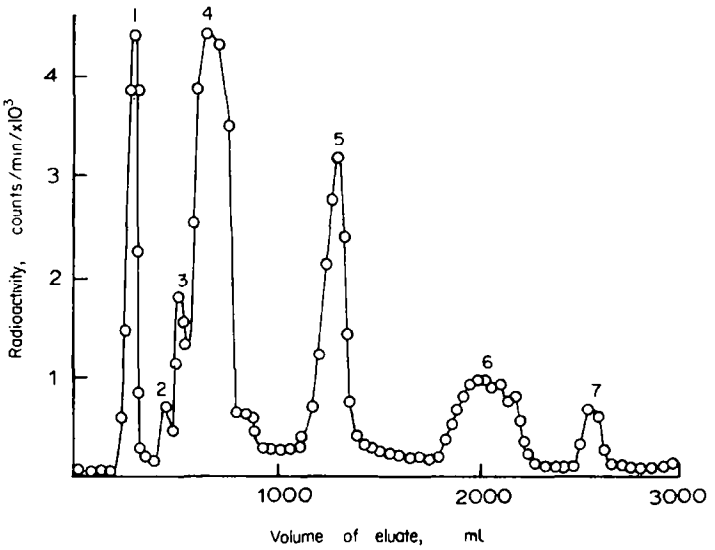

FIG. 3. SEPARATION OF BASES FROM *C. vulvaria* AFTER FEEDING WITH METHIONINE.

Chromatography on a column (1·1 cm × 100 cm) of Dowex 50 (W8) (H^+ form) of an extract of cuttings of *C. vulvaria* L. which had received 25 μc (0·24 mg) of L-methionine-Me-^{14}C for 48 hr. The column was equilibrated with N-hydrochloric acid and 2200 ml of the acid followed by 800 ml of 2·5 N acid was used for the elution. Other experimental details as described in the text. Peaks: 1, 5, 6, and 7 unknowns: peak 2, N-methylethanolamine; peak 3 N,N-dimethylethanolamine; peak 4, choline and betaine.

Peak 6 contained two radioactive compounds. The more heavily labelled compound had the following R_f values: 0·30 (5% ammonia in 95% ethanol), and 0·40 (butan-1-ol–acetic acid–water, 12:3:5). This spot gave a faint pink with Dragendorff's reagent, a brown colour with iodine, and a faint blue a few days after spraying with iodoplatinic acid. No colour reaction was given with the phosphomolybdic acid/stannous chloride reagent. The other more lightly labelled compound had the following R_f values in the same two solvent: 0·16 and 0·35 and gave positive reactions with all of the spray reagents used for the location of the quaternary ammonium compounds. The radioactive compound in peak 7 did not react with any of the spray reagents used.

DISCUSSION

It would appear from the results of the present investigation that trimethylamine is not normally formed by the breakdown of choline in the tissues of *Chenopodium vulvaria*. A

possible objection to the validity of this assumption is that the radioactive choline might have been metabolized to a compound other than trimethylamine before it reached a possibly localized site of production of trimethylamine. This objection might be pertinent if production of trimethylamine were restricted to the epidermal glands which have a higher concentration of trimethylamine than the leaf tissues.[9]

Furthermore, experiments indicated that choline-Me-^{14}C fed to plants was rapidly oxidized to betaine but some free radioactive choline always remained in the leaves. In addition feeding of sodium formate-^{14}C and methionine-Me-^{14}C gave rise to radioactive choline in the leaves of *C. vulvaria*, but little or no radioactivity was incorporated into trimethylamine isolated from the leaves. Feeding of choline-Me-^{14}C to cuttings of *C. vulvaria* which were maintained in an atmosphere of nitrogen led to a reduction in the rate of oxidation of choline to betaine with the result that the level of radioactive choline in the leaves remained high (Table 3), but even under these conditions trimethylamine isolated from the plant showed little radioactivity. Evidence from the present experiments (Table 3) and other workers[12,16–18] indicates that choline is rapidly oxidized to betaine in plant tissues. However, attempts to demonstrate the presence of enzyme systems catalysing this oxidation have so far been unsuccessful.[12,18] Radioactive trimethylamine-N-oxide was produced when trimethylamine-^{14}C was fed to cuttings of *C. vulvaria* despite the fact that trimethylamine-N-oxide does not appear to be present in detectable amounts in the tissues of this plant. This finding would account for the minute traces of radioactive trimethylamine-N-oxide observed after feeding with choline-Me-^{14}C, since traces of trimethylamine-^{14}C were unavoidably present in the choline administered, as a result of spontaneous radiosensitive decomposition.

Baker *et al.*[19] have recently observed that when Sorghum (*Sorghum vulgare* Pers.), alfalfa and wheat seedlings were fed with trimethylamine-^{14}C, 3 to 12 per cent of the radioactivity was subsequently found in a compound which was identified as trimethylamine-N-oxide. However these workers were unable to demonstrate the synthesis of trimethylamine-N-oxide *in vitro* by homogenates or acetone powders of eighteen different species of plants.

At present there is no indication as to whether the enzyme system responsible for the oxidation of trimethylamine is specific for trimethylamine or whether a single non-specific enzyme is responsible for the oxidation of a number of substrates. Aliphatic mono-amines are known to be slowly oxidized by partially purified enzyme preparations from the seedlings of peas (*Pisum sativum* L.[20,21]). Another possibility is that the feeding of trimethylamine to tissues of *C. vulvaria* which do not normally contain trimethylamine might bring about the *de novo* induction of an enzyme which was capable of oxidizing trimethylamine to trimethylamine-N-oxide. Although well known amongst micro-organisms, this type of enzyme induction and adaptation has only recently been recorded in higher plants.[22]

Feeding of betaine-Me-^{14}C to plants of *C. vulvaria* did not give rise to any radioactivity in trimethylamine isolated from the tissues. Betaine is particularly resistant to breakdown either by enzymes or by chemical means. Many previous workers have been unable to demonstrate a biological system which was capable of producing trimethylamine from

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[22] M. M. R. K. Afridi and E. J. Hewitt, *J. Exp. Botany* **15**, 251 (1964).

betaine.[5,9,23–25] Similarly the suggestion by Guggenheim[26,27] that methylamine could undergo successive methylations by formaldehyde to yield dimethylamine and trimethylamine was not supported by the results of the experiments in the present study in which methylamine-^{14}C was fed to cuttings of *C. vulvaria*. This theory is perhaps more attractive in the case of species such as *Heracleum sphondylium* L. and *Chaerophyllum aromaticum* L. in which methylamine, dimethylamine and trimethylamine have been recorded simultaneously.[28] Methylamine was not detected in *Chenopodium vulvaria* during the present study nor was it revealed in this species by gas chromatography.[29]

Radioactively labelled phosphoryl choline was metabolized *in vivo* by cutting of *Chenopodium vulvaria* with the production of labelled choline, betaine and traces of trimethylamine but the low level of radioactivity of the trimethylamine suggested that it had arisen from the radiosensitive decay of the parent molecule or of the "free" choline liberated either directly from the phosphoryl choline, or from a phospholipid formed from it. Prostatic and intestinal phosphatases are known in mammals which rapidly and quantitatively hydrolyse phosphoryl choline[30] but there is little evidence for the existence of similar enzymes in plant material. On the other hand, there is considerable evidence for the existence of phospholipases in the tissues of various plants which catalyse the hydrolysis of choline from lecithin.[31–33]

Feeding of sodium formate-^{14}C to plants of *C. vulvaria* resulted in the appearance of slight traces of radioactivity in trimethylamine isolated from the tissues but considerable labelling of both choline and betaine occurred. When the choline was later degraded chemically nearly all of the labelling was found to be incorporated into the N-methyl groups, thus confirming the findings of Kirkwood and Marion.[34] It is possible that formate contributes to the synthesis of the methyl group of methionine,[35] which then enters the choline molecule via S-adenosyl methionine,[36] or a one-carbon compound derived from formate may be directly responsible for the methylation of the ethanolamines thus leading to the formation of choline.

Feeding of methionine-Me-^{14}C to cuttings of *C. vulvaria* gave rise to small amounts of radioactivity in trimethylamine isolated after a period of 32 hr. There are considerable difficulties in interpreting the results as providing evidence for the conclusion that methionine directly participates in the synthesis of trimethylamine by the stepwise methylation of a precursor which contains nitrogen. The incorporation of radioactive labelling into trimethylamine was very low (0·073–0·130 per cent) and this perhaps suggests the existence of several intermediate compounds. The radioactive methyl groups of the methionine were also incorporated into both choline and betaine and into several unidentified compounds.

In the absence of any conclusive evidence for the identity of the immediate precursor of

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[27] M. GUGGENHEIM, In *Handbuch der Pflanzenphysiologie* VIII (Edited by W. RUHLAND). Springer-Verlag, Berlin (1958).
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trimethylamine in *C. vulvaria* some types of compound which are potential precursors of trimethylamine may be suggested.

The aliphatic and heterocyclic quaternary ammonium compounds which contain an "onium" pole are quite widely distributed in plants.[27,37] The presence of an onium pole in these compounds renders the molecule susceptible to nucleophilic attack which often leads to the production of trimethylamine. Although there is no conclusive evidence that a compound of this type exists in *C. vulvaria*, several unidentified radioactive compounds which reacted with the spray reagents used for the location of quaternary ammonium bases were isolated from extracts of the tissues after feeding with methionine-Me-^{14}C. Furthermore several alkaloids containing N-methyl groups have been recorded in other members of the Chenopodiaceae.[38–40] The only evidence that any of these compounds give rise to trimethylamine in biological material comes from research on the degradation of ergothioneine by the bacteria *Alcaligenes faecalis*[41,42] and *Eschericia coli*.[43]

EXPERIMENTAL

Materials and Methods

Seeds of *Chenopodium vulvaria* were collected from plants growing outdoors in the University Botanic Garden, Hull. Seeds of *C. rigidum* L. were obtained as a generous gift from Dr. B. G. Cumming of the Plant Research Institute, Ottawa, Canada. All seeds were dried and stored at 0–4° for at least two months prior to germination. The plants were grown in John Innes Compost (No. 2). Where possible the plants were kept under conditions of long day illumination in the greenhouse to delay the onset of flowering.

Extraction of trimethylamine. The method used in the early experiments was substantially as reported by Cromwell[9] except that the extract before vacuum distillation was made alkaline by the addition of 0·3 M phosphate buffer at pH 8, instead of excess of $Mg(OH)_2$. In the later experiments trimethylamine was isolated from extracts of leaves by the method of AYREY *et al.*[44]

Micro-estimation of trimethylamine and quaternary ammonium compounds. Trimethylamine and the quaternary ammonium compounds were estimated by an adaptation of the method described by Kushner[45] for the micro-estimation of choline. Samples, containing up to 50 μg of amine, were made up to 2 ml with 2 N HCl. One millitre of a reagent (10 g of resublimed iodine and 12·4 g of KI in 1 l. of water) was then added to the solution which was shaken and placed in an ice-bath for 20 min. Ten ml of 1:2-dichloroethane were then added and the two layers mixed by bubbling a fine stream of nitrogen through them for exactly 30 sec. The absorptivity of the organic layer was determined in a Unicam S.P. 600 at a wavelength of 365 mμ within 10 min, and compared with standard curves obtained previously for each of the amines.

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Methylamine was estimated spectrophotometrically with the 1-fluoro-2,4-dinitrobenzene reagent.[46]

Trimethylamine-N-oxide content of extracts was determined by preliminary reduction of the oxide with acidified titanous chloride[47] followed by estimate of the free amine by the periodide method described above.

Radioactively labelled compounds. Choline chloride-Me-^{14}C, methylamine hydrochloride-^{14}C, L-methionine-Me-^{14}C, sodium formate-^{14}C and paraformaldehyde-^{14}C were purchased from the Radiochemical Centre, Amersham, Bucks.

Trimethylamine(^{14}C) was synthesized from paraformaldehyde-^{14}C and ammonium chloride.[48] The product was purified by passage through a column (1·8 cm × 94 cm) of Dowex 50 W8 (H^+ form), eluting with N HCl. Collected fractions containing the peak of trimethylamine-^{14}C were combined evaporated to dryness *in vacuo* at 50°, and the compound isolated as the reineckate[18] (yield 47 per cent). The product was chromatographically homogeneous in five different solvent systems, and had m.p. 272–4° (authentic trimethylamine m.p. 271–5°). Infra-red spectra of the product were in close agreement with that obtained from authentic trimethylamine HCl.

Trimethylamine-N-oxide-^{14}C was prepared by treating trimethylamine-^{14}C with 4% hydrogen peroxide.[49] The product was purified and converted to the hydrochloride by passage through a column of Dowex-50 W-X8 (H^+ form, 200–400 mesh).[50] The purified product has a m.p. of 215–217° (authentic TMA N-oxide has m.p. 217–220°), and i.r. spectra of the product were in close agreement with that from authentic trimethylamine-N-oxide HCl and published data.

The method of Riley[51] was employed for the synthesis of phosphoryl choline-Me-^{14}C from choline-Me-^{14}C. The product, phosphoryl choline chloride (Me-^{14}C) Ca salt, was dissolved in a small volume of distilled water and recrystallized by the addition of three volumes of absolute ethanol (yield 39·6 per cent). The identity and purity of the product were checked by i.r. spectroscopy and paper chromatography.

Administration of radioactive compounds to plants. Solutions containing known amounts of the radioactive compounds (3–12 μc) in small volumes of sterile distilled water were administered to cuttings of *C. vulvaria* as described previously.[52] In some experiments the vessels containing the excised cuttings and the radioactive solutions were placed under a bell-jar through which a continuous stream of nitrogen was maintained during the experimental period.

Discs (1·8 cm in dia.) punched from leaves of *C. vulvaria* which were surface sterilized by immersion in a 1% (v/v) solution of Dettol for 5 min were vacuum infiltrated with sterile solutions of the labelled compounds in 0·01 M phosphate-citrate buffer pH 6·8 to which a few drops of 1% Mannoxol O.T. (British Drug Houses Ltd.) were added as a surfactant.

Sterile excised roots of *C. vulvaria* were grown in a nutrient medium[53] with the following modifications: 10^{-5} M EDTA-ferric complex was added instead of $Fe_2(SO_4)_3$, 40 g/l. of sucrose instead of 20 g/l., and each flask also received 5 μg of 3-indolylacetic acid. Roots from

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several flasks were transferred to a 100-ml flask which contained 10 ml of the nutrient solution and the (^{14}C) labelled compound.

It is known that choline chloride-Me-^{14}C is extremely sensitive to radiation decomposition by a free radical chain mechanism to trimethylamine and acetaldehyde.[13] Decomposition can only be arrested at $-80°$ and 150°[14] and therefore no attempt was made to purify this compound prior to feeding to plants of *C. vulvaria*. Samples of choline-chloride-Me-^{14}C were used as a control for radiation decomposition in every experiment.

Measurement of radioactivity. All determinations of radioactivity were carried out as described previously.[52]

Infra-red spectroscopy. All i.r. spectra were obtained with KBr pellets using a Perkin–Elmer "Infra-cord" spectrophotometer.

Preparation of extracts for measurement of radioactivity in trimethylamine and quaternary ammonium compounds. The plant material was ground with a small volume of 0·1 N HCl. After filtration the residue was reground in 90% ethanol (acidified with a few drops of 1 N HCl), and then extracted for 2 hr (Soxhlet) with the ethanolic solution. The two extracts were combined and applied to a column (1·7 cm × 20 cm) of Dowex resin 50 W × 8 (H^+ form, 200–400 mesh), and the amines and quaternary ammonium compounds eluted with 2·5 N HCl, and evaporated to dryness *in vacuo* at 50°. In addition, precipitation by ammonium reineckate,[18] iodine-potassium iodide,[54] and sodium tetraphenylboron[55] were also employed for the isolation of these compounds from plant extracts.

Ion-exchange column chromatography of quaternary ammonium compounds. Extracts containing radioactively labelled betaine, dimethyl glycine, choline and trimethylamine were fractionated on Zeo-Karb 226 by a modification of the method described by Blau.[56] (Zeo-Karb 226 (H^+ form, 100 mesh beads) was kindly supplied in the graded form by Dr. K. Blau). The resin was regenerated and buffered at pH 7·3. The compounds were dissolved in a phosphate-citrate buffer pH 5·3 for application to the columns and eluted with the same buffer. Two millilitres of 2 N HCl were added to each fraction collected to prevent possible losses of trimethylamine due to volatility at pH 7·3. Fractions of the eluate containing an amine peak were combined and evaporated to dryness *in vacuo* at 50°. The residues were either desalted by means of one of the precipitation procedures previously described or by passage through a column (2·8 cm × 21 cm) of Zeo-Karb 226 in the free acid form.[56]

Extracts containing betaine and trimethylamine-N-oxide which were not resolved by the Zeo-Karb 226 resin were fractionated using Dowex resin 50 (H^+ form, 8 per cent cross-linkage, 200–400 mesh) and 1 N HCl as the eluent.[57]

Extracts containing phosphoryl choline, betaine, choline and trimethylamine were fractionated on columns (0·9 × 59 cm) of Aminex-MS (fraction C, Bio-Rad Laboratories, Berkeley, California).[54] The eluting buffers were changed after 350 ml had passed through the column.

Paper Electrophoresis

The method used was a slight modification of that reported by Brockhuysen *et al.*[58] and the pH of the 0·3 M acetic acid/pyridine buffer was 4·00. A voltage of 10 V/cm was applied

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for 90 min. All electrophoretograms were dried for 24 hr in a current of circulating air at room temperature to remove all traces of pyridine which would otherwise interfere with the location of the quaternary ammonium bases. The relative mobilities of the quaternary ammonium compounds in this system are given in Table 2.

Ion-exchange resin paper chromatography. Chromatography of the quaternary ammonium bases was carried out on Amberlite SA-2 ion exchange paper (British Drug Houses Ltd.) using 1 N HCl as the developing solvent. Chromatography was also carried out on Amberlite WA-2 ion exchange paper (British Drug Houses Ltd.) impregnated with 0·2 M phosphate–citrate buffer pH 7·3, and the papers developed with the same buffer at pH 5·2. The R_f values of the quaternary ammonium compounds in these two systems are given in Table 2.

Paper chromatography The following solvent systems (all single phase) were used, and Whatman No. 1 paper employed, unless otherwise stated: butan-1-ol-conc. HCl-water (7:2:1, by vol);[56] butan-1-ol–acetic acid–water (4:1:2, by vol); butan-1-ol–formic acid–water (77:10:13, by vol);[50] 2 methyl-propan-1-ol–formic acid–water (6:1:1, by vol.); butan-1-ol–ethanol–ammonia (sp. gr. 0·88) (8:1:3, by vol); butan-1-ol–acetic acid–water–pyridine (15:3:12:10, by vol);[56] pyridine–pentan-1-ol–water (3:2:2, by vol);[56] 95 vol of 95% (v/v) ethanol and 5 vol of ammonia (sp. gr. 0·88);[16] butan-1-ol–acetic acid–water (5:4:1, by vol); butan-1-ol–ethanol–acetic acid–water (8:2:3:1, by vol);[7] pyridine–butan-2-ol–water (6:3:1, by vol).[59]

The reagents used for the detection of the spots of the quaternary ammonium compounds were as follows: 0·2% iodine in light petrol (b.p. below 40°); phosphomolybdic acid-stannous chloride;[60] Dragendorff's reagent;[60] 0·2% dipicrylamine in 50% (v/v) acetone–water;[60] potassium iodoplatinate;[61] potassium ferrocyanide-cobalt chloride;[60] and tropaeolin 00.[60]

Degradation of (^{14}C) *choline reineckate.* In order to determine the position of (^{14}C) labelling in the molecule, samples of radioactively labelled choline isolated from extracts of tissues which had been fed with various (^{14}C) compounds were degraded to trimethylamine.[62]

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