The Metabolism of Fluoroacetate in Lettuce

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1. Whole lettuce plants were incubated with (1) [1-¹⁴C]acetate, (2) fluoroacetate followed by [1-¹⁴C]acetate, (3) fluoro[1-¹⁴C]acetate, (4) fluoro[2-¹⁴C]acetate or (5) *S*-carboxy-[¹⁴C]methylglutathione. 2. Fluoroacetate did not affect the expiration of ¹⁴CO₂ from [1-¹⁴C]acetate and only a small amount of ¹⁴CO₂ was produced from either fluoro[1-¹⁴C]acetate or fluoro[2-¹⁴C]acetate in 43 h. 3. Fluoroacetate at 50 mg/kg wet wt. doubled the plant citrate concentration after 43 h incubation, and depending on the age and size of the plant 50-100% of the compound was metabolized. 4. With both fluoro[1-¹⁴C]acetate and fluoro[2-¹⁴C]acetate all the radioactivity except that in the CO₂ was found in the water-soluble acid fraction. About 2% was in fluorocitrate and the remainder, apart from unchanged fluoroacetate, was in a number of compounds devoid of fluorine but containing nitrogen and sulphur. These were peptide-like and could be separated by chromatography on an amino acid analyser. 5. Identical compounds were obtained from the spontaneous reaction between iodo[2-¹⁴C]acetate and glutathione, the major product being *S*-carboxy-methylglutathione. 6. *S*-Carboxymethylcysteine was also isolated and its mass spectrum compared with a commercial sample. 7. Reaction rates of all the monohaloacetates with glutathione were studied at pH 7 at 25°C. No reaction was observed with fluoroacetate. 8. The metabolism of fluoroacetate by lettuce is discussed in relation to that of aliphatic and aromatic halogen compounds, including fluoroacetate, by mammalian liver and to the metabolism of fluoroacetate by different plants reported by other workers.

Organic fluorine compounds are much more widely distributed in living organisms than was once thought. They have recently been isolated from such common plants as soya bean, lucerne and tea (Cheng *et al.*, 1968; Lovelace *et al.*, 1968; Hall, 1969) as well as from rarer ones such as species of *Dichapetalum*, *Acacia*, *Palicourea* and *Gastrolobium* (Marais, 1944; Murray *et al.*, 1961; Oelrichs & McEwan, 1961; de Oliveira, 1963; Aplin, 1967). The isolation of organic fluorine compounds from animals has been less common than from plants, since such compounds are toxic to animals because they are broken down *in vivo* to fluoroacetate. This undergoes 'lethal synthesis' in mitochondria to fluorocitrate, which inhibits aconitate hydratase (EC 4.2.1.3) and blocks the tricarboxylic acid cycle (Buffa *et al.*, 1951).

Fluorine, presumably as inorganic fluoride, is probably an essential trace element in animals, but very little is known about how it is transported to bones and teeth, and the possibility should not be overlooked that organic fluorine compounds are involved. The metabolic concentrations of such fluorocarbon derivatives would be far below those that block the tricarboxylic acid cycle even in the most sensitive species of animal. This hypothesis is supported by the measurement of a non-diffusible fluoro compound in normal human blood serum by Taves

(1968), and the discovery of a similar compound in the urine of workers engaged in the manufacture of hydrofluoric acid (Cernik *et al.*, 1970). The possibility that a fluorocarbon compound may be involved in the utilization of fluorine led us to examine the metabolism of fluoroacetate, one of the simplest of such compounds, at a concentration insufficient to block the tricarboxylic acid cycle. To isolate sufficient of the metabolites for identification we started with a plant system because of its relative insensitivity to fluoroacetate poisoning, which would allow the use of a relatively higher concentration of fluoroacetate. The lettuce was chosen for no other reason than its convenient size and its availability throughout the year from a market garden close to Babraham.

Materials and Methods

Fluoroacetate

Pure sodium fluoroacetate was obtained by neutralizing with NaOH fluoroacetic acid prepared from compound 1080 (Monsanto Chemical Co., St. Louis, Mo., U.S.A.) by the method of Pattison (1959). The purity of the compound was checked by g.l.c. Sodium fluoro[1-¹⁴C]acetate and sodium fluoro[2-¹⁴C]acetate were synthesized and purified by the method of Ward & Huskisson (1966).

Fig. 1. Apparatus for the incubation of lettuce with either [^{14}C]acetate, or fluoroacetate and [^{14}C]acetate, or fluoro[^{14}C]acetate

The lettuce, after injection of metabolites as detailed in the text, was placed in the 20cm-diam. desiccator with its roots immersed in 300ml of water. Air from a cylinder was bubbled through the water. The effluent gas was first dried in a CaCl_2 tube, then bubbled via a sintered-glass dispersion disc through a tube containing 21 ml of 2-methoxyethanol-ethanolamine (2:1, v/v) followed by another tube containing 6ml of the same mixture. The gas escaped through a soap-bubble flow-meter used to set the air flow to approx. 50ml/min. The apparatus was illuminated by four white fluorescent tubes (61 cm; 20 W) placed 30cm above it.

Plant material

Experimental lettuces (*Lactuca sativa* L.) were all of the cabbage type but were of many varieties and at different stages of growth. They had been grown either in the open or under glass in both winter and summer. Some 50 lettuces were examined and all produced the same end-products of fluoroacetate metabolism.

Lettuce incubations

Each lettuce (50–200 g) was carefully dug from the ground and taken to the laboratory with its roots immersed in water. Within 30min the plant was washed free from soil, and 0.1 ml of a sterile aqueous solution of the compound under study was injected into the stem at the base of the leaves with a sterile Hamilton syringe. The injection point was sealed by 1 drop of DePeX mounting medium (G. T. Gurr Ltd., London S.W.6, U.K.) and the lettuce placed in a desiccator (20cm diam.) with its roots immersed in 300ml of water (Fig. 1). Air from a cylinder was passed via a Dreschel head in the top of the desiccator and bubbled through the water. It then flowed, together with any volatile metabolic products, through a CaCl_2 drying tower and into a tube for the collection of CO_2 . This tube contained 21 ml of 2-

methoxyethanol-ethanolamine (2:1, v/v) and the gas entering was dispersed by a sintered-glass disc. The effluent passed into a second tube, similar to but smaller than the first, containing 6ml of 2-methoxyethanol-ethanolamine (2:1, v/v). Finally, the gas passed through a soap-bubble flow-meter, which was used in adjusting the air entering the system to 40–50ml/min. The lettuce was illuminated throughout the experiment by four white fluorescent tubes (61 cm; 20 W) placed 30cm above it. This system was used for four different incubation procedures as follows: (1) 43 h incubation with 100 μCi of sodium [$1\text{-}^{14}\text{C}$]acetate (1 mg/kg wet wt. of lettuce); (2) 24 h incubation with sodium fluoroacetate (50 mg/kg wet wt. of lettuce) followed by 43 h incubation with 100 μCi of sodium [$1\text{-}^{14}\text{C}$]acetate (1 mg/kg wet wt. of lettuce); (3) 43 h incubation with 100 μCi of sodium fluoro[$1\text{-}^{14}\text{C}$]acetate (50 mg/kg wet wt. of lettuce); (4) 43 h incubation with 100 μCi of sodium fluoro[$2\text{-}^{14}\text{C}$]acetate (50 mg/kg wet wt. of lettuce).

Another series of incubations were conducted in which the CO_2 -collecting tubes were replaced by a 4.3-litre ion chamber and a vibrating-reed electrometer (Cary model 401; Cary Instruments, Monrovia, Calif., U.S.A.). In these experiments the air flow was increased to approx. 1 litre/min. This system was used for the following incubations: (1) 43 h incubation with 100 μCi of sodium [$1\text{-}^{14}\text{C}$]acetate (1 mg/kg

wet wt. of lettuce); (2) 43 h incubation with 100 μ Ci of sodium [14 C]acetate (50 mg/kg wet wt. of lettuce); (3) 24 h incubation with sodium fluoroacetate (50 mg/kg wet wt. of lettuce) followed by 43 h incubation with 100 μ Ci of sodium acetate (50 mg/kg wet wt. of lettuce); (4) 43 h incubation with 2.5 μ Ci of *S*-carboxy[14 C]methylglutathione (40 mg/kg wet wt. of lettuce).

Extraction of radioactive metabolites from fluoro- 14 C]acetate-treated lettuces

After incubation the lettuce was homogenized in a precooled Waring Blendor for 1 min with 400 ml of ethanol at 0°C. The mixture was boiled for 10 min and filtered through a sintered-glass funnel (pore size 100–120 μ m). The residue was further extracted by boiling for 10 min and filtering successively with 400 ml of ethanol–water (1:4, v/v), 400 ml of water and 400 ml of ethanol–water (4:1, v/v). The combined filtrates were evaporated to dryness at reduced pressure in a rotary evaporator. The residue was dissolved in 300 ml of water and extracted three times with an equal volume of ether and once with an equal volume of chloroform. Low-speed centrifugation was necessary to separate the phases. The organic phases were discarded and the aqueous phase was concentrated to about 50 ml. The opaque liquid was cleared by centrifugation at 38000g (r_{av} , 7 cm) for 30 min at 2°C. It was transferred to the top of a column [90 cm $\times$ 2.5 cm diam.; void vol. (V_0) 110 ml] of Amberlite IR-120(H) (H^+ form) and eluted with water at 150 ml/h until 500 ml had been collected in 10 ml fractions. Elution was continued with aq. NH_3 (sp.gr. 0.88)–water (1:9, v/v) until the effluent from the column was alkaline (about 650 ml), then 500 ml more was collected.

Extraction of radioactive metabolites from the eluate from the Amberlite IR-120(H) column

The first 500 ml of eluate was made slightly alkaline with aq. NH_3 solution, concentrated to 10 ml in a rotary evaporator and centrifuged at 38000g (r_{av} , 7 cm) for 30 min at 2°C to remove insoluble matter. The solution was acidified with dilute H_2SO_4 and extracted continuously with 50 ml of diethyl ether for 8 h. The ethereal solution was dried over Na_2SO_4 and evaporated to 5 ml, with care being taken to avoid loss of volatile compounds. This was achieved by using a 100 ml flask fitted with a 22 cm air condenser in the reflux position. The ethereal solution in the flask was immersed in water and the temperature raised until ether vapour just escaped from the condenser. The pale-yellow solution was saturated with diazomethane before being subjected to g.l.c.

The remaining eluate was made slightly alkaline

with aq. NH_3 solution and evaporated to dryness at 40°C in a rotary evaporator. It was transferred, dissolved in 3 ml of water, to the top of a column (95 cm $\times$ 2.5 cm diam.; V_0 130 ml) of Sephadex G-25 (fine grade). The column was eluted with water and the effluent collected in 10 ml fractions until all the radioactivity had emerged. The fractions containing the two partially resolved radioactive peaks were pooled together and evaporated to dryness at 40°C. Purification of the radioactive components was continued by chromatography on a smaller column (75 cm $\times$ 1 cm diam.; V_0 15 ml) of Amberlite IR-120(H) (H^+ form). The amber-coloured gum was dissolved in the minimum volume of water (1–2 ml), loaded on to the column and eluted in 10 ml fractions with water. Tubes in which the radioactive peaks emerged were pooled. The solutions were made slightly alkaline with aq. NH_3 solution and evaporated to dryness in a rotary evaporator. Final purification was effected by paper chromatography. Each radioactive metabolite was applied in aqueous solution as a band near the shorter edge of a piece of Whatman no. 1 paper (53 cm $\times$ 46 cm). Downward solvent development at 2°C was used with methanol–100% formic acid–water (56:9:5, by vol.). The radioactive bands were detected by radioautography, cut out and eluted with water. The concentrated eluates were then re-chromatographed in the same way with methanol–aq. NH_3 (sp.gr. 0.88)–water (21:3:11, by vol.). The radioactive bands were detected, eluted with water and concentrated to colourless gums.

Radioactivity counting technique

The radioactivity of ^{14}C was measured with a Panax GX9 water-cooled liquid-scintillation counter; the counting medium depended on the material of which the radioactivity was to be counted. For non-aqueous solutions and the collection of components from the gas chromatograph (see below), toluene containing 2,5-diphenyloxazole (0.4%) and 1,4-bis-(5-phenyloxazol-2-yl)benzene (0.01%) was used. For aqueous solutions the toluene was replaced by toluene–ethanol (7:3, v/v) (Hall & Cocking, 1965). For the measurement of $^{14}CO_2$ the gas was first absorbed in 2-methoxyethanol–ethanolamine (2:1, v/v), then 1 ml of this solution was added to 5 ml of toluene–2-methoxyethanol (2:1, v/v) containing 2,5-diphenyloxazole (0.55%) (Jeffay & Alvarez, 1961).

G.l.c.

A modified Pye Panchromatograph was fitted with a flame ionization detector for analytical work and with an argon ionization detector for preparative work and collection for radioactivity measurements. Methyl fluoroacetate was measured as described by

Ward & Huskisson (1966). For comparative measurements of all the metabolites that could be subjected to g.l.c., a column (152cm×0.5cm diam.) of 10% (w/w) polyethylene glycol adipate was used. It was maintained at 50°C for 12min, then temperature-programmed at 12°C/min to 180°C. For measurements of citrate and fluorocitrate a column (152cm×0.5cm diam.) of 15% (w/w) polydiethylene glycol succinate at 180°C was used. The stationary-phase support employed throughout was Gas-Chrom Z (80–100 mesh) (Applied Science Laboratories Inc., State College, Pa., U.S.A.). Methyl esters were prepared by bubbling diazomethane gas through a solution of the acids in diethyl ether until it was permanently yellow. For mixtures that were yellow before methylation a moistened universal indicator paper reddened with HCl vapour was held above the methylation tube. An excess of diazomethane gas indicating saturation of the solution could be detected by the colour change of the paper to yellow. The gas generator used was that of Roper & Ma (1957) except that Diazald (Ralph N. Emmanuel Ltd., Wembley, Middx., U.K.) and 2ml of methanol replaced the *N*-methyl-*N*-nitrosourea. Two methods were used for collection of components. For the more-volatile components the heated outlet from the chromatograph was connected to a glass column (35cm×1cm diam.) fitted with a coarse sintered-glass disc at its lower end containing 7ml of diethyl ether or counting medium. For less-volatile components an electrostatic precipitator was used (Scott *et al.*, 1964). Argon carrier gas was essential for the efficient functioning of this precipitator.

Amino acids and peptides

These were measured and separated by using the method and apparatus described by Moore *et al.* (1958) and Spackman *et al.* (1958). The column (91cm×1cm diam.) was packed with 8%-cross-linked sulphonated polystyrene spherical beads (20–25µm diam.). The column temperature was 30°C and a buffer gradient from pH2.5 to 3.25 was used. For analysis of radioactive compounds a scintillation flow counter was incorporated into the effluent from the bottom of the column. This consisted of a 5cm-diam. close-wound spiral of glass tubing (3mm bore, 4mm outside diam.) packed with anthracene crystals (30–60 B.S. mesh). The spiral was mounted against the face of an E.M.I. 6097B photonmultiplier connected to a suitable ratemeter and pen recorder. An efficiency of 15% for the counting of ¹⁴C radioactivity was attained.

Citrate determinations

The fresh lettuce plant was homogenized for 1 min in a precooled Waring Blender with 400ml of water

at 0°C and the mixture was freeze-dried. Then 0.5 g of dried plant was mixed with 1g of diethyl ether-extracted Gooch-crucible asbestos and made into a stiff paste with 2ml of 2.5M-H₂SO₄. A layer (2cm deep) of 5mm-diam. glass beads was poured into a 60ml downward-displacement liquid/liquid extraction tube (Quickfit type EX10/23) and covered with a layer of ether-extracted muslin. The plant paste was placed on the muslin and covered by a similar layer of muslin. The rest of the tube was filled with glass beads. The paste was extracted with diethyl ether for 16h. The ether extract was evaporated to 5ml, transferred to a stoppered tube and shaken with an equal volume of water for 1 min. The tube was cooled until the water layer was frozen, then the ether layer was poured off and discarded. After thawing, the water layer was extracted twice more with ether in the same way, before evaporation to dryness. The extract was now free from fatty acids. About 5ml of methanol–diethyl ether (1:9, v/v) was added to the tube and the contents were saturated with diazomethane gas. After a few minutes, to ensure complete methylation, excess of diazomethane was boiled off and the solution (3–5ml) quantitatively transferred to a tube containing an accurately weighed amount (about 7mg) of methyl stearate. About 3–5µl was subjected to g.l.c., with a flame ionization detector, and the responses of the citrate and stearate peaks were measured.

If the chromatograph detector gives a linear

Fig. 2. Relative response of argon ionization and flame ionization g.l.c. detectors for trimethyl citrate and methyl stearate

The responses were obtained by injecting 3–5µl of solutions containing weighed amounts of both citrate and stearate in diethyl ether. The weight of citrate injected did not exceed 10µg and the weight of stearate injected did not exceed 3µg. o, Argon ionization detector; Δ, flame ionization detector.

response over the range used for stearate and citrate, then:

$$\text{Wt. of citrate injected} = K \left(\frac{\text{citrate response}}{\text{stearate response}} \right) (\text{wt. of stearate injected}) \quad (1)$$

where K is a constant depending on the relative response values for the esters. Fig. 2 shows that a linear response is obtained over a wide range for the flame ionization detector but not for the argon detector. Since:

$$\text{Wt. of any ester injected} = \frac{(\text{wt. of ester in solution})(\text{volume injected})}{(\text{volume of solution})} \quad (2)$$

then substituting in eqn. (1) for the weights of citrate and stearate injected and simplifying gives eqn. (3):

$$\text{Wt. of citrate in solution} = K \left(\frac{\text{citrate response}}{\text{stearate response}} \right) (\text{wt. of stearate in solution}) \quad (3)$$

This shows that the weight of citrate may be calculated without knowledge of the total volume of the solution or the volume injected. This greatly decreases errors in the values obtained for citrate, since weights may be measured more accurately than volumes under these circumstances. The value of K may be calculated from the responses obtained with a solution containing weighed amounts of both citrate and stearate. Little difference in accuracy was obtained whether detector response was measured as peak height or as area calculated by an electronic integrator.

Measurement of reaction rates of monohaloacetates with glutathione

A conical flask containing 25 ml of freshly prepared aqueous glutathione (0.1 M) was continuously stirred and maintained at 25°C in a water bath. The pH of the solution was measured with suitable electrodes and adjusted to pH 7 with 0.1 M-NaOH from a burette. A solution of 25 ml of previously neutralized 0.1 M-iodoacetic acid was added to the flask and the timing of the reaction started. The pH of the solution was constantly maintained within the range 6.8–7.2 by the addition of 0.1 M-NaOH from the burette, the time of each addition being noted. A graph was plotted of time against volume of NaOH added. Similar reactions were conducted with bromoacetic acid, chloroacetic acid and fluoroacetic acid in place of iodoacetic acid.

Synthesis of S-carboxy[¹⁴C]methylglutathione

The method used was identical with that for the measurement of the reaction rate between iodoacetate

and glutathione. The reaction mixture was composed of 25 ml of iodo[2-¹⁴C]acetate (0.1 M; 50 μCi)

and 25 ml of 0.1 M-glutathione. The solution was evaporated to dryness at the end of the reaction. The product was largely freed from NaI by elution with water from a column (75 cm × 1 cm diam.; V_0 15 ml) of Amberlite IR-120(H) (H^+ form). The eluate was

further purified by chromatography on a column (65 cm × 1 cm diam.; V_0 12 ml) of 8%-cross-linked

sulphonated polystyrene (60–70 μm spherical beads) with volatile eluting buffers with a gradient from pH 2.5 to 3.25. The buffers were prepared from freshly distilled components as follows: 87 ml of acetic acid plus 22 ml of 100% formic acid were dissolved in 1 litre of water and adjusted with pyridine to pH 2.50 and pH 3.25. The column was loaded with the compound dissolved in the acid mixture with no pyridine added (pH approx. 1.8).

Fluorine analyses

The method of Hall (1968) was employed.

Chemicals

The radioactive chemicals, except fluoro[¹⁴C]-acetate, were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. S-Carboxymethylcysteine was obtained from BDH Chemicals Ltd., Poole, Dorset, U.K.

Mass spectroscopy

The derivatives were analysed dissolved in dichloromethane on an AEI model MS902 mass spectrometer.

Results and Discussion

Plant incubations

Lettuces were incubated as described in the Materials and Methods section. In all experiments the radioactivity was rapidly translocated throughout

Table 1. Radioactivity escaping from lettuces incubated with either [^{14}C]acetate, or fluoroacetate and [^{14}C]acetate, or fluoro[^{14}C]acetate

In the desiccator in Fig. 1 air was bubbled through 300 ml of water in which the roots of the plant were suspended. The effluent gas was first dried with CaCl_2 , then CO_2 was absorbed in tubes containing 2-methoxyethanol-ethanolamine (2:1, v/v). The air flow was maintained at 50 ml/min.

Incubations	Plant wet wt. (g)	Plant dry wt. (g)	Radioactivity in incubation water after 1 h (μCi)	Radioactivity in incubation water after incubation (μCi)	Radioactivity in expired CO_2 (μCi)
For 43 h with 100 μCi of [^{14}C]acetate (1 mg/kg wet wt.)	135	10.24	3.1	0.1	10.6
	63	4.01	8.2	1.2	12.4
	105	5.40	9.4	0.5	19.4
For 24 h with fluoroacetate (50 mg/kg wet wt.), then 43 h with 100 μCi of [^{14}C]acetate (1 mg/kg wet wt.)	62	4.38	9.5	3.8	17.7
	51	3.63	8.3	1.7	15.4
For 43 h with 100 μCi of fluoro-[^{14}C]acetate (50 mg/kg wet wt.)	69	5.05	2.2	0.6	0.4
	47	3.27	5.1	2.1	0.3
	83	5.70	4.1	3.7	0.8

the plant. Some radioactivity was found in the incubation water shortly after injection, but much of this was taken up through the roots during the incubation. In the incubations in which expired CO_2 was absorbed in 2-methoxyethanol-ethanolamine, it was efficiently absorbed in the first collecting tube. The second never contained more than 0.001 % of the absorbed radioactive CO_2 . Experiments with [^{14}C]acetate alone were conducted to ensure that the conditions were suitable for metabolic studies. In these plants radioactivity was found in different classes of compounds, notably long-chain fatty acids (Stumpf & James, 1963), organic acids and amino acids; the conditions were therefore considered suitable for the study of the metabolism of fluoroacetate. Similar results were obtained with lettuces treated first with non-radioactive fluoroacetate then with [^{14}C]acetate. Plants varied considerably both in size and in stem/leaf ratio; nevertheless the radioactivity in the expired CO_2 (Table 1) was much the same for [^{14}C]acetate-treated lettuces whether or not they were pretreated with non-radioactive fluoroacetate. In the incubations in which expired $^{14}\text{CO}_2$ from [^{14}C]acetate was continuously monitored by a Cary ion chamber, radioactivity appeared within seconds of injection. Similar results were obtained for both the rate and quantity of $^{14}\text{CO}_2$ expired whether the plants were incubated with 100 μCi of [^{14}C]acetate at 1 mg/kg or 50 mg/kg or whether they were preincubated with fluoroacetate at 50 mg/kg before incubation with 100 μCi of [^{14}C]acetate at 50 mg/kg (Fig. 3). It was concluded that nearly all the acetate that had been directly converted into acetyl-CoA and had entered the tricarboxylic acid cycle had been oxidized to CO_2 and that fluoroacetate at 50 mg/kg did not affect the

ability of the tricarboxylic acid cycle to deal with the quantities given. In lettuces treated with [^{14}C]fluoroacetate only a little radioactivity appeared in the CO_2 after 43 h (Table 1). A small amount of $^{14}\text{CO}_2$ was also expired when the metabolite of fluoroacetate in lettuce, *S*-carboxy[^{14}C]methylglutathione, was similarly incubated, and it is likely that this is the pathway by which fluoroacetate is metabolized to CO_2 in lettuce.

Citrate concentrations

Citrate concentrations in five plants treated with [^{14}C]acetate were $13.9 \pm 2.6 \mu\text{mol/g}$ dry wt. (mean $\pm$ S.E.M.) and in five plants treated with fluoro[^{14}C]acetate were $30.2 \pm 5.4 \mu\text{mol/g}$ dry wt. Two plants pretreated with fluoroacetate before receiving [^{14}C]acetate contained 26.2 and $29.6 \mu\text{mol}$ of citrate/g dry wt. and were similar to those treated with fluoro[^{14}C]acetate. It therefore appears that fluoroacetate given to the lettuce at 50 mg/kg wet wt. approximately doubles the citrate concentration in the plant. By comparison, in a fluoroacetate-poisoned rat the citrate concentration in the kidney may increase more than 50-fold (Buffa & Peters, 1949). Most of the plant citrate is stored in the vacuole, away from the mitochondrial sites of the tricarboxylic acid-cycle enzymes, so it is likely that at these mitochondrial sites the concentration of citrate may be very much greater. Even though this is evidence for the inhibition of the tricarboxylic acid cycle the degree of this inhibition is insufficient to affect the ability of the cycle to deal with the quantities of acetate given, since the expiration of CO_2 derived from acetate by the plant remains similar in fluoroacetate-treated and untreated plants.

Fig. 3. Rate of expiration of $^{14}\text{CO}_2$ from lettuce plants incubated after injection with $[1\text{-}^{14}\text{C}]\text{acetate}$

Each lettuce plant (wet wt. 130–160g) was incubated with the metabolite in a 20cm-diam. desiccator with its roots immersed in 300ml of water. Air was passed through the desiccator at approx. 1 litre/min, then through a CaCl_2 drying tube. It finally flowed through a 4.3-litre Cary ion chamber connected to a vibrating-reed electrometer. ○, Incubation after injection of $100\mu\text{Ci}$ of sodium $[1\text{-}^{14}\text{C}]\text{acetate}$ at 1mg/kg wet wt. of lettuce; □, incubation after injection of $100\mu\text{Ci}$ of sodium $[1\text{-}^{14}\text{C}]\text{acetate}$ at 50mg/kg wet wt. of lettuce; ●, incubation after pre-injection of 50mg of sodium fluoroacetate/kg for 24h before injection of $100\mu\text{Ci}$ of sodium $[1\text{-}^{14}\text{C}]\text{acetate}$ at 50mg/kg wet wt. of lettuce.

Location and properties of radioactive metabolites in either fluoro $[1\text{-}^{14}\text{C}]\text{acetate}$ - or fluoro $[2\text{-}^{14}\text{C}]\text{acetate}$ -treated plants

No difference was observed between the metabolism of fluoro $[1\text{-}^{14}\text{C}]\text{acetate}$ and fluoro $[2\text{-}^{14}\text{C}]\text{acetate}$. All the radioactivity other than that in the incubation

water or expired as CO_2 was found within the plant and could be completely extracted with the ethanol–water mixtures. After the ethanol–water extract had been evaporated and dissolved in water, very little radioactivity could be extracted into diethyl ether (approx. 0.06% of the initial radioactivity) or chloroform (approx. 0.08% of the initial radioactivity).

Preliminary experiments on the crude extract in aqueous solution showed that the radioactivity could be eluted by water from a cationic-exchange column but was completely adsorbed on an anionic-exchange column, indicating that it was associated with acidic material. When the solution of the crude extract was acidified with H_2SO_4 and extracted with ether for 8h, up to 50% of the radioactivity was found in the diethyl ether. Fluorine analysis revealed that, within the limits of the method, all the fluorine injected as fluoroacetate was also found in the ether. The indigenous fluoride in the plants was approx. $5\mu\text{g/g}$ dry wt. and contributed only about 3% to the total. Approximately one-half the fluorine was organically bound, as measured by subtracting the value for acid-labile fluoride before ashing from the value for acid-labile fluoride after ashing. The remainder was inorganic. Separate extractions of NaF alone established that it passes into ether under the conditions used on the plant extract. It appeared from these analyses that considerable scission of the C–F bond had occurred during the incubation.

The radioactivity in the diethyl ether extract after acidification was found by g.l.c. (Ward & Huskisson, 1966) to be largely associated with unchanged fluoroacetate. With higher temperatures and different columns (Table 2) a small amount of radioactivity (about 2% of the total) was found in fluorocitrate. Together fluoroacetate and fluorocitrate accounted for all of the ether-extractable radioactivity, and the large amounts of malate and citrate present were non-radioactive, unlike those found in plants treated with $[1\text{-}^{14}\text{C}]\text{acetate}$. After the incubations there was a close correlation between the value for organically bound fluorine and that present in the extracted fluoroacetate and fluorocitrate.

A more careful examination of the behaviour of the radioactivity in the crude extract of fluoro $[1\text{-}^{14}\text{C}]\text{acetate}$ -treated plants on the first and larger Amberlite IR-120(H) cationic-exchange column referred to in the Materials and Methods section showed that in most plants about one-half was eluted rapidly by water but the remainder was partially adsorbed and did not start to emerge until later. This phenomenon was utilized as a crude fractionation procedure. Most of the plant pigments were eluted at the front with all the fluorine-containing compounds and some of the radioactivity and this allowed the remaining radioactive materials to be rapidly eluted by aq. NH_3 solution in a purer state. About 90% of the radioactivity emerging at the front could be extracted

Table 2. *G.l.c. retention volumes of methyl esters of organic acids*

Retention volumes are relative to that of methyl palmitate on glass columns (152 cm $\times$ 0.5 cm diam.) at 180°C. The stationary-phase support was Gas-Chrom Z (80–100 mesh). The retention volumes of the methyl esters of linoleate and linolenate are given because of their close proximity to those of citrate and fluorocitrate respectively on a polyethylene glycol adipate column.

Methyl ester	Retention volume on 10% (w/w)	Retention volume on 15% (w/w)
	polyethylene glycol adipate	polydiethylene glycol succinate
Succinate	0.19	0.42
Malate	0.62	1.28
Fluoromalate	0.85	1.80
Linoleate	2.69	2.40
Citrate	2.67	4.90
Linolenate	3.51	3.20
Fluorocitrate	3.63	6.64

into ether as above, and was found by g.l.c. to be associated with the fluorocitrate and unchanged fluoroacetate.

The radioactive material that was partially adsorbed on the second, smaller, Amberlite IR-120(H) column could not be extracted into ether under acidic conditions. It was very soluble in water but insoluble in chloroform, acetone and light petroleum (b.p. 40–60°C) as well as ether, and contained no fluorine. Lettuce treated with fluoro[1-¹⁴C]acetate and fluoro[2-¹⁴C]acetate gave similar results, suggesting that both of the carbon atoms but not the fluorine of fluoroacetate were contained in this fraction. It was freed from high-molecular-weight impurities on a Sephadex G-25 column eluted with water, and incomplete separation of the radioactivity into two components was effected (Fig. 4). The first component was partially excluded by the gel. This is sometimes found when an acid is introduced and eluted with water because of the presence of some carboxyl groups in Sephadex, but the second component emerged with the small molecules. The ratio of the size of these two components varied in different experiments, but the first to emerge was always the larger.

The combined radioactive components were now sufficiently pure to separate well on an Amberlite IR-120(H) column eluted with water (Fig. 5). Four peaks were obtained, designated R-1, R-2, R-3 and R-4. In addition, some radioactivity, approximately equivalent to that found in peak R-1, was retained on the column and could be eluted with aq. NH₃ solution. This proved to be component R-4 that was incompletely eluted from the Amberlite IR-120(H) column by water. The relative sizes of peaks R-2, R-3 and R-4 varied widely between individual plants, though peak R-3 was always the largest and peak R-1 accounted for about 5% of the radioactivity. Component R-1 appeared to be a breakdown product of

Fig. 4. Removal of high-molecular-weight impurities from radioactive metabolites of fluoro[¹⁴C]acetate in lettuce on a column (95 cm $\times$ 2.5 cm diam.; V_0 130 ml) of Sephadex G-25 eluted with water

The elution range of high-molecular-weight and low-molecular-weight compounds is illustrated by Blue Dextran and NaCl respectively.

components R-2 and R-3 on the column, for whenever the latter two were rechromatographed separately more of component R-1 was formed. Component R-4, on the other hand, could be rechromatographed without breakdown.

Components R-2, R-3 and R-4 when dry were pale-amber-coloured gums and were sufficiently pure to run without tailing on paper chromatograms. The two solvent systems were used alternately until products of constant specific radioactivity were obtained, two chromatograms in each solvent being generally

Fig. 5. Chromatography of radioactive metabolites of fluoro[^{14}C]acetate in lettuce on a column (75 cm $\times$ 1 cm diam.; V_0 15 ml) of Amberlite IR-120(H) ion-exchange resin eluted with water for 1200 ml, then with aq. NH_3 solution (sp.gr. 0.88)–water (1:9, v/v)

adequate. The relevant R_F values are given in Table 3.

The purified components contained nitrogen but no phosphorus. The i.r. spectra of components R-2 and R-3 showed peaks at 3700, 1715, 1660 and 1390 cm^{-1} typical of amino acids, with an additional peak at 1530 cm^{-1} characteristic of the secondary amide group of peptides. In the i.r. spectrum of component R-4 the peak at 1530 cm^{-1} was replaced by one at 1490 cm^{-1} , suggesting that the peptide bond was absent. All three components gave a purple colour with ninhydrin, and they were analysed by using a Moore & Stein (Moore *et al.*, 1958) amino acid analyser. The radioactive components, being acidic, were eluted very rapidly and closely with buffer gradients starting at pH 3.25. A wide separation was achieved by starting at a lower pH, and all the relevant compounds could be eluted between pH 2.5 and 3.25. Component R-2 was eluted as a double-humped peak about halfway between urea and hydroxyproline, component R-3 emerged just after hydroxyproline and component R-4 emerged almost identically with aspartate. Component R-1, when prepared by the breakdown of component R-2 or component R-3 on an Amberlite IR-120(H) column, was ninhydrin-negative and gave a radioactive peak close to the front on the Moore & Stein column. Fig. 6 illustrates the elution of components R-2, R-3 and R-4 and their hydrolysis products on the amino acid analyser. With these three components the products of hydrolysis varied with the conditions used. Under mild conditions and in the absence of air (1 h at 130°C in an evacuated sealed tube with 6M-HCl) components R-2 and R-3 behaved identically, producing radioactive component R-4 and non-radioactive glutamate and glycine in equimolar quantities. Component R-4 was unaffected by these conditions. Under stronger conditions and in an open tube further breakdown of all three components took place. The amount of breakdown was enhanced if formic acid was present and if refluxing was carried out in a sand bath with a water condenser rather than in a Kjeldahl rack with an air condenser. The most severe conditions used were 48 h refluxing on a sand bath with a water condenser in conc. HCl–100% formic acid (1:1, v/v), but no coloration due to pyrolysis was

Table 3. R_F values for paper chromatography of components R-2, R-3 and R-4

The acid solvent was methanol–100% formic acid–water (56:9:5, by vol.). The alkaline solvent was methanol–aq. NH_3 (sp.gr. 0.88)–water (21:3:11, by vol.). Downward solvent development at 2°C was used. Each value is the mean $\pm$ S.E.M. for four similar chromatograms.

Component ...	R_F		
	R-2	R-3	R-4
Acid solvent	0.548 ± 0.010	0.307 ± 0.007	0.638 ± 0.003
Alkaline solvent	0.682 ± 0.010	0.731 ± 0.012	0.678 ± 0.012

Fig. 6. Chromatography of components R-2, R-3 and R-4 and their hydrolysis products on a Moore & Stein amino acid analyser fitted with a radioactivity detector

Each chromatogram shows peaks of the intensity of ninhydrin colour (increasing upwards) and radioactivity (increasing downwards). The position of hydroxyproline is shown as a marker. The buffer gradient was pH 2.5–3.25 and the column temperature was 30°C. Conditions of hydrolysis are given in the text.

observed. With components R-2 and R-3 these conditions produced ninhydrin-negative peaks near the front, a small amount of radioactive component R-4, non-radioactive glutamate and radioactive glycine. The glycine/glutamate molar ratio was slightly greater than 1:1. With component R-4 partial breakdown occurred, producing a ninhydrin-negative radioactive peak near the front.

The mild hydrolysis of components R-2 and R-3

indicated that they were both derivatives of a non-hydrolysable substance R-4 to which equal numbers of glutamate and glycine residues had been attached by peptide bonds. The consequences of vigorous hydrolysis, on the other hand, were difficult to explain. Possibly degradation and oxidation took place.

The presence of equimolar amounts of glutamate and glycine in components R-2 and R-3 suggested that these components might be derivatives of

glutathione, in which case component R-4 would be a derivative of cysteine. Consequently all three components were examined for the presence of sulphur, and all gave a positive Lassaigne's test (Feigl, 1947) for organically bound sulphur. With this test it was essential to use a borosilicate-glass ignition tube and to wash the sodium metal free of naphtha with dry ether, otherwise the blank gave a positive result. None of the components gave a positive nitroprusside test until they were fused with NaOH (freshly prepared from sodium metal and water) in a Pyrex tube. The fusion mixtures were also capable of catalysing the iodine-azide reaction (Feigl, 1947). These results indicated that all three components contained bivalently linked sulphur but precluded the presence of a thiol group or disulphide bond.

The absence of a thiol group made special precautions unnecessary when preparing volatile derivatives for structural studies with a mass spectrometer, and the *N*-trifluoroacetyl derivative of the methyl ester of component R-4 was readily prepared by the method of Darbre & Islam (1968). It was sufficiently pure to be introduced into the mass spectrometer on a direct probe. Fig. 7 shows the mass spectrum of the derivative of component R-4, from which it was inferred that component R-4 itself is *S*-carboxymethylcysteine [$\text{HO}_2\text{C}-\text{CH}(\text{NH}_2)-\text{CH}_2-\text{S}-\text{CH}_2-\text{CO}_2\text{H}$]. The accurate mass of the molecular ion was 303.03860 ($\text{C}_5\text{H}_{12}\text{F}_3\text{NO}_5\text{S}$ requires 303.03882). An important fragmentation occurred between the α - and β -carbon atoms of the cysteine, producing an ion at 119.01617 ($\text{C}_4\text{H}_7\text{O}_2\text{S}$ requires 119.01667), and another was formed at 184.00355 by the removal of both esterified carboxyl groups ($\text{C}_5\text{H}_5\text{F}_3\text{NOS}$ requires 184.00439). The strongest ion, at m/e 158, was

produced by a keten formed at the carboxyl group of the cysteine, of structure $\text{O}=\text{C}=\text{CH}-\text{CH}_2-\text{S}-\text{CH}_2-\text{CO}-\text{O}-\text{CH}_3$. The accurate mass of this ion was 158.00425 ($\text{C}_6\text{H}_6\text{O}_3\text{S}$ requires 158.00376).

Since component R-4 is *S*-carboxymethylcysteine, it follows that either component R-2 or component R-3 should be *S*-carboxymethylglutathione, but this could not be confirmed by mass-spectrometric analysis. The attempted preparation of the *N*-trifluoroacetyl methyl esters of both components gave on mass spectroscopy a mixture of compounds, one or more of which had a higher molecular weight than the 503 expected for the *S*-carboxymethylglutathione derivative. G.l.c. before mass spectrometry was unsuccessful. Hydrolysis of the prepared derivatives failed to reproduce either the original components or their further hydrolysis product *S*-carboxymethylcysteine, showing that structural changes had occurred on preparation of the derivatives.

Synthesis of *S*-carboxy[^{14}C]methylglutathione

To confirm that components R-2 and R-3 were structurally related to glutathione their preparation from sodium iodo[^{14}C]acetate and glutathione was attempted. These two compounds reacted with great facility at pH 7 and room temperature, and the radioactive products were identical with the radioactive products from lettuces treated with fluoro[^{14}C]acetate. On the column of Amberlite IR-120(H) an elution pattern of radioactivity similar to that shown in Fig. 5 was obtained. The same four components, which were identical with components R-1–R-4, appeared on the amino acid analyser, peak R-3 being much the largest. After purification on a column of

Fig. 7. Mass spectrum of the *N*-trifluoroacetyl dimethyl ester of compound R-4 (*S*-carboxymethylcysteine)

For details see the text.

sulphonated polystyrene and preparation of a volatile derivative as before, the synthetic compound R-4 gave a mass spectrum identical with those of both the compound isolated from lettuce and a commercial sample of *S*-carboxymethylcysteine. Mass spectroscopy of the synthetic compounds R-2 and R-3 was unsuccessful, as with the same components from lettuce.

General Discussion

The major product of the reaction of iodoacetate with glutathione is identical with component R-3 from lettuce, and in both cases the products of hydrolysis are equimolar amounts of glutamate and glycine together with *S*-carboxymethylcysteine; hence this component is very likely to be *S*-carboxymethylglutathione. Component R-2, which is eluted as an unresolved double component on the amino acid analyser, is also hydrolysed to form the same compounds. It is tentatively suggested that component R-2 consists of the two possible ring structures formed, with the elimination of water, between the free amino group of the glutamate and either the carboxyl group of the carboxymethyl group or the carboxyl group of the glycine. As can be seen with atomic models, these two ring structures are very compact and their formation would account for the very low ninhydrin-colour yield from component R-2, and, because of their similar pK values, for the rapid elution and composite nature of component R-2 on the amino acid analyser. The chemistry of *S*-carboxymethylglutathione is complex and will need further investigation. Of particular interest is the formation of [^{14}C]glycine during vigorous hydrolysis when either carbon atom of the carboxymethyl group is labelled. This reaction must involve the scission of a C-S bond and the transference of a primary amino group, and was so unexpected as to delay us in arriving at the true structure of compounds R-3 and R-4.

The production of compounds R-2 and R-4 (*S*-carboxymethylcysteine) during the preparation of *S*-carboxymethylglutathione from iodoacetate and glutathione suggests that they may be either by-products of the reaction or artifacts produced during removal of NaI on the column of Amberlite IR-120(H). Consequently some pure radioactive compound R-3 (*S*-carboxymethylglutathione) isolated from fluoroacetate-treated lettuce was passed down a column (75 cm $\times$ 1 cm diam.; V_0 15 ml) of Amberlite IR-120(H) and eluted with 2 litres of water. The eluate was evaporated and chromatographed on the column of sulphonated polystyrene used for the preparation of synthetic *S*-carboxymethylglutathione. Of the eluted radioactivity 9% was in compound R-2, 2.4% was in compound R-4 (*S*-carboxymethylcysteine) and the remainder in compound R-3 (*S*-carboxymethylglutathione). The

Fig. 8. Reaction rates of monohaloacetates with glutathione at pH 7 at 25°C

Each haloacetate (25 ml; 0.1 M) was neutralized to pH 7 and added to 25 ml of a previously neutralized 0.1 M solution of glutathione. The pH of the solution was maintained within the range 6.8–7.2 by the neutralization of the hydrogen halide formed with 0.1 M-NaOH from a burette. Individual plots are not given, as very many small additions of NaOH were required to maintain the solution within the limits pH 6.8–7.2. Fluoroacetate is shown as a broken line for clarity. Although continuous monitoring of the fluoroacetate reaction was discontinued after 5.3 h, the pH of the solution was still unchanged after 20 h.

spontaneous appearance of compounds R-2 and R-4 during the purification of compound R-3 suggests that only *S*-carboxymethylglutathione is a metabolite of fluoroacetate in lettuce, the other components being produced during the isolation of this compound. More experiments are required to prove this possibility.

The facility with which iodoacetate reacts with glutathione led us to examine the reaction rates of all the monohaloacetates with glutathione at physiological pH and temperature. Fig. 8 shows that iodoacetate reacts almost completely in 2 h, bromoacetate reacts nearly as quickly, chloroacetate reacts much more slowly and no reaction at all was detected between fluoroacetate and glutathione. This suggests

that an enzyme is necessary to catalyse the reaction between fluoroacetate and glutathione in lettuce and that the reaction is unlike the spontaneous one between fluoropyruvate and thiols (Avi-Dor & Mager, 1956).

The metabolism of fluoroacetate by lettuce very closely resembles the metabolism of many aliphatic and aromatic halogen compounds by mammalian liver (Boyland, 1962). Here the halogen is split off and a conjugate formed with glutathione is excreted, often as a mercapturic acid. There is evidence that this detoxifying reaction may occur in fluoroacetate-poisoned rats, guinea pigs and pigeons, where the accumulation of citrate in the liver is many times lower than in the other organs tested (Buffa & Peters, 1949; Gal *et al.*, 1956). Gal *et al.* (1961) also noticed that 1–1.5% of fluoroacetate administered to rats was defluorinated.

Many precautions were taken to ensure that the fluoro[^{14}C]acetate used for our experiments was pure, and this is reflected in the biochemical simplicity of the metabolism observed. There is some evidence of interference with the tricarboxylic acid cycle by fluoroacetate giving an increased citrate concentration in the plant and the formation of a small amount of fluorocitrate. This interference seems to have little effect, possibly because the plant utilizes a mechanism for the rapid defluorination of fluoroacetate but in the process it immobilizes amino acids that could be usefully employed elsewhere.

Our results are not in very close agreement with those obtained by Preuss *et al.* (1968). These workers found incorporation of radioactivity into lipids, water-soluble fractions and evolved CO_2 in fluoro-[^{14}C]acetate-treated seedlings of *Acacia georginae*, peanut, castor bean and kidney bean. Although it is to be expected that *A. georginae*, a plant known to biosynthesize and store large quantities of fluoroacetate, would metabolize the compound differently from lettuce, it is surprising that peanut, castor bean and kidney bean do so too.

The chemically highly stable C–F bond is fairly easily broken by a number of enzyme systems including those in soil micro-organisms (Goldman *et al.*, 1968; Kelly, 1965) and rat intestinal mucosa (Barnett *et al.*, 1967). The present study indicates another way in which this bond may be broken. There is now known to be a range of living systems that are not only capable of synthesizing organic fluorine compounds but also of catabolizing them. Fluorine is likely to be an essential trace element for animals, and there is a wide choice of possible mechanisms for its absorption and transport to the tissues that require it.

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