*Biochem. J.* (1965) **95**, 724

# Fluoride Metabolism in *Acacia georginae* Gidyea

By R. A. PETERS, L. R. MURRAY and M. SHORTHOUSE
*Department of Biochemistry, University of Cambridge*

(*Received* 22 *July* 1964)

1. The metabolism of fluoride in seedlings and small plants of *Acacia georginae* has been studied with the idea of finding the conditions under which the plant makes fluoroacetate in the laboratory. 2. Individual seedlings vary in the extent to which they take up fluoride and convert it into a form other than inorganic which is here called 'organic' fluoride, F(org.). The differences between the toxicity of *A. georginae* Gidyea trees may therefore be genetic in origin. 3. The uptake of fluoride from solutions 0·525–1·05mM (10–20p.p.m.) was not large. In 1–4 days it reached 8 p.p.m. in the aerial parts and 16 p.p.m. in the roots. Unlike the distribution of the halogen in grass, total fluoride was greater than inorganic fluoride. It was almost a rule that more 'organic' fluoride was present in the roots than in the aerial parts. 4. With higher concentrations of fluoride 10·5–15·75mM (200–300 p.p.m.) much larger amounts of fluoride were taken up, especially by the roots, and much more apparent organic fluoride was formed. 5. pH had a large influence upon the intake, this being lowest at an initial pH 8·4 and highest at pH 4·0. The pH outside this range was not investigated.

Some observations have been made with a view to clarifying the biochemical paths for the synthesis of the C–F bond. 6. There is no evidence that chloride is an intermediary in synthesis. 7. Succinate is not accumulated in fluoride-stressed plants, suggesting that succinate dehydrogenase is not inhibited. 8. Enolase does not appear to be inhibited *in vivo*.

The fluoride metabolism of the Australian *Acacia georginae* has been studied because of its toxicity to the local cattle (Bell, Newton, Everest & Legg, 1955) due to fluoroacetate (Murray, McConnell & Whittem, 1961; Oelrichs & McEwan, 1961). There is urgency in this, as losses of animals on a farm may reach £50 000. The best hope of finding some means of averting toxicity lies in getting knowledge of the path of synthesis of the C–F bond. An essential preliminary to this was to study the means of inducing a maximum synthesis of C–F compounds under laboratory conditions, and of examining hypotheses as to possible enzymic paths involved.

## EXPERIMENTAL

*Growth of plants.* Seeds of *Acacia georginae* from the Northern Territory (Alice Springs) were grown in the hothouse in pots in a local black loam at about 32°, and also in a nutrient solution of the following composition (g./100l.): $KNO_3$, 20·2; $Ca(NO_3)_2$, 65·6; $NaH_2PO_4,2H_2O$, 20·8; $MgSO_4,7H_2O$, 36·9; ferric citrate, 2·45; $MnSO_4,4H_2O$, 0·223; $CuSO_4,5H_2O$, 0·024; $ZnSO_4,7H_2O$, 0·029; $H_3BO_3$, 0·186; $(NH_4)_6Mo_7O_{24},H_2O$, 0·0035; $Al_2(SO_4)_3,16H_2O$, 0·0186; $NiSO_4,7H_2O$, 0·0028; $CoSO_4,7H_2O$, 0·0028. In some experiments the plants were grown under sterile conditions in an identical medium stiffened with agar. To reduce the fungus infection, the seeds were soaked in 0·1% mercuric chloride solution for 5min. or less before growing.

*Methods.* Chemicals used were AnalaR where possible. Fluoride was estimated by Hall's (1963) method in which diffusion from perchloric acid (Singer & Armstrong, 1959) in polythene bottles and collection of the fluoride on filter paper is followed by a modified colorimetric method of Belcher, Leonard & West (1959). According to Hall (1963, p. 81), the spread between duplicate fluoride determinations was: 0·5–1·0$\mu$g. ± 2·5%; 0·2$\mu$g. ± 3·0%; 0·1$\mu$g. ± 11% when diffusion occurred to the filter paper from pure fluoride solutions in the 66% perchloric acid. The errors can be expected to be larger on diffusion from plant extracts. The maximum fluoride that can be measured by this method without modification is approx. 1·5$\mu$g./bottle, so that aqueous extracts for diffusion of 1·0ml. should be diluted to contain 0·3–1·0$\mu$g. As a rule the results quoted are the average of satisfactory duplicates. In our hands, tests with 1·0$\mu$g. of F gave with duplicate errors of ±1·2%, agreeing with those of Hall (1963).

Total fluoride was estimated after combustion. Table 1 gives the recoveries of fluoride after combustion at 400° in the presence of lithium hydroxide and magnesium. Between 10 and 100$\mu$g. the values lie within ±5%. When less was present in the platinum crucibles the error was greater. Most of the determinations were done with amounts above 5$\mu$g. in the crucibles. Some of the earlier results were obtained with calcium oxide at 600°; but this method was changed to that of Hall (1963) owing to the difficulty of

Table 1. *Accuracy of estimations of total fluoride, obtained by combustion in platinum crucibles and subsequent diffusion*

For experimental details, see the text.

| Amount of F in crucible ($\mu$g.) | Spectrophotometer readings $E_{570m\mu}$ | Recovery of F (% of original) |
|---|---|---|
| 100 | 0·204 | 99 |
| | 0·208 | |
| 75 | 0·165 | 105 |
| | 0·165 | |
| 50 | 0·205 | 99·5 |
| | 0·210 | |
| 25 | 0·100 | 96 |
| | 0·100 | |
| 10 | 0·201 | 95 |
| | 0·200 | |
| 5 | 0·166 | 92 |
| | 0·157 | |
| 2 | 0·095 | 112 |
| | 0·095 | |
| 2 | 0·097 | 112 |
| | 0·093 | |

getting calcium oxide free from fluorine. In some cases the estimation of organically combined fluoride, F(org.), was made by Ramsey & Clifford's (1949) method, i.e. after passing the solution through a silica column to remove inorganic fluorine.

Two controls with 1·0 $\mu$g. of F were set up for each set of determinations, and the spectrophotometer reading was usually between 0·210 and 0·190 (final volumes 4·3 ml.), being lower than the values obtained by Hall (1963). The various factors affecting the maximum colour obtained with 1·0 $\mu$g. of F are not yet clear. The colour obtained at pH 4·6 was increased some 10% with a rise of 0·1 pH, agreeing with Hall (1963), and decreased 22% with a fall of 0·1 pH.

In the original method, the filter paper for the collection of fluoride was inserted into a polythene stopper, and the whole sealed with wax. It was simpler, and there was no loss of accuracy, if a small rubber stopper was used with an $\frac{1}{8}$ in. hole drilled in the smaller end into which the filter paper was inserted; the wax was then unnecessary.

After exposure to the fluoride solutions, or growth in soil, the plants were washed quickly (15 sec. each washing) in about six changes of water, then ground in cooled 1% perchloric acid in a mortar. In some experiments extraction was with perchloric acid (1·0%) at 80° for 10 min., and a few experiments were made with 1·0% sulphuric acid as extracting agent.

The usual amount of perchloric acid was approx. 10 ml.; but this amount was not critical. In cases where weights of 0·1–0·3 g. were extracted, the amounts of perchloric acid were reduced to 2 ml. After extraction with perchloric acid, the solids were washed three times with 2 ml. amounts of water, which were added to the original acid extract. Separation of the solid residue was made by either filtration or centrifugation.

All the analytical results are expressed as $\mu$g. of F/g. of fresh tissue. The moisture content was for leaves 80 ± 5% and for roots approx. 50%.

## RESULTS

*Plants grown in soil.* The first point to determine was whether plants grown in the hothouse in Cambridge made fluoroacetate or F(org.). Plants of sufficient size for analysis, aerial parts being approx. 1·0 g. and roots 0·2–0·4 g., were taken after growth for 10 weeks. The roots and aerial parts (the complete plant above soil level) were then analysed for total F and F(org.), the latter by the method of Ramsey & Clifford (1949), and finally diffusion. In three plants the total F was 13·6, 13·2, and 15·7 $\mu$g./g. in the roots, and 0·6, 0·8 and 0·7 $\mu$g./g. in the corresponding aerial parts. No organically bound F could be detected in either the roots or the aerial parts of these plants. They had nevertheless been exposed to some fluoride in the soil, which at pH 7·9 contained on a dry basis 1·8% of calcium soluble in 0·1 N-hydrochloric acid. The total F content was 190 $\mu$g./g. by combustion. The water-soluble fluoride, determined by shaking 1·0 g. of dry soil with 50 ml. of water for 30 min. and analysis of the filtrate, was 2·6 $\mu$g./g.

The plants were then exposed to more fluoride. Each pot was watered daily for one week with a saturated solution of calcium fluoride (15 p.p.m.) until drainage occurred through the bottom of the pot; the soil was permitted to drain after each watering ensuring maximum contact with the saturated calcium fluoride. Such plants showed an increase of total F in both roots and aerial parts of approximately 20-fold. Thus two plants gave 344 and 324 $\mu$g./g. in the roots and 10·8 and 15·7 $\mu$g./g. in the aerial parts. The increase of total F was accompanied by a synthesis of F(org.) in the aerial parts of 0·3–0·4 $\mu$g./g., but of none in the roots. The experiments showed that, with increased exposure to F, more entered the plants, and some was converted into F(org.).

*Plants grown in water culture.* Confirmation of the synthesis of organically combined F under laboratory conditions was found with the plants grown in water culture, by using relatively low concentrations of F. In 15 experiments in which small plants were exposed for varying periods to concentrations of F of 0·525–1·05 mM (10–20 $\mu$g./ml.), the uptake of F was close to that reported for grass (Peters & Shorthouse, 1964); the results of two typical experiments are given in Table 2. The difference from the grass experiments was that in most cases more F was estimated by combustion (total F) than inorganic F, indicating a synthesis of F(org.) (Expt. 1). Expt. 2 illustrates the gradual uptake of F during 72 hr. In this series of experiments it was usual to find more F in the roots than

Table 2. *Uptake of fluoride from low concentrations by* Acacia georginae

The weights of seedlings used in Expt. 1 were: aerial parts 0·24g., roots 0·125g.; in Expt. 2 aerial parts respectively 0·76g., 0·37g., 0·46g., 0·31g., 0·38g.; roots 0·17g., 0·1g., 0·12g., 0·12g., 0·13g. At least two seedlings were used for each determination. For other details see the text.

| | | | Inorganic F ($\mu$g./g. wet wt.) | Total |
|---|---|---|---|---|
| Expt. 1 | Plants grown in water culture for 14 days: stood in $F^-$ (10$\mu$g./ml., 0·525mM) for 18hr.; vol. 20ml. | Aerial parts | 3·6 | 6·6 |
| | | Roots | 10·3 | 17·0 |

| | | | Inorganic F | | | | |
|---|---|---|---|---|---|---|---|
| | | Time (hr.) ... | 0 | 24 | 48 | 72 | 120 |
| Expt. 2 | Plants grown in water culture for 30 days; stood in $F^-$ (20$\mu$g./ml., 1·05mM); vol. 125ml. | Aerial parts | 1·2 | 2·3 | 7·7 | 8·1 | 5·5 |
| | | Roots | 1·4 | 1·5 | 4·5 | 11·3 | 16·2 |

Table 3. *Individual variation in the uptake of fluoride*

The weights taken for analysis were: Expt. 3: A, 0·35g., B and C, 0·29g., D, 0·38g. Expt. 4: 1, 0·46g., 2, 0·82g., 3, 0·64g., 4, 0·37g. For other details, see the text.

| | | Seedlings or whole plants | Inorganic fluoride ($\mu$g. of F/g. wet wt.) |
|---|---|---|---|
| Expt. 3 | Seedlings A, B, C, D grown in the same container in water culture; after 8 days stood in $F^-$ (10$\mu$g./ml.) for 24 days; vol. 200ml. | A | 10·0 |
| | | B | 21·0 |
| | | C | 25·2 |
| | | D | 15·5 |

| | | | Fluoride ($\mu$g. of F/g. wet wt.) | |
|---|---|---|---|---|
| | | | Inorganic | Total |
| Expt. 4 | Plants 1, 2, 3, 4 grown in one pot in the hothouse and watered with saturated $CaF_2$ for 54 days (aerial parts only analysed) | 1 | 82·0 | 81·3 |
| | | 2 | Nil | 3·3 |
| | | 3 | 11·0 | 14·7 |
| | | 4 | Nil | 9·4 |

in the aerial parts; in nine experiments out of ten the inorganic F was higher in the roots and in eight out of ten the total F was higher in the roots.

The concentrations given are the initial concentrations of F used in the experiments of Tables 2 and 3; we found that some 25% of F might disappear in 24hr. from such solutions, presumably adsorbed even by the Pyrex glass. This would mean that at the end of Expt. 1 the concentration of F would be approx. 7·5$\mu$g./ml., apart from the negligible amount taken up by the seedlings of 3·7$\mu$g./ml. from the original 200$\mu$g. in the beaker. When larger amounts of F were present initially, as reported below, the percentage diminution is less. For instance an initial concentration of 100$\mu$g. of F/ml. in a volume of 125ml. fell to 98$\mu$g. of F/ml. within 2hr. The changes in the low concentrations must be reckoned with in any further detailed study where a 30% fall is important; in the present context they are not important.

*Individuality of fluoride metabolism.* It has been observed in the Northern Territory (Australia) (L. R. Murray, personal communication) that even in one area individual trees may vary in toxicity without showing botanical differences. As such trees may be contiguous, the variation is unlikely to be due to variations in content of fluoride in the soil, or of unequal water stress (drought). It was of interest therefore to study the behaviour of seedlings grown under identical conditions together. Table 3 gives the results, Expt. 3 for seedlings grown in the same container, and Expt. 4 for four plants grown in the hothouse. In Expt. 3 the inorganic fluoride found varied from 10·0 to 25·2$\mu$g./g., an amount outside experimental error, even though the amounts taken for analysis were only 0·1g. In Expt. 4 only the aerial parts were analysed, as the roots could not be separated. Again considerable variation was shown. These two experiments suggest that, even from the start, there may be biochemical differences in toxicity, suggesting a genetic origin. It may be mentioned that the biochemistry of individual seedlings of other plants is known to vary (Mikoajczyk & Nowacki, 1961; Epstein & Jefferies, 1964).

*Effect of higher concentrations and of* pH *on uptake of fluoride.* The experiments with exposure to low concentrations have shown that fluoride can be taken up at these concentrations by the seedlings, and that F(org.) can be formed. The amounts of this were low, however. With higher concentrations of 200–300 $\mu$g. of F/ml. (10·5–15·75 mM) much more F(org.) can be formed, up to 100 times the concentration.

It has been reported (see Sutcliffe, 1962) that changes of pH may alter the absorption of salts, e.g. nitrate, chloride and phosphate. An extensive study of the effect of pH on the inhibition of oxygen uptake of yeast by fluoride was made by Simon & Beevers (1952). They concluded that the p$K$ for HF (3·4) was influencing the results; the inhibitory action increased as the pH was changed from 7·0 to 3·0. No estimations were made of the amounts of fluoride actually taken up. The large influence of pH in our experiments was discovered accidentally during an attempt to repeat on *A. georginae* the observations made on yeast by Chung & Nickerson (1953). They found that the addition of glucose 1-phosphate antagonized the inhibitory effect of fluoride. To our surprise glucose 1-phosphate (10 mM) much decreased the uptake of fluoride from 200 and 300 $\mu$g./ml., as compared with F alone. In Expt. 1, Fig. 1, in which plants were exposed to 200 $\mu$g./ml. for 36 hr. with or without glucose 1-phosphate, the difference in both uptake and the formation of organic fluoride for the roots was some 12 times. The initial pH for the phosphate compound was 8·4 and for the fluoride alone 6·6. If the pH for the phosphate compound is started at 6·6, little difference is seen with the added phosphate, as shown in Expt. 2, Fig. 1, where the concentration of F was also 200 $\mu$g./ml., but the exposure 48 hr. The conclusion that the difference was mainly due to pH and not to phosphate was also confirmed in another experiment, and in Expt. 3, Fig. 2, where phosphate itself was added at an initial pH of 8·3.

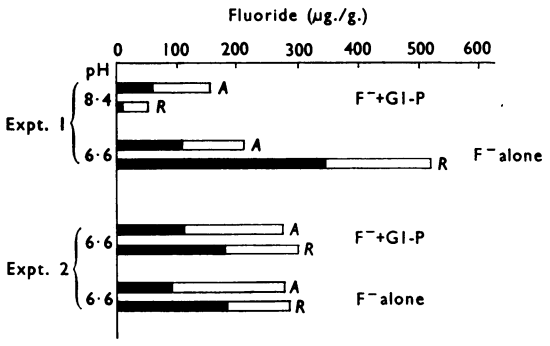


Fig. 1. Comparison of the amounts of fluoride taken up by plants of *A. georginae* (approx. 6 in. high) from solutions of sodium fluoride (200 $\mu$g./ml., 11·5 mM) with or without glucose 1-phosphate (10 mM) at varying pH. With fluoride alone the pH was 6·6. Estimations were made of inorganic fluoride by diffusion and of total fluoride after combustion in the presence of lithium hydroxide at 400° in Expt. 2 and calcium oxide at 600° in Expt. 1. At the end of the exposure (Expt. 1, 36 hr.; Expt. 2, 48 hr.) the plants were washed six times with 2 ml. of water, for 15 sec. in all. They were then ground and extracted as follows: in Expt. 1 with cold 1% perchloric acid, 1·0 g. wet wt. to 12 ml.; in Expt. 2 with cold and hot perchloric acid, the extracts being combined. *A*, Aerial parts (those above the roots); *R*, roots; □, organic fluoride; ■, inorganic fluoride; G 1-P, glucose 1-phosphate (10 mM).

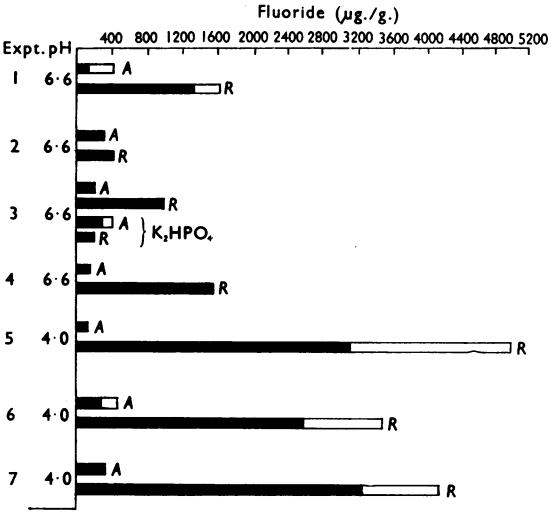


Fig. 2. Comparison of the amounts of fluoride taken up from solutions of sodium fluoride, 300 $\mu$g./ml. (15·75 mM), at pH 6·6 and pH 4·0 (acidification with nitric acid). Expt. 3 was also exposed to dipotassium hydrogen phosphate. Estimations were as in Fig. 1; combustion was with calcium oxide at 600° in Expts. 1 and 2, and the rest were with lithium hydroxide at 400°. The method of extraction was as follows: Expt. 1, Cold perchloric acid 1%. Expt. 2, Warm perchloric acid 1%. Expt. 3, Cold followed by warm perchloric acid combined. Expts. 4, 5, 6, Hot perchloric acid. Expt. 7, N-Sulphuric acid at 80° for 10 min. The final volumes varied from 20 to 37 ml. Where no 'organic' fluoride is shown, the amount found for total fluoride on combustion did not exceed the inorganic fluoride by diffusion within experimental error, except in Expt. 4. In Expt. 4 the estimated total fluoride for the roots was less than that found for the inorganic fluoride by diffusion alone by 235 $\mu$g./g. We cannot account for this deficit of 15% by experimental error of the estimation; but it could have been due to an unrecognized fluctuation of temperature in the oven. The periods of exposure to fluoride were for Expts. 1 and 2, 36 hr. and for the rest 48 hr. Organic fluoride is reckoned as total minus inorganic fluoride. *A*, Aerial parts; *R*, roots; □, organic fluoride; ■, inorganic fluoride.

In Fig. 2, the behaviour of *A. georginae* at initial pH values of 6·6 and 4·0 has been compared. The plants were kept in a fluoride concentration of 300 $\mu$g./ml. for 3 days, then extracted by the method described in the protocol to the Figure. In several experiments the inorganic and total fluoride were the same within the limits of error, but at pH 4·0 the amounts of F taken up by the roots was very much greater, and there was a relatively large formation of F(org.). In Expt. 5, Fig. 2, a parallel experiment was done in phosphate ($K_2HPO_4$) plus fluoride at pH 5·5. The uptake in the aerial parts was approximately the same; in the roots the values were 917 (inorganic) and 1530 (total) $\mu$g. of F/ml., so that there was a synthesis of F(org.), but this was less than at pH 4·0. The influence of pH on the uptake of fluoride was clear, but the amount of F(org.) formed does not always follow the uptake of fluoride. Other factors are evidently at work.

*Residual fluoride.* Though our main objective was to discover the conditions for the synthesis of F(org.), we noticed other points in the metabolism of fluoride in the course of the work. The total F found in the extracting fluid in these experiments (Fig. 2) did not represent all the fluoride that had been taken up, because an analysis of the solid material left behind always revealed the presence of some inorganic fluoride. With the aerial parts this did not amount to more than 20–150 $\mu$g./g. of solid as a general rule; but in the roots it might be much greater, even after an extraction of the solid with warm perchloric acid (1·0%). In Expt. 6, Fig. 2, for instance, the solid left gave a value on combustion of 8000 $\mu$g./g. It was extracted with chloroform–methanol (2:1, v/v), the extract containing only 5 $\mu$g. of F, the residue then giving an estimation of 6000 $\mu$g. of inorganic F. Further, the solid taken without extraction of the lipids released inorganic F by diffusion (7500 $\mu$g./g.). Hence in spite of our extraction with 1% perchloric acid, fluorine appears to be left behind in a form released as inorganic F on treatment with strong perchloric acid, after the solid is dried down. We do not yet know the state of this F.

Since we get the largest formation of F(org.) in the roots after exposure to fluoride at a pH approaching 4·0, it seems likely that the C–F bond is synthesized in the root itself; but it would depend on the speed at which transfer could take place from leaf to root. We have tried to find out whether illumination affects the rate of synthesis, and have obtained rather conflicting results.

The further question arises whether all the apparent F(org.) is fluoroacetate. In one experiment, the F(org.) from the roots in Expt. 6, Fig. 2, was examined with the thioindigo reaction (Ramsey & Patterson, 1951) and the fluoroacetate reaction was obtained, though less strongly than for an equal amount of fluoroacetate alone.

## DISCUSSION

The influence of pH on the absorption of F with *A. georginae* follows well the results obtained by Simon & Beevers (1952) for the inhibition of the respiration of yeast by fluoride. In our work their observations on yeast are extended by using *A. georginae* to the proof that the fluoride is actually absorbed by the plant and that this follows the concentration of undissociated molecules. It is not therefore merely an effect on the surface of the cell. Yet the surface may be magnifying locally the concentration of undissociated ions, because Peters (1931, 1963) found that there was an apparent shift in pH of 3·0 units to the alkaline side for a weak acid in the interface. Hence, at pH 4·0, HF in the interface is probably little dissociated, the p$K$ for this being about 6·4 instead of 3·4 in bulk solution. The form in which the fluoride acts inside the cells after penetration is not decided by this type of experiment. In cell extracts, fluoride is used as an inhibitor at reactions near the neutral point. Those who have worked on variations of internal pH in a yeast cell on exposure to solutions outside of varying pH (Conway & O'Malley, 1946) consider that the actual pH inside the cell is little changed.

At first we thought that there was evidence of an adaptive enzyme, formed slowly in the plant in fluoride solutions. We have discarded this hypothesis as we find no marked differences between plants grown in ordinary soil and soil watered with calcium fluoride.

### *Some observations on chloride metabolism and the effects of fluoride on certain enzymes*

We have examined two of the more obvious hypotheses about possible routes of synthesis of the fluoroacetate: (*a*) that organo-chlorine compounds are an intermediate; (*b*) that synthesis follows a block in either succinate dehydrogenase or enolase (Miller, 1958; Malmström, 1961).

*Chlorine metabolism.* There are numerous reports on the total concentrations of chlorine in the higher plants, but apparently organic chloride has not yet been detected in any plant. In particular *A. georginae* might metabolize chlorine to yield monochloroacetic acid, which is non-toxic, relative to fluoroacetate. Competition between the halogens both in uptake and in subsequent metabolism could then incidentally lead to practical measures to control losses of stock.

Plants (grown in both soil and nutrient solution) were kept for varying times (24 hr.–2 weeks) with their roots in nutrient solutions containing extra

KCl and $Na^{36}Cl$ in a known ratio. The plants were removed and washed in water, and the roots and aerial parts separately ground in 0·1N-sulphuric acid. The acid extract was exhaustively extracted with ether, and organic acids were extracted from the ether with sodium hydroxide solution. Chloride was then precipitated from the sulphuric acid extract by the addition of silver sulphate solution. Chlorine, as organic ether-soluble chlorine and as chloride, was determined by radioactive counting of the fractions after drying on planchets, and calculation from the known ratio of $^{35}Cl$ to $^{36}Cl$.

Although high concentrations of chlorine were taken up (values up to 600 $\mu$g./g. were found in both roots and aerial parts), in no experiment was any ether-soluble organic chlorine detected. All the chlorine in the plants could be accounted for as inorganic chloride.

Recent field investigations in Central Australia (L. R. Murray, unpublished work) have revealed consistently high amounts (3mg./g.) of chloride in leaves of *A. georginae*, in which the total concentrations of fluorine varied from 0·4 to 14 $\mu$g./g. Thus, in the field at least, chlorine does not seem to affect significantly the uptake of fluorine.

Epstein and colleagues (Epstein, 1953; Elzam, Rains & Epstein, 1964, and personal communication) found with barley roots that bromide inhibits chloride absorption competitively, but that fluoride did not affect the absorption of chloride. There is thus no evidence that an organically combined chloride is an intermediate in the formation of fluoroacetate.

*Succinate dehydrogenase.* Since inorganic fluoride blocks several isolated enzymes, a possible hypothesis about the initiation of formation of the C–F bond *in vivo* is that this starts with the inhibition of some enzyme. For instance, a block of succinate dehydrogenase might lead to the attachment of HF to the double bond of fumarate. The reverse reaction can take place (V. Desreux, personal communication). To investigate possible inhibition of succinate dehydrogenase, the concentration of succinic acid was measured in 'normal' and 'water-stressed' plants by the method of Rodgers (1961). Stressed plants watered with calcium fluoride solution and then exposed to dry conditions contained approximately twice the concentration of succinic acid in both aerial parts and roots; but this relatively small accumulation suggests that any inhibition would not be severe enough to trigger off an adaptive mechanism, especially when compared with the large increase in citrate with inhibition of aconitase.

*Enolase.* We confirmed that acetone-dried powders of pea (*Pisum sativum*) seeds prepared by the method of Evans (1955) were active. The enolase activity was measured according to the spectrophotometric procedure of Miller (1958); the enolase present was inhibited by fluoride plus phosphate (5–6mM). As reported for enolase from muscle (Peters, Shorthouse & Murray, 1964), phosphorofluoridate was not inhibitory to the enolase from pea seeds. The enolase activity prepared from acetone-dried powders of the aerial parts and roots of young, soil-grown plants of *A. georginae* was very low, being only 2–5% of the activity from the pea seeds. Low activities were found in both normal and fluoride-stressed plants, which could favour an inhibition.

The inhibition of purified enolase from pea seed by fluoride plus phosphate was largely removed by dialysis of the inhibited enzyme solution against tris buffer for 24hr. However, similar dialysis of tris extracts of acetone-dried powders from fluoride-stressed plants (10 weeks watering with calcium fluoride solution) produced no increase in enzyme activity. This can be interpreted as evidence for the view that there is no significant inhibition by fluoride of enolase *in vivo*, but the activity of the extracts from *A. georginae* was very low.

Some remarks are needed about enolase and its possible inhibition in plants *in vivo*. According to an estimate by L.R.M., the concentration of fluoride necessary *in vivo* must be 0·5mM (0·5p.p.m.). It could be higher in localized concentrations, but in any case seems to be much less than the usual amount of fluoride in Warburg experiments (approx. 23·6mM). One case is reported (Aisenberg & Potter, 1955) in rat liver and kidney homogenates where acetate activation was inhibited by 0·1mM-sodium fluoride without affecting pyruvate oxidation, oxidation rate or the concentration of adenine nucleotides. Bonner & Thimann (1950) and Ordin & Skoe (1963), with *Avena* coleoptiles, observed an inhibition of growth, thought to be due not to inhibition of enolase but rather to inhibition of phosphoglucomutase; the latter conclusion has been stressed in work by Yang & Miller (1962, 1963).

We thank Shell Research and the Wellcome Trustees for grants for technical assistance, the Wellcome Trustees for grants for expenses, and the Tropical Products Research Organization for the loan of apparatus; also Professor F. G. Young, F.R.S., for facilities provided. We are grateful to Professor Blackman, F.R.S., and to Dr Loughman for advice on water culture, to Mr Barrett for help with sterile cultures; also to several colleagues in the Laboratory for helpful advice. The seeds were kindly provided by the staff of the Laboratory at Alice Springs. We thank Professor H. Godwin, F.R.S., the Director of the Botanical Gardens in Cambridge and the staff for growth of plants in the hothouse.

## REFERENCES

Aisenberg, A. C. & Potter, V. R. (1955). *J. biol. Chem.* **216**, 737.

Belcher, R., Leonard, M. A. & West, T. S. (1959). *J. chem. Soc.* p. 3577.

Bell, A. T., Newton, L. G., Everest, S. L. & Legg, J. (1955). *Aust. vet. J.* **31**, 249.

Bonner, W. D. & Thimann, K. V. (1950). *Amer. J. Bot.* **37**, 66.

Chung, C. W. & Nickerson, N. J. (1953). *J. biol. Chem.* **208**, 395.

Conway, E. J. & O'Malley, E. (1946). *Biochem. J.* **40**, 59.

Elzam, O. E., Rains, D. W. & Epstein, E. (1964). *Biochem. biophys. Res. Commun.* **15**, 273.

Epstein, E. (1953). *Nature, Lond.*, **171**, 83.

Epstein, E. & Jefferies, R. L. (1964). *Annu. Rev. Pl. Physiol.* **15**, 169.

Evans, A. J. (1955). *Plant Physiol.* **30**, 437.

Hall, R. J. (1963). *Analyst*, **88**, 76.

Malmström, Bo. (1961). In *The Enzymes*, vol. 5, chapter 29. Ed. by Boyer, P. D., Lardy, H. & Myrbäck, K. New York: Academic Press Inc.

Mikoajczyk, J. & Nowacki, E. (1961). *Genet. polon.* **2**, 55.

Miller, G. W. (1958). *Plant Physiol.* **33**, 199.

Murray, L. R., McConnell, J. D. & Whittem, J. H. (1961). *Aust. J. Sci.* **24**, 41.

Oelrichs, P. B. & McEwan, T. (1961). *Nature, Lond.*, **190**, 808.

Ordin, L. & Skoe, S. P. (1963). *Plant Physiol.* **38**, 416.

Peters, R. A. (1931). *Proc. Roy. Soc.* A, **133**, 140.

Peters, R. A. (1963). *Biochemical Lesions and Lethal Synthesis*, 210. London: Pergamon Press Ltd.

Peters, R. A. & Shorthouse, M. (1964). *Nature, Lond.*, **202**, 21.

Peters, R. A., Shorthouse, M. & Murray, L. R. (1964). *Nature, Lond.*, **191**, 1331.

Ramsey, L. L. & Clifford, P. A. (1949). *J. Ass. offic. agric. Chem.* **32**, 788.

Ramsey, L. L. & Patterson, W. I. (1951). *J. Ass. offic. agric. Chem.* **34**, 827.

Rodgers, K. (1961). *Biochem. J.* **80**, 240.

Simon, E. W. & Beevers, H. (1952). *New Phytol.* **51**, 163.

Singer, L. & Armstrong, W. D. (1959). *Analyt. Chem.* **31**, 105.

Sutcliffe, J. F. (1962). *Monogr. pure appl. Biol.* **1**, 52.

Yang, S. F. & Miller, G. W. (1962). *Plant Physiol.* **37** (Suppl.), lxix.

Yang, S. F. & Miller, G. W. (1963). *Biochem. J.* **88**, 509.