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EXPERIMENTS ON THE FLUOROACETATE METABOLISM OF *DICHAPETALUM CYMOSUM* (GIFBLAAR)

J. N. ELOFF* and B. VON SYDOW†

Margaretha Mes Institute for Plant Physiology and Biochemistry, University of Pretoria, Pretoria, South Africa

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Abstract—It was shown that application of fluoroacetate to leaf disks of *Dichapetalum cymosum* (Gifblaar) did not lead to an inhibition of oxygen uptake or accumulation of citrate, in contrast to the 'control plant' *Parinarium capense* which lacks fluoroacetate. The addition of fluorocitrate did, however, inhibit the oxygen uptake of both plants and caused an accumulation of citrate. From the results it was deduced that either citrate synthetase or acetate thiokinase from *D. cymosum* had different affinities for the fluorinated derivative and the 'normal' metabolite. The addition of fluoropyruvate to leaf disks caused a decrease in oxygen uptake and no change in the citrate concentration. From this it was deduced that fluoropyruvate inhibited pyruvate oxidase in both plants. It was concluded that the tolerance of *D. cymosum* to such high concentrations of fluoroacetate may be ascribed to the fact that the 'lethal synthesis' of fluorocitrate does not take place in the plant most probably because citrate synthetase has different affinities for fluoroacetylcoenzyme A and acetylcoenzyme A.

INTRODUCTION

IN 1944 Marais[1] showed that the toxic principle of the South African plant *Dichapetalum cymosum* (Gifblaar) is fluoroacetate. The results obtained by Martius[2] and by Peters and his co-workers[3] proved that the toxicity of fluoroacetate to animals is due to an *in vivo* synthesis of fluorocitrate which competitively inhibits aconitase. Treble, Lamport and Peters[4] demonstrated that plant aconitase is also inhibited by fluorocitrate and they made the following remark: "... the mystery remains why *D. cymosum* and *Acacia georginae* which make fluoroacetate are not poisoned by their own product". It has been shown that very young leaves of Gifblaar may contain up to about 50 μg of fluoroacetate per mg of nitrogen.[5] It has also been shown that Gifblaar has an active Krebs cycle.[6] In this communication the reason why fluoroacetate does not inhibit the metabolism of the plant is investigated.

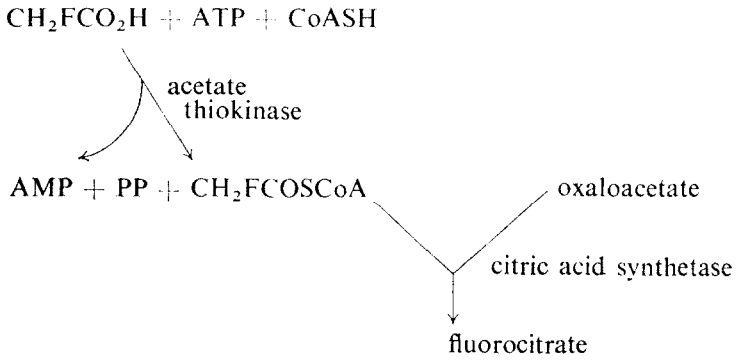
One possibility is that the fluoroacetate could be compartmentalized from the mitochondria by being located in the vacuole. The fact that the fluoroacetate concentration/mg dry wt. decreases with ageing[5] and the total vacuole volume increases with ageing more or less rules out this possibility. In the case of very young leaves it seems likely that the vacuoles could not contain all the fluoroacetate present in the leaves.

* Present address: Botany Department, University of the Orange Free State, Bloemfontein, South Africa.

† Present address: Botanisches Institut der Universität, 355 Marburg/Lahn, Pilgrimstein 4, West Germany.

[1] J. S. C. MARAIS, *Onderstepoort J. Vet. Sci. Animal Ind.* **20**, 67 (1944).
[2] C. MARTIUS, *Ann.* 561, 227 (1949).
[3] R. A. PETERS, *Advan. Enzymol.* **18**, 113 (1957).
[4] D. H. TREBLE, D. T. A. LAMPORT and R. A. PETERS, *Biochem. J.* **85**, 113 (1962).
[5] B. VON SYDOW, *Flora* **160**, 196 (1969).
[6] J. N. ELOFF, *Z. Pflanzenphysiol.* (in press).

The metabolism of fluoroacetate in other organisms may be represented as follows:



If Gifblaar does contain an active Krebs cycle as has been shown elsewhere[6,7] one of the following possibilities may explain the resistance of Gifblaar to fluoroacetate poisoning:

(1) The transformation of fluoroacetate to fluoroacetylcoenzyme A does not take place because the affinity of Gifblaar thiokinase for acetate is different from the affinity for fluoroacetate. The fact that the labelling pattern found when feeding acetate-^{14}C to young Gifblaar leaves was consistent with the metabolism of acetate through the Krebs cycle[7] indicates that the plant is equipped with acetate thiokinase.

(2) The transformation of fluoroacetylcoenzyme A to fluorocitrate does not take place because citrate synthethase has different affinities for fluoroacetylcoenzyme A and acetylcoenzyme A.

(3) Fluoroacetate is transformed to fluorocitrate and the aconitase in the plant is able to metabolize fluorocitrate, most probably with the subsequent liberation of fluorine as inorganic fluorine in a later stage of the metabolism.

Respiration studies were undertaken with Gifblaar in the presence of fluoroacetate, ethylfluorocitrate, fluorocitrate and fluoropyruvate to test the various possibilities. *Parinarium capense* Harv., a non-poisonous plant associated with Gifblaar in the veld and morphologically similar to Gifblaar was chosen as a control in all experiments. One of the most marked effects of fluorocitrate poisoning is the increase in citrate concentration due to inhibition of aconitase.[8] Therefore the citrate concentration was also determined in different experiments to verify the results obtained by respiration studies.

RESULTS AND DISCUSSION

The results obtained when the influence of fluoroacetate on the respiration of Gifblaar was examined by one of us are fully reported elsewhere.[5] On the whole, addition of fluoroacetate led to inhibition of the respiration of *P. capense* but had no effect or in some cases even a stimulation on the respiration of Gifblaar leaf disks.

The triethyl ester of fluorocitric acid was prepared by a Reformatsky-type reaction according to the method of Rivett.[9] When this compound was used in respiration studies it was found that the ester had no or very little effect on the respiration of either Gifblaar or *P. capense* leaf disks, most probably because it is not easily hydrolyzed *in vivo*. It has been

[7] J. N. ELOFF, D.Sc. Thesis, Potchefstroom University for C.H.E., South Africa (1967).
[8] G. KALNITSKY, *Arch. Biochem. Biophys.* **17**, 403 (1948).
[9] D. E. A. RIVETT, *J. Chem. Soc.* 3710 (1955).

found that ethyl fluorocitrate is not poisonous to animals.[10] There are many problems associated with the hydrolysis of ethyl fluorocitrate,[11] and when barium fluorocitrate became commercially available (Calbiochem), this compound was used for further work.

As the barium fluorocitrate did not dissolve readily in the buffer (0·3M KH_2PO_4) it was dissolved by acidifying slightly and resetting the pH with 50% KOH. The oxygen uptake of leaf disks of *P. capense* and Gifblaar floating on the different solutions was determined with the aid of a Warburg apparatus. Three concentrations of barium fluorocitrate were used (0·01M, 0·0001M, 0·0M-control) and the experiment was repeated twice with four replicates in each case.

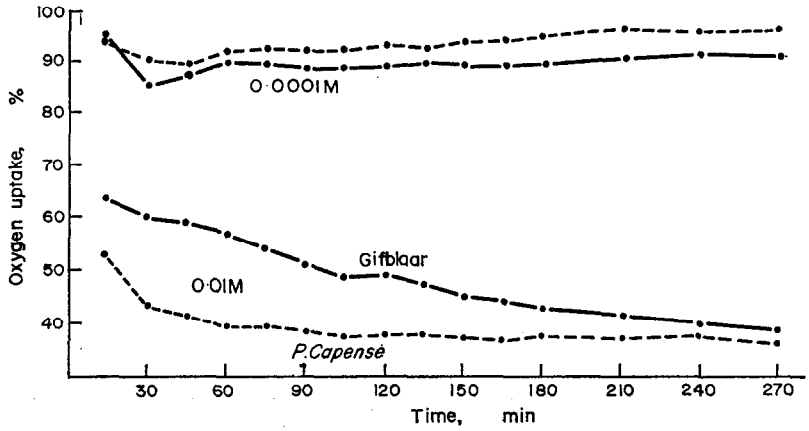


FIG. 1. THE INFLUENCE OF BARIUM FLUOROCITRATE ON THE PERCENTAGE OXYGEN UPTAKE (CONTROL = 100%) OF LEAF DISKS OF GIFBLAAR (● —— ●) AND *Parinarium capense* (● - - - - - ●). Control rate of oxygen uptake was 125·0 μl O_2/hr/mg protein for Gifblaar and 67·2 μl O_2/hr/mg protein for *P. capense*.

From the results presented in Fig. 1 it is clear that barium fluorocitrate strongly inhibited the oxygen uptake of both Gifblaar and *P. capense* leaf disks. The higher concentration had a bigger effect than the lower concentration in each case. To ensure that the effect was not due to an artifact, the experiment was repeated with equimolar concentrations of $BaCl_2$, free fluorocitric acid and citric acid. Fluorocitric acid had nearly quantitatively the same inhibitory effect as barium fluorocitrate, but $BaCl_2$ and citric acid had no effect on the oxygen uptake whatsoever.

The results indicate that Gifblaar has an active Krebs cycle, that aconitase is inhibited by fluorocitrate and that fluoroacetate cannot be transformed to fluorocitrate *in vivo* in Gifblaar. Therefore either acetate thiokinase has a different affinity for acetate than for fluoroacetate or citric acid synthethase has a different affinity for fluoroacetylcoenzyme A than for acetylcoenzyme A.

In order to discriminate between these possibilities, respiration studies with fluoroacetylcoenzyme A would have been appropriate. The chemical synthesis of this compound

[10] F. L. M. PATTISON and R. A. PETERS, in *Handbuch der experimentelle Pharmakologie* (edited by O. ELCHLER, A. FARAH, H. HERKEN and A. D. WELCH), Vol. XX/I, p. 401, Springer-Verlag, Berlin (1966).
[11] P. F. V. WARD and R. A. PETERS, *Biochem. J.* **78**, 661 (1961).

has been described,[12,13] but fluoroacetylcoenzyme A is very unstable and the reagents for the synthesis are very expensive. It could well be, fluoroacetylcoenzyme A being a large molecule (mol. wt. 831), that it will not readily be taken up by leaf disks. Although fluoroacetylcoenzyme A was not commercially available, fluoropyruvate could be obtained commercially. An attempt was therefore made to synthesize fluoroacetylcoenzyme A *in vivo* by feeding fluoropyruvate. If fluoropyruvate is transformed by Gifblaar to fluoroacetylcoenzyme A and the block of fluorocitrate formation from fluoroacetate were at the acetate thiokinase site only, the addition of fluoropyruvate would lead to an inhibition of respiration due to the synthesis of fluorocitrate. On the other hand, if the block of fluorocitrate formation from fluoroacetate were at the citrate synthetase site, addition of fluoropyruvate would not lead to an inhibition of oxygen uptake in Gifblaar because the fluoropyruvate would not be transformed to fluorocitrate. The influence of fluoropyruvate on the oxygen uptake of leaf disks of *P. capense* and Gifblaar was determined as in the previous experiment.

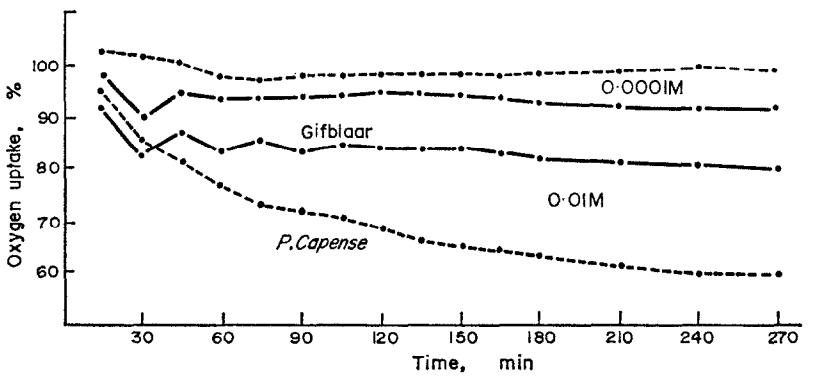


FIG. 2. THE INFLUENCE OF FLUOROPYRUVATE ON THE PERCENTAGE OXYGEN UPTAKE (CONTROL = 100%) OF LEAF DISKS OF GIFBLAAR (● —— ●) AND *Parinarium capense* (● - - - - ●). Control rate of oxygen uptake was 132·7 μl O_2/hr/mg protein for Gifblaar and 76·1 μl O_2/hr/mg protein for *P. capense*.

From the results in Fig. 2 it is clear that fluoropyruvate inhibited the oxygen uptake of both *P. capense* and Gifblaar leaf disks and that the higher concentration had a bigger effect than the lower concentration in both cases. If fluoropyruvate is transformed to fluoroacetylcoenzyme A by pyruvate oxidase and fluoropyruvate as such does not inhibit respiration, these results indicate that fluorocitrate is formed from fluoroacetylcoenzyme and therefore the block in fluorocitrate synthesis from fluoroacetate in Gifblaar does not lie at the citrate synthetase site but rather at the acetate thiokinase site.

One of the marked effects of fluoroacetate poisoning is the accumulation of citrate due to the competitive inhibition of aconitase by the *in vivo* synthesis of fluorocitrate.[8] To verify the results obtained with the respiration studies it was decided to determine the influence of fluoroacetate, fluoropyruvate and barium fluorocitrate on the citrate accumulation of leaf disks of Gifblaar and *P. capense*. The method of McArdle[14] was used, but due to interfering compounds present in the plant extracts, the extracts had to be purified by passing through activated charcoal, cation- and anion exchange resins. The experiment was

[12] R. O. BRADY, *J. Biol. Chem.* **217**, 213 (1955).
[13] A. MARCUS and W. B. ELLIOTT, *J. Biol. Chem.* **218**, 823 (1956).
[14] B. MCARDLE, *Biochem. J.* **60**, 647 (1955).

repeated four times with Gifblaar and three times with *P. capense* leaf disks. Very big differences were found in the citric acid content of the control treatments. (Gifblaar: 651–1398 μg citric acid/10 mg N. *P. capense:* 108–366 μg citric acid/10 mg N.) These differences may be ascribed to different pool sizes of citric acid present in the plants at sampling time due to external and/or endogenous factors. A statistical analysis was made and the lowest significant differences were determined as shown in Fig. 3.

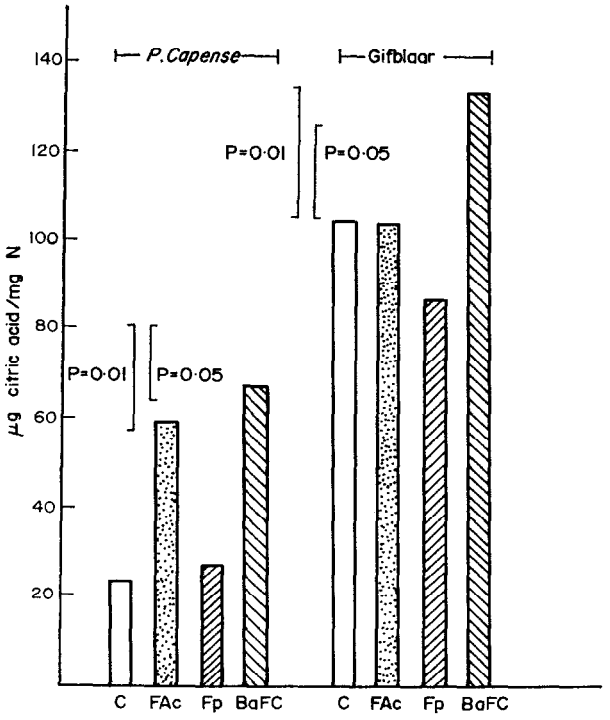


FIG. 3. THE INFLUENCE OF DIFFERENT PRETREATMENTS ON THE CITRIC ACID CONCENTRATION OF GIFBLAAR AND *Parinarium capense.*
C = control, FAc = fluoroacetate, FP = fluoropyruvate, BaFAc = barium fluorocitrate.

The most striking aspect of the results shown in Fig. 3 is that addition of fluoropyruvate to both Gifblaar and *P. capense* did not lead to an increase in citrate concentration although the oxygen uptake was inhibited. This obviously indicates that some other enzyme system, most probably pyruvate oxidase was inhibited and that fluoropyruvate was not transformed to fluorocitrate in either Gifblaar or *P. capense*. In has been shown that fluoropyruvate inhibits mammalian pyruvate oxidase.[15]

From the results it is clear that the addition of fluoroacetate to *P. capense*, while inhibiting the oxygen uptake[5] led to a statistically significant accumulation of citrate which indicates that an *in vivo* synthesis of fluorocitrate took place thereby inhibiting aconitase. As could be expected the addition of fluorocitrate to leaf disks of *P. capense* decreased the oxygen uptake and increased the citrate concentration. The fluoroacetate metabolism of *P. capense* is thus the same as that of other organisms.[3]

[15] A. TRAUB and Y. GINSBURG, *Exptl. Cell Res.* **17**, 246 (1959).

The addition of fluoroacetate to leaf disks of Gifblaar did not inhibit the oxygen uptake and in some cases stimulated the oxygen uptake.[5] The fact that addition of fluoroacetate to leaf disks did not lead to an increase in citrate concentration indicates that fluoroacetate was not transformed to fluorocitrate by Gifblaar. The fact that addition of barium fluorocitrate to leaf disks strongly inhibited the oxygen uptake and led to an increase in citrate concentration further substantiates the viewpoint that fluoroacetate is not transformed to fluorocitrate in Gifblaar leaves.

In these experiments fully grown leaves were used in order to obtain enough leaf disks for each treatment from the same leaf. (Each leaf had the same number of leaf disks represented in the different treatments.) It has been shown [6,7] that, as in other plants, the Krebs cycle plays a much more important role in young than in old leaves. If very young leaves were used, the effects found would most probably have been much greater.

From the results it seems that the main difference between Gifblaar and other organisms are due to the fact that acetate thiokinase and/or citrate synthetase from Gifblaar has a different affinity for the fluorinated and the 'normal' metabolite. At this point it is not possible to say which one of these enzyme systems differ from that of other organisms. In a related species, *D. toxicarium*, ω-fluoro-oleic acid and ω-fluoropalmitic acid has been found.[16,17] These compounds were most probably synthesized from fluoroacetate via fluoroacetylcoenzyme A and fluoromalonylcoenzyme A. It seems possible that fluoroacetylcoenzyme A would also be formed in *D. cymosum* and that the blockage to fluorocitrate synthesis from fluoroacetate therefore lies at the citrate synthetase level.

The fact that Gifblaar leaves have a relatively high citric acid content may indicate that a small amount of fluorocitrate is present in the plant, but because fluorocitrate competitively inhibits aconitase the high substrate concentration allows the transformation of enough citric acid to maintain the metabolism of the plant.

EXPERIMENTAL

Respiration Studies with Warburg Respirometer

Fully grown healthy leaves were picked in the veld at approximately 8–9 a.m. and at least 10 uniform leaves were selected. Twelve leaf disks of 8 mm dia. were punched out of each of the leaves and placed in 12 petri dishes containing distilled water. The petri dishes were shaken gently to and fro to remove damaged cell material and, after blotting dry with filter paper the leaf disks from each petri dish were carefully transferred to 12 13-ml Warburg flasks containing 3·0 ml of the different test solutions. In the inner compartment of the Warburg flasks 0·2 ml 10% KOH was placed. Runs were at 30° in the dark and flasks were shaken with an amplitude of 2 cm and a frequency of 80 c/min. After an equilibration time of 15 min the stopcocks were closed and the manometer-readings were taken every 15 min for 4·5 hr with the aid of a low-powered green lamp. The leaf disks were rinsed and the dry weight and N content were determined according to the micro Kjeldahl method.[18]

Determination of Citrate

When the method of McArdle[14] was applied to solutions of citric acid the method worked satisfactorily and gave reproducible results. When applied to plant extracts the results were erratic and very high values were obtained. The plants were extracted with MeOH–$CHCl_3$–H_2O (12:5:3) according to the method of Bieleski and Turner.[19] After defatting the extract it was separated in cationic, anionic and uncharged fractions by passing through Dowex 50 and Dowex-1 exchange resins.[20] The citric acid was determined on the formic acid eluate of the Dowex-1 column after evaporating the formic acid *in vacuo*. The results were,

[16] R. A. PETERS, R. J. HALL, P. F. V. WARD and N. SHEPPARD, *Biochem. J.* **77**, 17 (1960).
[17] P. F. V. WARD, R. J. HALL and R. A. PETERS, *Nature* **201**, 611 (1964).
[18] E. C. HUMPHRIES, in *Modern Methods of Plant Analysis* (edited by K. PAECH, M. V. TRACEY and H. F. LINSKENS), Vol. I, p. 479 Springer-Verlag, Berlin (1956).
[19] R. L. BIELESKI and R. A. TURNER, *Anal. Biochem.* **17**, 278 (1966).
[20] N. K. BOARDMAN, in *Modern Methods of Plant Analysis* (edited by K. PAECH, M. V. TRACEY and H. F. LINSKENS), Vol. V, p. 159, Springer-Verlag, Berlin (1962).

however, not satisfactory and usually unrealistically high. According to Weil-Malherbe and Bone[21] compounds such as salicyclic acid and other aromatic compounds interfere with the determination of citric acid. According to them the interference of these compounds may be eliminated by prior bromination. When this was tried a reduction in the citric acid content was encountered but some practical problems were encountered with the bromination. The aromatic compounds were subsequently removed by passing the extract through a small column of acid-washed activated charcoal[20] before separating the extract in cationic, anionic and uncharged fractions. When the citric acid concentration was determined in the anionic fraction reproducible values were obtained.

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[21] H. Weil-Malherbe and A. D. Bone, *Biochem. J.* **45**, 377 (1949).