Biochemical Studies on the Effect of Fluoride on Higher Plants

3. THE EFFECT OF FLUORIDE ON DARK CARBON DIOXIDE FIXATION

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A study by Yang & Miller (1963) indicated that leaves from fluoride-fumigated soya-bean plants contained greater concentrations of organic acids and amino acids than did leaves from control plants. The accumulation of these acids could have resulted from a variety of alternative or combined factors, such as an increase in the rate of glycolysis or of CO₂ fixation, which could result in increased rates of synthesis of these acids, blockage in utilization of these acids for protein synthesis and respiration, an increased rate of protein degradation etc.

Yang & Miller (1963) found a low respiratory quotient in fluoride-fumigated leaves. This low respiratory quotient would be expected if the accumulated amino acids and organic acids were derived from sugars. The accumulation of amino acids and organic acids from the degradation of protein would not account for the low respiratory quotient. Carboxylation of pyruvate or phosphoenolpyruvate would permit a net synthesis of dicarboxylic acids, and, if coupled with the reactions of the Krebs cycle and with transamination or amination, would permit the accumulation of any member of the cycle and related amino acids. Three such carboxylases are now known to be present in the tissues of higher plants. These are:

(a) 'Malic enzyme' (Ochoa, Mehler & Kornberg, 1948), which catalyses the reaction:

(b) Phosphoenolpyruvate carboxykinase (Tchen & Vennesland, 1954; Mazelis & Vennesland, 1957), which catalyses the reaction:

(c) Phosphoenolpyruvate carboxylase (Bandurski & Greiner, 1953), which catalyses the reaction:

This investigation was initiated to determine whether the accumulation of organic acids and amino acids in fluoride-necrotic leaves was due to

the increased rate of dark CO₂ fixation, and how fluoride might affect the carboxylating enzyme systems.

EXPERIMENTAL

Dark fixation of ¹⁴CO₂. Soya beans were grown and fumigated with HF as described by Yang & Miller (1963). At the end of fumigation, one control plant and one fumigated plant that showed slight necrotic symptoms were put into a 6 l. desiccator. After the introduction of ¹⁴CO₂ [generated from 10 mg. of Ba¹⁴CO₃ (0.0359 mc/mg.) with lactic acid], the desiccator was kept air-tight and in complete darkness for 4 hr.

Analysis of radioactive organic acids and amino acids. At the end of the dark ¹⁴CO₂ fixation, the desiccator was opened and the leaves from each plant were cut off and extracted with hot 80% (v/v) ethanol. The ethanol extracts were further fractionated with ion-exchange resins Amberlite IR-120 (H⁺ form) and Dowex 1 (CO₃²⁻ form) into the neutral fraction (effluent plus washing) which contained sugars, the cation fraction which contained amino acids, and the anion fraction which contained organic acids. Samples of the three fractions were counted for total radioactivity. The anion fraction was further chromatographed with a silica-gel column, and the fractions obtained were titrated with standard alkali. The neutralized solutions were subsequently evaporated to dryness and counted for radioactivity. The cation fraction was subjected to two-dimensional paper chromatography, and the positions of radioactive amino acids were detected by radioautography. Radioactivity in each spot was counted after elution with water. The procedures of chromatography were as outlined by Yang & Miller (1963).

Determination of ethanol-insoluble nitrogen and soluble protein. Leaves were dried at 80° and extracted three times with boiling 80% (v/v) ethanol for 30 min. The nitrogen content of the residue was estimated by the Kjeldahl method (Chibnall, Rees & Williams, 1943) and denoted as ethanol-insoluble nitrogen. Fresh leaves were ground and extracted with 20 mM-tris-HCl buffer, pH 8.0. The protein content of the extract that had been dialysed for 2 days against distilled water was estimated by Folin's phenol reagent (Lowry, Rosebrough, Farr & Randall, 1951) and referred to as soluble protein.

Phosphoenolpyruvate-carboxylase assays. Homogenates of control and necrotic leaves were prepared by grinding in a mortar 1 part (by wt.) of fresh leaves with 3 parts (by vol.) of 0.1 M-tris-HCl buffer, pH 8.0. The homogenates were strained through three layers of cheese-cloth and centrifuged at 1500g for 5 min. The supernatant solutions

were then dialysed against 1 mM-tris buffer containing EDTA (1 mM), pH 8.0, for 6 hr., and then against 1 mM-tris-HCl buffer, pH 8.0, for 20 hr. with two changes of the buffer solution. After dialysis, the homogenates were diluted with distilled water to twice the original volume. The undialysed as well as the dialysed solution was used as enzyme solution. Since there was usually some loss of activity after dialysis, only the freshly-prepared undialysed homogenate was used for quantitative assays of the enzyme activities of control and necrotic leaves. The reaction mixture contained: 1 μ mole of phosphoenolpyruvate; 120 μ moles of tris-HCl buffer, pH 8.0; 2 μ moles of $\text{KH}^{14}\text{CO}_3$, containing 52000 counts/min. (20 μ c) of radioactivity; 0.4 μ mole of NADH; and 0.2 ml. of undialysed or 0.4 ml. of dialysed enzyme solution in a total volume of 1 ml. The mixture was incubated for 15 min. at 37°. The reaction was stopped by adding 0.1 ml. of 10 mM-2,4-dinitrophenylhydrazine in 1.5N-HCl, which also expelled unfixed HCO_3^- ions as CO_2 . Samples (0.2–0.4 ml.) of the resultant mixture were transferred to Pyrex planchets and dried at room temperature over conc. H_2SO_4 and NaOH under reduced pressure in a desiccator, and counted with a Geiger-Müller counter to an accuracy of $\pm 3\%$ (6% efficiency). This method was essentially described by Huffaker, Hall, Shannon, Wallace & Rhoads (1959) as being a modification of that employed by Bandurski & Greiner (1953).

The protein content of the dialysed homogenates was determined by using Folin's phenol reagent (Lowry *et al.* 1951).

RESULTS

Dark $^{14}\text{CO}_2$ fixation. Table 1 shows the distribution of ^{14}C in sugars, amino acids and organic acids separated on ion-exchange resins. About four times as much ^{14}C was fixed in necrotic leaves as in control leaves. Under the present experimental conditions, ^{14}C appeared almost entirely in organic acid and amino acid fractions; little was found in the sugar fraction.

The amounts of various organic acids and their radioactivities are shown in Table 2. The results indicated that $^{14}\text{CO}_2$ was fixed mostly into malonic acid, malic acid and citric acid. These acids were also those mainly involved in the increases of organic acids in necrotic leaves. Radioactivity was found in all organic acids.

The amounts of various amino acids and their radioactivities are shown in Table 3. Unlike the organic acids, radioactivity was found only in four amino acids: aspartic acid, glutamic acid, serine and asparagine. This difference indicated that the transformation among the organic acids was very fast, whereas the amino acid turnover was very slow. Most of the radioactivity was distributed between aspartic acid and glutamic acid in control leaves, and in asparagine, in addition to aspartic acid and glutamic acid, in necrotic leaves.

The compounds with high specific radioactivity (amount of radioactive acid/amount of total acid) were either first primary products of the dark CO_2 fixation or products of faster reactions. Malic acid among organic acids and aspartic acid among amino acids showed the highest specific radioactivities. Malonic acid and citric acid among organic acids and glutamic acid among amino acids also showed high specific radioactivities. The accumulation of ^{14}C in the C_4 dicarboxylic acids, malic acid and aspartic acid, suggested the addition of CO_2 to a C_3 unit. Citric acid and glutamic acid could have been the coupled products in the Krebs cycle derived from the C_4 dicarboxylic acid. These

Table 1. *Distribution of ^{14}C in different fractions separated on ion-exchange resins in control and fluoride-necrotic soya-bean leaves after a 4 hr. exposure to $^{14}\text{CO}_2$ in a dark system*

Experimental details are given in the text. Results are expressed as counts/min./g. fresh wt. of leaf.

Treatment	Control	HF (0.03 p.p.m.)
Fraction		
Sugars	50	190
Amino acids	5190	32420
Organic acids	9340	28220
Total	14580	60830

Table 2. *Total organic acids and ^{14}C -labelled organic acids in control and fluoride-necrotic soya-bean leaves after a 4 hr. exposure to $^{14}\text{CO}_2$ in a dark system*

Experimental details are given in the text. Total acids are expressed as μ equiv./g. fresh wt. of leaf, radioactive acids as counts/min./g. fresh wt. of leaf and specific radioactivities as counts/min./ μ equiv. of acid.

Treatment	Total acid		Radioactive acid		$10^{-2} \times \text{Sp. radioactivity}$	
	Control	HF (0.03 p.p.m.)	Control	HF (0.03 p.p.m.)	Control	HF (0.03 p.p.m.)
Organic acid						
Fumaric acid	3.3	3.3	230	360	0.7	1.1
Succinic acid	1.7	2.9	110	410	0.6	1.4
Malonic acid	13.8	20.3	5310	10900	3.8	5.4
Malic acid	3.0	9.7	1380	7900	4.6	8.2
Citric acid	5.0	8.6	1070	6440	2.1	7.5
Total	26.8	44.8	8100	26010		

Table 3. *Total amino acids and ^{14}C -labelled amino acids in control and fluoride-necrotic soya-bean leaves after a 4 hr. exposure to $^{14}\text{CO}_2$ in a dark system*

Experimental details are given in the text. Total amino acids are expressed as $\mu\text{moles/g.}$ fresh wt. of leaf, radioactive amino acids as counts/min./g. fresh wt. of leaf and specific radioactivities as counts/min./ μmole of amino acid. Tr., Trace amounts too low for accurate determination; N.D., not detected.

Treatment ... Amino acid	Total amino acid		Radioactive amino acid		$10^{-2} \times \text{Sp. radioactivity}$	
	Control	HF (0.03 p.p.m.)	Control	HF (0.03 p.p.m.)	Control	HF (0.03 p.p.m.)
Asparagine	1.3	16.6	240	5760	1.9	3.8
Aspartic acid	2.6	6.5	2550	14510	9.8	22.3
Glutamic acid	4.2	5.9	1460	8500	3.5	14.5
Serine	1.5	2.7	160	690	1.1	2.6
Glutamine	Tr.	0.5	N.D.	N.D.		
Threonine	0.3	1.3	N.D.	N.D.		
Alanine	0.5	0.7	N.D.	N.D.		
Tyrosine	Tr.	0.6	N.D.	N.D.		
γ -Aminobutyric acid	0.4	0.6	N.D.	N.D.		
Phenylalanine	0.2	0.9	N.D.	N.D.		
Leucine + isoleucine	0.1	2.2	N.D.	N.D.		
Pipecolic acid	N.D.	2.0	N.D.	N.D.		
Total	11.1	40.5	4410	29460		

results support the conclusions that dark CO_2 fixation in higher plants is principally through the carboxylation of a C_3 acid, phosphoenolpyruvate or pyruvate. Although considerable asparagine was found in a soya bean, its radioactivity was low. An increased rate of dark CO_2 fixation in necrotic leaves is also manifested by the higher specific radioactivities of organic acids and amino acids in that tissue.

The results in Tables 2 and 3 indicate a close correlation between the increases in total organic acids and amino acids and the increased rate of dark CO_2 fixation found in fluoride-necrotic leaves compared with leaves of normal plants. The accumulation of organic acids and amino acids in necrotic leaves could be attributed to the increased rate of dark CO_2 fixation.

Changes in alcohol-insoluble nitrogen and soluble protein. If the accumulation of amino acids were partly attributable to the decreased rate of protein synthesis from amino acids or to the increased rate of protein degradation, it would be expected that the protein content of the fluoride-necrotic leaves would be lessened. As shown in Table 4, both the alcohol-insoluble nitrogen and soluble protein contents on a fresh-weight basis were slightly higher in necrotic leaves than in control leaves. These increases could not be attributed totally to the higher dry weight of the necrotic leaves, since the increases both in alcohol-insoluble nitrogen and in soluble protein in necrotic leaves as compared with the control leaves were greater than the increases in dry weight. Apparently neither the decreased rate of protein synthesis nor the increased rate of protein degradation contributed substantially to the

Table 4. *Alcohol-insoluble nitrogen and soluble protein in control and fluoride-necrotic soya-bean leaves*

Experimental details are given in the text. Results are expressed as mg./g. fresh wt. of leaf.

Treatment	Dry wt.	Alcohol-insoluble nitrogen	Soluble protein
Control	191	5.0	25.4
HF (0.03 p.p.m.)	208	6.9	34.9

accumulation of amino acids in the fluoride-fumigated leaves.

$^{14}\text{CO}_2$ fixation in leaf homogenate. Amounts of $^{14}\text{CO}_2$ fixed by soya-bean homogenate, with pyruvate and phosphoenolpyruvate as substrates in the presence of other cofactors, are listed in Table 5. Under these experimental conditions, carboxylation was much greater with phosphoenolpyruvate than with pyruvate. The addition of NADH to the phosphoenolpyruvate reaction mixture increased the fixation by about 30 %. The radioactive products of the reaction mixture, to which 2,4-dinitrophenylhydrazine-hydrochloric acid had been added to stop the reaction, were tentatively identified by radioautography of a paper chromatogram developed with the solvent system pentan-1-ol-formic acid-water (Buch, Montgomery & Porter, 1952). In this solvent system the hydrazone of oxaloacetate moved very fast and separated from the free acids. Without NADH in the reaction mixture, only oxaloacetate hydrazone was found. With NADH, most of the radioactivity was found in malic acid and very little

in oxaloacetate hydrazone. The results indicated the presence of malate dehydrogenase in the homogenate. NADH might act to increase the $^{14}\text{CO}_2$ fixation through its effect in transforming the unstable oxaloacetate into the stable malic acid. The inhibitory effect of ATP or ADP on CO_2 fixation in the phosphoenolpyruvate- Mn^{2+} ion system (Table 5) was contrary to the finding of Huffaker & Wallace (1959), who reported that the addition of ATP in the phosphoenolpyruvate- Mn^{2+} ion system increased CO_2 fixation by about 45%. The present results suggested that most of the phosphoenolpyruvate-carboxylating activity in this enzyme preparation was due to phosphoenolpyruvate carboxylase, and there was little, if any, phosphoenolpyruvate carboxykinase, which requires ADP.

Table 5. $^{14}\text{CO}_2$ fixation in soya-bean homogenates as influenced by different substrates and cofactors

Each reaction mixture contained: 1 μmole of phosphoenolpyruvate, 2 μmoles of $\text{KH}^{14}\text{CO}_3$ (52000 counts/min.), 120 μmoles of tris-HCl buffer, pH 8.0, MgCl_2 and MnCl_2 as indicated, 1 μmole of CaCl_2 , 0.4 μmole of NADH, 0.4 μmole of NADPH, 4 μmoles of ATP, 4 μmoles of ADP, 2 μmoles of pyruvate, enzyme and water in a total volume of 1 ml. Incubation was at 37° for 15 min. The reaction was stopped by adding 0.1 ml. of 10M-2,4-dinitrophenylhydrazine in N-HCl. Samples of 0.4 ml. were used for radioactivity measurements after drying. Results are expressed as counts/min. Expt. A, with 0.2 ml. of undialysed homogenate; Expt. B, with 0.4 ml. of dialysed homogenate.

Reaction system with $\text{KH}^{14}\text{CO}_3$	Total ^{14}C fixed
Expt. A. Homogenate alone	0
+ Phosphoenolpyruvate	820
+ Phosphoenolpyruvate + MgCl_2 (10 μmoles)	1280
+ Phosphoenolpyruvate + NADH	1140
+ Phosphoenolpyruvate + MgCl_2 (2 μmoles) + NADH	1400
+ Phosphoenolpyruvate + MgCl_2 (10 μmoles) + NADH	1700
+ Phosphoenolpyruvate + MnCl_2 (1 μmole) + NADH	1480
+ Phosphoenolpyruvate + MnCl_2 (1 μmole) + NADH + ADP	600
+ Phosphoenolpyruvate + MnCl_2 (1 μmole) + NADH + ATP	250
+ Phosphoenolpyruvate + CaCl_2 + NADH	1100
+ Pyruvate	0
+ Pyruvate + MnCl_2 (1 μmole)	0
+ Pyruvate + MnCl_2 (1 μmole) + NADH	10
+ Pyruvate + MnCl_2 (1 μmole) + NADPH	140
Expt. B. Homogenate alone	0
+ Phosphoenolpyruvate + NADH	210
+ Phosphoenolpyruvate + MgCl_2 (2 μmoles) + NADH	920
+ Phosphoenolpyruvate + MgCl_2 (10 μmoles) + NADH	1360
+ Phosphoenolpyruvate + MnCl_2 (1 μmole) + NADH	1160
+ Phosphoenolpyruvate + MnCl_2 (5 μmoles) + NADH	940
+ Phosphoenolpyruvate + CaCl_2 + NADH	100

Further, this phosphoenolpyruvate-carboxylase activity is inhibited by ADP or ATP. Experimental evidence supporting this supposition is discussed below. The absence of phosphoenolpyruvate carboxykinase in this preparation was further confirmed by the absence of the exchange reaction. Phosphoenolpyruvate carboxylase required Mg^{2+} or Mn^{2+} ions for maximum activity when dialysed homogenates were used (Table 5). Activity without these metal ions was only one-sixth of that in the presence of optimum concentrations of Mg^{2+} ion (10 mg. ions/l.) or Mn^{2+} ion (1 mg. ion/l.). Though the activation with Mn^{2+} ions was a little smaller than that with Mg^{2+} ions, the optimum concentration of Mn^{2+} ions required for activation was much lower than that of Mg^{2+} ions; Ca^{2+} ions were found to be inhibitory.

Malic-enzyme activity in the crude leaf homogenate was indicated by the requirement for pyruvate and NADPH (Table 5). However, the activity was low compared with that of phosphoenolpyruvate carboxylase. Since phosphoenolpyruvate carboxylase was the most active carboxylating enzyme in the soya-bean homogenate, comparative activity measurements of this enzyme in control and fluoride-necrotic leaves and the effect of fluoride on the enzyme activity *in vitro* were studied.

Activity of phosphoenolpyruvate carboxylase in homogenates of control and necrotic soya-bean leaves. Fig. 1 illustrates the relation of total ^{14}C fixed to enzyme concentration. There was a slight departure from linearity between the total ^{14}C fixed and the enzyme concentration; however, it is possible to get a corrected value for the ^{14}C fixed which is proportional to the enzyme concentration, as shown in Fig. 1. The activities of phosphoenolpyruvate carboxylase in homogenates from control and necrotic soya-bean plants are given in Table 6. Greater fixation of CO_2 was always obtained with homogenates from necrotic soya-bean leaves. The contaminating fluoride in the reaction mixture with an homogenate of fluoride-necrotic leaves was estimated to be about 0.5 μmole . The addition of 1 μmole of fluoride to the reaction mixture usually slightly increased the fixation of $^{14}\text{CO}_2$, but the extent of this activation varied from one enzyme preparation to another. This stimulation effect could not be attributed to the stabilization of labile reaction products during the drying period, since the addition of fluoride at the conclusion of the enzyme reaction, so that it was present during the drying period only, did not increase the radioactivity appreciably. Nevertheless, it is clear from the results in Table 6 that the increased carboxylase activity found in homogenates from necrotic soya-bean leaves could not be attributed to the pre-existing fluoride in the homogenate, since the

addition of fluoride to the homogenate of control leaves did not increase the activity to the level of that of necrotic leaves.

Inhibition of phosphoenolpyruvate carboxylase by adenosine triphosphate and adenosine diphosphate. The results in Table 5 indicate that phosphoenolpyruvate carboxylation is inhibited by ATP and ADP. Possible causes of this inhibition are that either ATP and ADP are themselves inhibitors of the enzymic reaction, or that ATP and ADP may participate in other competing reactions for phosphoenolpyruvate and thus decrease the available concentration of substrate. The reactions that might compete for phosphoenolpyruvate involve the reactions catalysed by phosphoenolpyruvate phosphatase and pyruvate kinase, which requires ADP. The activity of pyruvate kinase plus phosphoenolpyruvate phosphatase was measured by incubating phosphoenolpyruvate with leaf homogenate, and the pyruvate formed was estimated by the method described by Kachmar & Boyer (1953). The results showed that both ATP and ADP inhibited the transformation of phosphoenolpyruvate into pyruvate. Therefore the enzyme responsible was judged to be phosphatase and not pyruvate kinase. This phosphatase was also found

to be inhibited appreciably by fluoride. It seems clear then that neither ATP nor ADP competed for phosphoenolpyruvate, but they inhibited the hydrolysis of phosphoenolpyruvate and thus provided a higher concentration of phosphoenolpyruvate. From these results it is concluded that the reaction catalysed by phosphoenolpyruvate carboxylase was inhibited directly by ADP or by ATP. The inhibition of phosphoenolpyruvate carboxylase by ADP and ATP is shown in Table 7.

Table 6. *Phosphoenolpyruvate-carboxylase activity in homogenates of control and fluoride-necrotic soya-bean leaves*

The reaction mixture contained: 1 μ mole of phosphoenolpyruvate, 2 μ moles of $\text{KH}^{14}\text{CO}_3$ (52000 counts/min.), 0.4 μ mole of NADH, 10 μ moles of MgCl_2 , 120 μ moles of tris-HCl buffer, pH 8.0, KF as indicated, and 0.2 ml. of undialysed homogenate, equivalent to 50 mg. of fresh leaf, in a total volume of 1 ml. The protein contents of the homogenates were: Expt. A, control, 1.56 mg.; HF, 2.06 mg.; Expt. B, control, 1.28 mg.; HF, 1.86 mg. The results are expressed as corrected counts/min. (see Fig. 1).

	Amount of KF added (μ moles)	Treatment	
		Control	HF (0.03 p.p.m.)
Expt. A	0	800	2000
	1	1020	2260
	10	840	2000
Expt. B	0	750	1860
	1	900	1860

Table 7. *Inhibition of phosphoenolpyruvate carboxylase by adenosine triphosphate and adenosine diphosphate*

The reaction mixture contained: 1 μ mole of phosphoenolpyruvate, 2 μ moles of $\text{KH}^{14}\text{CO}_3$ (30000 counts/min.), 120 μ moles of tris-HCl buffer, pH 8.0, 10 μ moles of MgCl_2 , 1 μ mole of MnCl_2 , 0.4 μ mole of NADH, 4 μ moles of ADP, 4 μ moles of ITP, ATP as indicated, and 0.4 ml. of dialysed homogenate in a total volume of 1 ml. Incubation was at 37° for 15 min. Results are expressed as counts/min.

Reaction system with $\text{KH}^{14}\text{CO}_3$	Total ^{14}C fixed
Phosphoenolpyruvate + NADH + MgCl_2	700
Phosphoenolpyruvate + NADH + MgCl_2 + ADP	590
Phosphoenolpyruvate + NADH + MgCl_2 + ATP	600
(2 μ moles)	
Phosphoenolpyruvate + NADH + MgCl_2 + ATP	530
(4 μ moles)	
Phosphoenolpyruvate + NADH + MgCl_2 + ATP	450
(8 μ moles)	
Phosphoenolpyruvate + NADH + MnCl_2	500
Phosphoenolpyruvate + NADH + MnCl_2 + ADP	220
Phosphoenolpyruvate + NADH + MnCl_2 + ATP	430
(2 μ moles)	
Phosphoenolpyruvate + NADH + MnCl_2 + ATP	90
(8 μ moles)	
Phosphoenolpyruvate + NADH + MnCl_2 + ITP	100

Fig. 1. Dependence of ^{14}C fixation on concentration of phosphoenolpyruvate carboxylase in soya-bean leaves. Each reaction mixture contained: 1 μ mole of phosphoenolpyruvate, 2 μ moles of $\text{KH}^{14}\text{CO}_3$ (52000 counts/min.), 0.4 μ mole of NADH, 10 μ moles of MgCl_2 , 120 μ moles of tris-HCl buffer, pH 8.0, with variation in the volume of undialysed homogenate to give a total volume of 1 ml. Incubation was at 37° for 15 min. The corrected value (counts/min.) can be obtained from the measured value on the ordinate (already corrected for self-absorption), which is proportional to the enzyme concentration on the abscissa. For example: a measured radioactivity of 2000 counts/min. is corrected to 2500 counts/min.

ADP was a less effective inhibitor than ATP in the presence of either Mg^{2+} or Mn^{2+} ions. The inhibitory effect of these nucleotides on phosphoenolpyruvate carboxylase might be due to binding of the activating metal ions rather than to a direct effect on the enzyme.

DISCUSSION

The following results support the conclusion that the accumulation of organic acids and amino acids in necrotic leaves resulted from the increased rate of the dark CO_2 fixation: (1) A close correlation existed between the increase in total organic acids and amino acids, and the increased rate of dark CO_2 fixation in the fluoride-necrotic leaves compared with that in the control leaves; (2) equal or greater amounts of protein associated with necrotic leaves; (3) a lower respiratory quotient in necrotic leaves (Yang & Miller, 1963).

Phosphoenolpyruvate-carboxylase activity was higher in necrotic leaves than in control leaves. An increased rate of dark CO_2 fixation does not only depend on enzyme activity, however. The present results do not exclude other possibilities such as the higher phosphoenolpyruvate concentration in the tissue resulting from higher respiratory and glycolysis rate, or the higher CO_2 concentration in the tissue due to easier penetration into the necrotic leaves. The mechanisms of a fluoride-induced increase in dark CO_2 fixation and its relation to the cause of fluoride necrosis has not been studied. From the increased rate of dark CO_2 fixation, which results in an increased free amino acid pool in the fluoride-fumigated leaves, it is very likely that some abnormal protein or enzymes are actively synthesized in the fluoride-fumigated plants by utilizing these abnormally accumulated free amino acids at an early stage of fluoride-induced necrosis. These, in turn, could induce the subsequent abnormal metabolism which leads finally to necrosis.

The increased dark CO_2 fixation in fluoride-necrotic leaves makes it unlikely that enolase, which was reported to be sensitive to fluoride inhibition *in vitro* (Miller, 1958), was inhibited appreciably *in vivo*.

Wallace, Mueller, van Noort & North (1960) reported a similar relationship between increased dark CO_2 fixation and increased organic acids in cases of iron-deficiency chlorosis and virus-induced chlorosis.

Two possibilities might explain the stimulation of phosphoenolpyruvate-carboxylase activity in crude soya-bean homogenates by fluoride, namely

the inactivation of inhibitory metal ions and inhibition of the hydrolysis of phosphoenolpyruvate. Nevertheless, at higher concentration (10 mM) fluoride was slightly inhibitory to the carboxylase (Table 6). It seems likely that fluoride might stimulate the carboxylation of phosphoenolpyruvate *in vitro* as well as *in vivo* by inhibiting the phosphoenolpyruvate-phosphatase activity which competes for the substrate.

SUMMARY

1. Dark CO_2 fixation and phosphoenolpyruvate-carboxylase activity were studied in fluoride-necrotic and control soya-bean leaves.
2. Necrotic leaves had a higher rate of dark CO_2 fixation than control leaves both *in vivo* and *in vitro* (phosphoenolpyruvate carboxylase).
3. Results suggested that the accumulation of organic acids and amino acids in necrotic leaves resulted from an increased rate of dark CO_2 fixation.
4. The possible role of fluoride in stimulating the carboxylation and its implication to necrosis are discussed.

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