Biochemical Studies on the Effect of Fluoride on Higher Plants

1. METABOLISM OF CARBOHYDRATES, ORGANIC ACIDS AND AMINO ACIDS

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Fluoride occurs naturally in plants, but its presence has attracted attention primarily in certain industrial areas where concentrations are elevated above normal by accumulation from the atmosphere. Certain plant species have been observed to be injured as a result of the accumulation of excessive fluoride from the atmosphere. The general symptoms of fluoride injury are necrotic lesions and burning, which appear first in the leaf tips and margins.

The exact mechanism by which fluoride causes damage to plants is little understood. Nevertheless, certain physiological processes are known to be markedly affected by fluoride. For example, a decrease in chlorophyll (McNulty & Newman, 1961), a diminished rate of photosynthesis (Thomas & Hendricks, 1956), decreased plant growth (Bonner & Thimann, 1950) and increased respiration in growing plants (McNulty & Newman, 1957) have been reported. An effect of fluoride on a physiological process may be a reflection that one or more enzyme systems are affected by fluoride.

The effects of fluoride on higher plants could be more clearly determined if we knew more specifically what metabolic steps are affected by it. When one or more normal metabolic steps are disturbed, some metabolic intermediates might occur either in relatively low or relatively high concentrations. In addition, some compounds not representing common metabolic components might appear in detectable quantities. These compounds could either be the cause or the product of abnormal metabolism. Quantitative and qualitative analyses of such accumulated metabolites could be valuable in implicating the possible site of fluoride action.

In the present investigation, the metabolic changes in carbohydrates, organic acids and amino acids that accompanied the development of necrosis resulting from atmospheric fluoride fumigation were studied.

EXPERIMENTAL

Materials. Soya bean (*Glycine max* var. Hawkeye) was germinated and grown in containers of soil in the greenhouse. Plants were placed in fumigation chambers when 6–8 in. tall. The fumigation chambers were constructed as described by Ross, Wiebe & Miller (1962). Treatments

consisted of fumigations with air which was contaminated with 0.00006 p.p.m. of HF ('control'), and with 0.03 p.p.m. of HF in air ('HF'). The leaves were harvested after 3–5 days of fumigation, depending on when marginal burning first became evident. After fumigation about 200 µg. of fluoride accumulated/g. of fresh leaf. The fluoride content was determined, after drying at 80° for 2 days, by the method outlined by Nielson (1958).

Extraction and separation of sugars, organic acids and amino acids. Soluble sugars, organic acids and amino acids were extracted, from fresh leaves (10–20 g.) from each treatment, three times with hot 80% (v/v) ethanol. The extracts were combined and the pH was adjusted to 6–7. After concentration to about 100 ml. *in vacuo* at 50°, the extract was passed through successive ion-exchange resin columns (2 cm. × 20 cm.) of Amberlite IR-120 (H⁺ form) and Amberlite IR-4B (OH⁻ form), and washed with water. The eluate and washings that contained sugars were combined, concentrated *in vacuo* at 50° to about 20 ml. and deproteinized with 2 ml. of saturated neutral lead acetate solution. Excess of lead acetate was removed with a mixture of Amberlite IR-120 (H⁺ form) and Amberlite IR-4B (OH⁻ form) by the bath method (Mori & Nakamura, 1959). Organic acids and amino acids were eluted from the anion-exchange and cation-exchange columns respectively with 2N-ammonia. The eluates were freed from an excess of ammonia and concentrated by evaporation under reduced pressure. Chloroform was added to the resulting solutions of sugars, organic acids and amino acids for preservation purposes and to remove contaminating pigments and other interfering substances.

Determination of total reducing sugars and non-reducing sugar. Total reducing sugars were determined by the method of Somogyi (1952) and calculated as glucose. Non-reducing sugar was determined by the difference in reducing power after and before hydrolysis with hydrochloric acid (Association of Official Agricultural Chemists, 1955).

Ion-exchange chromatography and analysis of sugars. Sugar-borate complexes were chromatographed with an analytical column (0.9 cm. × 5.0 cm.) of Dowex 1 (200–400 mesh, borate form) as described by Khym & Zill (1952). The effluent was collected in 15 ml. fractions by a fraction collector, and a 1 ml. sample from each fraction was used for sugar determination by the phenol-H₂SO₄ reagent (Dubois, Gilles, Hamilton, Rebers & Smith, 1956). The sugar concentration was determined from a standard curve made with known sugar samples at the same borate concentrations.

Partition chromatography and analysis of organic acids. Organic acids were separated by silica-gel partition chromatography and the fractions were titrated with 0.01 N-NaOH, with thymol blue as indicator. The general

procedures were similar to those of DeKock & Morrison (1958b) with the following modifications. The gradient-elution solvent system consisted of 320 ml. of chloroform, which had been washed with water and saturated with 0.1N-H₂SO₄, in a recipient bottle having a diameter of 8.5 cm., and 260 ml. of 50% (v/v) butan-1-ol in chloroform saturated with 0.1N-H₂SO₄ in a donor bottle having a diameter of 5.5 cm. The two bottles were connected with a siphon and the recipient bottle was connected to the silica-gel column for gradient elution. The dried sample applied to the column was dissolved in 0.1-0.5 ml. of 1.5N-H₂SO₄ to adjust the pH of the resulting solution to less than 2. If the resulting pH was higher than 2, the recovery of organic acids was poor, especially for chromatography of oxalic acid, which has pK_1 1.2. The frequently low recovery of oxalic acid by silica-gel column chromatography (DeKock & Morrison, 1958b) may possibly be attributable to a high pH of the sample rather than to the volatility of the acid. By the present method, recovery of individual acids was between 94 and 100%. Organic acids before column chromatography, as well as the fraction after elution and titration, were identified by paper chromatography (Buch, Montgomery & Porter, 1952).

Paper chromatography and determination of amino acids. The amino acids were separated by two-dimensional paper chromatography on sheets of Whatman no. 1 or no. 30 (18½ in. × 22½ in.) by using essentially the methods described by Levy & Chung (1953), with the modification that 0.01% of 8-hydroxyquinoline was added to the cresol-phenol-borate buffer solvent. The separated amino acids were detected on the air-dried chromatograms as described by Thompson & Morris (1959). Two standard papers with known amounts of the amino acids to be determined were run simultaneously and under the same conditions as the samples. The individual amino acid spots were cut out together with several blank areas, cut into several strips and extracted with 5 ml. of 50% (v/v) ethanol for 30 min. The extinction of the asparagine spot was read at 360 mμ and those due to all other amino acids at 570 mμ. The amount of each was estimated by comparison with the standards. At 360 mμ the yellow colour of asparagine has a high extinction, whereas the contaminating blue colour has a very low extinction. Leucine, isoleucine and phenylalanine were also determined separately by one-dimensional chromatograms in the butan-1-ol-acetic acid-water (4:1:5, by vol.; upper phase) solvent. In the two-dimensional chromatograms, these spots were diffuse and colour development with ninhydrin was inhibited. Replicate experiments showed that the accuracy of the method was always better than ±20%.

The amide nitrogen content in the amino acid fraction was determined by the method of Varner, Bullen, Vanecko & Burrell (1953).

Respiratory quotient. The respiratory quotient of detached leaves was measured directly with manometer flasks of Stanley & Tracewell (1955) at 30° in the dark.

RESULTS

Carbohydrates. As shown in Table 1, the values for sucrose obtained by the ion-exchange-resin chromatographic method and those for non-reducing sugar obtained by chemical methods are very close. The values for reducing sugars obtained by chemical methods, however, were higher than the sum of glucose and fructose obtained by chromatographic methods. Sucrose concentrations decreased remarkably, whereas those of reducing sugars increased in the leaves of fluoride-fumigated plants as compared with leaves from control plants. As a consequence, the ratio of non-reducing sugar (sucrose) to reducing sugar decreased. The effect of fluoride in this respect was also noted in leaves of *Chenopodium murale* after receiving 0.0005 or 0.005 p.p.m. of fluoride fumigation for 7 days, although no visual necrotic symptoms were evident in the plants receiving 0.0005 p.p.m. of hydrogen fluoride fumigation.

Two unidentified sugar fractions, not detectable in paper chromatograms, were found in ion-exchange-resin chromatograms between the fractions of sucrose and fructose. Pentose sugars were not detected in the sugar solutions by using the orcinol reagent of Drury (1948) nor by paper chromatography, in which only sucrose, glucose, and fructose were found.

Starch was analysed for amylose and amylopectin content in fluoride-fumigated and control plants. No difference was observed in total starch content, although the amylose content was higher in fumigated plants.

Organic acids. The organic acid content of control and fluoride-fumigated leaves from soya bean is shown in Table 2. There were greater concentrations of organic acids in fluoride-necrotic leaves

Table 1. *Free sugars in normal and fluoride-fumigated soya-bean leaves*

Sucrose, fructose and glucose were determined after separation with an ion-exchange-resin column; non-reducing sugar was determined directly by chemical methods. Experimental details are given in the text. Total sugar is non-reducing sugar plus reducing sugar. Results are expressed as mg./100 g. of fresh leaf.

Treatment	Sucrose	Fructose	Glucose	Non-reducing sugar	Reducing sugar	Total sugar	Non-reducing sugar:reducing sugar ratio
Control	216	22	18	208	45	253	4.6
HF (0.03 p.p.m.)	66	33	27	55	66	111	0.8
Control	229	12	10	217	25	242	8.7
HF (0.03 p.p.m.)	81	28	15	86	55	141	1.6

Table 2. *Organic acids in normal and fluoride-fumigated soya-bean leaves*

Experimental details are given in the text. Results are expressed as $\mu\text{equiv./g.}$ of fresh leaf. Tr., Trace amounts too low for accurate determination.

Organic acid	Treatment			
	Control	HF (0.03 p.p.m.)	Control	HF (0.03 p.p.m.)
Unidentified acid	Tr.	0.4	Tr.	0.4
Fumaric acid	5.1	5.0	4.9	7.8
Succinic acid	2.9	5.9	0.9	6.5
Malonic acid	22.1	32.5	11.0	27.8
Malic acid	9.5	20.0	6.2	12.6
Citric acid	7.9	12.5	2.7	3.4
Total	47.5	76.3	25.7	58.5
Citric acid:malic acid ratio	0.83	0.63	0.43	0.27

Table 3. *Free amino acids in normal and fluoride-fumigated soya-bean leaves*

Experimental details are given in the text. Results are expressed as $\mu\text{moles/g.}$ of fresh leaf. N.D., Not detected; Tr., trace amounts too low for accurate determination; —, not determined.

Amino acids	Treatment			
	Control	HF (0.03 p.p.m.)	Control	HF (0.03 p.p.m.)
Asparagine	2.9	7.2	1.3	16.6
Aspartic acid	1.0	2.7	2.6	6.5
Glutamine	0.4	1.0	Tr.	0.5
Serine	1.5	2.4	1.5	2.7
Glutamic acid	3.5	5.3	4.2	5.9
Threonine	0.3	1.4	0.3	1.3
Alanine	2.4	1.4	0.5	0.7
Tyrosine	0.2	0.4	Tr.	0.6
γ -Aminobutyric acid	1.0	0.9	0.4	0.6
Phenylalanine	0.2	0.5	0.2	0.9
Leucine + isoleucine	0.1	1.0	0.1	2.2
Pipecolic acid	N.D.	2.4	N.D.	2.0
Total	13.5	26.6	11.1	40.5
Amide nitrogen	3.66	7.52	—	—

than in control leaves. Striking increases occurred in the concentrations of malonic acid and malic acid, which were the major organic acid components in soya bean.

Amino acids. The free amino acid contents of control and necrotic leaves are shown in Table 3. The concentrations of most amino acids increased in necrotic leaves, but particularly striking increases occurred in those of aspartic acid, glutamic acid, asparagine and pipecolic acid. No detectable amount of pipecolic acid was present in the normal leaves, but a remarkable quantity of this acid accumulated in necrotic leaves. Pipecolic acid was identified by the R_F values in a paper chromatogram with the two solvent systems butan-1-ol-acetic acid-water (4:1:5, by vol.) and *m*-cresol-phenol-borate buffer, as well as by the colour developed with ninhydrin. Since pipecolic acid has an unusually high R_F of 0.82 in the *m*-cresol-phenol-borate buffer system, as compared with 0.79 for

proline which is the highest among the protein amino acids, it is easily isolated from the other amino acids. The colour of pipecolic acid developed with ninhydrin and eluted with 50% (v/v) ethanol was reddish-purple, as compared with the bluish-purple colour of most amino acids.

Respiratory quotient. As shown in Fig. 1 the respiratory quotient (CO_2/O_2) decreased in fluoride-fumigated leaves almost immediately after initiation of treatment. The quotient remained low until initial injury became evident. The quotient then increased and approximated that of control plants at the time of very severe necrosis.

DISCUSSION

Fluoride-fumigated leaves contained more reducing sugars and less sucrose than the normal leaves. This result suggested that sucrose synthesis might be inhibited by fluoride fumigation. Yang &

Fig. 1. Changes in the respiratory quotient of fluoride-fumigated (*F*) as compared with control (*C*) soya-bean leaves. Experimental details are given in the text. The ranges of respiratory quotient from three samples are indicated by arrows. With the fluoride-fumigated leaves, little or no visible injury was present during the first 4–5 days, but very severe burning and necrosis was evident after 6 days.

Miller (1963*a*) showed that phosphoglucumutase, which participates in sucrose biosynthesis in higher plants, was very sensitive to inhibition by fluoride and could account for the inhibition of sucrose synthesis in fluoride-fumigated plants.

The increase of total organic acids in fluoride-necrotic leaves was similar to that found in several types of chlorosis (Iljin, 1951*b*; Su & Miller, 1961). DeKock & Morrison (1958*b*) found that the citric acid:malic acid ratio increased in leaves made chlorotic by iron-deficiency, mutation or nickel-poisoning, whether or not the total organic acid content increased or decreased in the different types of chlorosis. In fluoride-necrotic leaves, however, the reverse was true; the concentration of malic acid was more markedly increased than was that of citric acid.

The concentration of total amino acids, like that of total organic acids, increased markedly in fluoride-fumigated leaves. Similar patterns have been found in several types of chlorosis (Bennett, 1945; Iljin, 1951*a*; DeKock & Morrison, 1958*a*). The amino acids that accumulated most noticeably in fluoride-fumigated leaves were asparagine, aspartic acid, serine, glutamic acid and pipecolic acid. The most marked increase was in the concentration of asparagine, and this accounted for at least 30 % of the total increase in amino acids.

The free amino acid pool in tissues depends on the relative contributions of degradation of storage protein, amino acid synthesis and amino acid utilizations for protein synthesis and for respiration. Yang & Miller (1963*b*) showed that the rate of dark CO_2 fixation increased remarkably in the fluoride-fumigated leaves and could account for this accumulation of organic acids and amino acids.

The rate of respiration in tissues is usually

controlled by the concentration of ADP. Webster & Varner (1955) reported that synthesis of asparagine from aspartate and ammonia required ATP and generated ADP. Therefore the increased rate of respiration in fluoride-necrotic leaves (increasing 30–100 % with soya bean) could be attributed partly at least to this energy-consuming process by which asparagine was formed and ADP was generated.

Though pipecolic acid has been detected in a wide variety of plants as a normal metabolite (Morrison, 1953), the acid accumulated in large quantities in fluoride-necrotic leaves but could not be detected in normal leaves. A simple feeding experiment was conducted by inserting young soya-bean plants in test tubes that contained several concentrations of synthetic DL-pipecolic acid, or pipecolic acid isolated from the fluoride-necrotic leaves (30 mM, for a week). Inability to induce necrosis by the feeding with pipecolic acid indicated that pipecolic acid was not a direct causative agent of necrosis. Diener & Dekker (1954) found that pipecolic acid accumulated in peach leaves that were affected by Western-X virus or arsenic-poisoning and did not accumulate in appreciable amounts in healthy leaves. S. F. Yang, G. W. Welkie & G. W. Miller (unpublished work) inoculated cucumber-mosaic virus in two varieties of cow-pea; one developed necrosis and the other developed chlorosis. Pipecolic acid was found to accumulate in necrotic cow-pea leaves, but no detectable amounts of pipecolic acid were found in chlorotic or healthy leaves. These results indicated a close relationship between the accumulation of pipecolic acid and development of necrosis regardless of whether induced by fluoride, arsenic or virus. However, the results do not exclude the possibility that pipecolic acid, when associated with other causative factors, might play a role in the process of necrosis development.

If the organic acids and amino acids that accumulated in fluoride-necrotic leaves were derived from sugars, the respiratory quotient would be expected to decrease owing to reabsorption of respired CO_2 or extra consumption of oxygen. The results illustrated in Fig. 1 supported this supposition. The respiratory quotient was low only before the leaves were severely burned. The respiratory quotient increased in severely burned leaves. This suggested that no accumulation of these acids occurred at this stage. It was very likely that the metabolic functions were exhausted and about to cease in severely burned leaves.

The abnormal metabolic processes of sugars, organic acids and amino acids associated with fluoride necrosis are apparent, but as yet it is not known how these metabolic changes are related to the causes of fluoride necrosis.

SUMMARY

1. Metabolic processes associated with free sugars, organic acids and amino acids in higher plants subjected to fluoride fumigation were studied quantitatively.

2. Fluoride-fumigated leaves contained more reducing sugars and less sucrose than the normal leaves. This result suggested inhibition of sucrose synthesis by fluoride.

3. Necrotic leaves contained increased total concentrations of organic acids, which were mostly attributable to malic acid, malonic acid and citric acid. The greater increase in malic acid relative to that of citric acid was the reverse of results observed in chlorotic tissue.

4. Necrotic leaves contained enhanced amounts of free amino acids. The greatest increase occurred in the concentration of asparagine and might be related to the increased respiratory rate of necrotic leaves. Pipecolic acid accumulated in large quantities in necrotic tissue and was not detected in normal leaves.

5. The accumulation of organic acids and amino acids in leaves during fluoride fumigation was evidenced by a lowered respiratory quotient.

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REFERENCES

- Association of Official Agricultural Chemists (1955). *Official Methods of Analysis*, 8th ed., p. 541.
Bennett, J. P. (1945). *Soil Sci.* **60**, 91.

- Bonner, W. D., jun. & Thimann, K. V. (1950). *Amer. J. Bot.* **37**, 66.
Buch, M. L., Montgomery, R. & Porter, W. L. (1952). *Analyt. Chem.* **24**, 489.
DeKock, P. C. & Morrison, R. I. (1958a). *Biochem. J.* **70**, 266.
DeKock, P. C. & Morrison, R. I. (1958b). *Biochem. J.* **70**, 272.
Diener, T. O. & Dekker, C. A. (1954). *Phytopathology*, **44**, 643.
Drury, H. (1948). *Arch. Biochem.* **19**, 455.
Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. & Smith, F. (1956). *Analyt. Chem.* **28**, 350.
Iljin, W. S. (1951a). *Plant & Soil*, **3**, 239.
Iljin, W. S. (1951b). *Plant & Soil*, **3**, 339.
Khyrn, J. X. & Zill, L. P. (1952). *J. Amer. chem. Soc.* **74**, 2090.
Levy, A. L. & Chung, D. (1953). *Analyt. Chem.* **25**, 396.
McNulty, I. B. & Newman, D. W. (1957). *Plant Physiol.* **32**, 121.
McNulty, I. B. & Newman, D. W. (1961). *Plant Physiol.* **36**, 385.
Mori, K. & Nakamura, M. (1959). *Bull. Agric. chem. Soc. Japan*, **23**, 389.
Morrison, R. I. (1953). *Biochem. J.* **53**, 474.
Nielsen, H. M. (1958). *Analyt. Chem.* **30**, 1009.
Ross, C. W., Wiebe, H. H. & Miller, G. W. (1962). *Plant Physiol.* **37**, 305.
Somogyi, M. (1952). *J. biol. Chem.* **195**, 19.
Stanley, R. G. & Tracewell, T. N. (1955). *Science*, **122**, 76.
Su, L. Y. & Miller, G. W. (1961). *Plant Physiol.* **36**, 415.
Thomas, M. D. & Hendricks, R. H. (1956). In *Air Pollution Handbook*, sect. 9, p. 22. New York: McGraw-Hill Book Co.
Thompson, J. F. & Morris, C. J. (1959). *Analyt. Chem.* **31**, 1031.
Varner, J. E., Bullen, W. A., Vanecko, S. & Burrell, R. C. (1953). *Analyt. Chem.* **25**, 1528.
Webster, G. C. & Varner, J. E. (1955). *J. biol. Chem.* **215**, 91.
Yang, S. F. & Miller, G. W. (1963a). *Biochem. J.* **88**, 509.
Yang, S. F. & Miller, G. W. (1963b). *Biochem. J.* **88**, 517.

Biochem. J. (1963) **88**, 509

Biochemical Studies on the Effect of Fluoride on Higher Plants

2. THE EFFECT OF FLUORIDE ON SUCROSE-SYNTHESIZING ENZYMES FROM HIGHER PLANTS

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Leaves of plants that were fumigated with hydrogen fluoride contained markedly decreased concentrations of sucrose and increased concentrations of reducing sugars compared with those in leaves of control plants (Yang & Miller, 1963). Presumably, sucrose synthesis was retarded in the

fluoride-fumigated leaves. Several authors (Cardini, Leloir & Chiriboga, 1955; Leloir & Cardini, 1955; Burma & Mortimer, 1956) have shown that UDP-glucose is involved in the biosynthesis of sucrose in higher plants. In green leaves sucrose is synthesized by reactions shown in Scheme 1.