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Effect of Fluoride on Respiratory Enzymes in Soybean Leaves

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EFFECT OF FLUORIDE ON RESPIRATORY ENZYMES IN SOYBEAN LEAVES

by

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INTRODUCTION

There are many reports relating the effects of fluorides on plant respiration. Fluoride has been regarded as an inhibitor of respiration. Warburg et al. (1942) demonstrated that fluoride inhibited enolase activity, therefore, decreased the respiration rate of yeast. Miller (1958), found a similar inhibition of enolase from pea seed. Bonner (1948), Bonner and Wildman (1946), and Laties (1949) reported that the fluoride ion in culture solutions reduced the respiration rate in Avena Coleoptile, spinach leaves and barley roots. Since the inhibition was reversed by the addition of pyruvate, they concluded that the inhibition of respiration was due to the inactivation of fluoride sensitive enolase. Chung and Nickerson (1954) studied yeast cells grown in presence of fluoride and concluded that the inhibition of growth was due to the inhibition of polysaccharide synthesis, and the site of inhibition was the enzyme phosphoglucomutase. Yang and Miller (1963) found divergent sucrose, reducing sugar contents in fluoride fumigated soybean leaves and concluded that the enzyme phophoglucomutase was the site of fluoride inhibition in sucrose synthesis. Phosphorylase (Rapp and Sliwinski, 1956), phosphatase (Lammer and Hafer, 1953; Pierpoint, 1957) and hexokinase (Melchior and Melchior, 1956) have also been reported to be fluoride sensitive *in vitro*.

In spite of so many enzymes in the Emden-Meyerhof-Parnas pathway that are sensitive to fluoride, many investigators have reported

accelerated oxygen uptake in fluoride treated plants. Weinstein (1961) found a 67 to 72 percent increase of respiration in hydrogen fluoride fumigated tomato and tendergreen bean leaves. Yu and Miller (unpublished data) also observed 30 to 80 percent increase in fluoride fumigated soybean leaves. Christiansen and Thimann (1950) grew pea stem sections and found that sodium fluoride at a concentration, which gave 50 percent depression in growth, stimulated oxygen uptake. Chung and Nickerson (1954) with yeast cells and Bonner and Thimann (1950) with oat coleoptile obtained similar results.

Applegate et al. (1960a, 1960b, 1960c) found that either sodium fluoride in solution culture or atmospheric fluoride fumigation at low concentrations stimulated oxygen uptake in bush bean seedlings.

Ross et al. (1962) applied glucose-1-C^{14} and glucose-6-C^{14} to control and fluoride treated Chenopodium murale L. and Polygonum orientale L. They found a decreased C_6/C_1 ration and concluded increased significance of the pentose phosphate shunt mechanism in fluoride sensitive plants, when subjected to fluoride treatment.

McNulty and Newman (1957) exposed bush beans and gladioli to atmospheric fluoride, and reported an increased oxygen uptake. They concluded that fluoride at low concentrations might cause an initial inhibition, followed by stimulation of respiration, with the appearance of damage. Hill et al. (1959) studied seven varieties of gladioli at low fluoride fumigation concentrations. Two varieties with necrotic injuries showed respiratory increases. The others remained uninjured and showed no respiratory response in spite of very high concentration of fluoride. Therefore, they concluded that the increased respiration was mainly caused by injury. The amount of

fluoride accumulated in the tissue did not appear to be a significant factor.

McNulty and Lord (1960) studied Chlorella pyrenoidosa and found high concentrations of fluoride inhibited, while lower levels stimulated oxygen uptake. The increase of oxygen uptake seemed to be correlated with the concentration of undissociated hydrogen fluoride in the suspending media. They also found a significant increase in phosphorylated nucleotides associated with the stimulation of oxygen consumption.

= Our present knowledge of the effect of fluoride on plant respiration is mainly based on studies of overall gas exchange and in vitro tests of some individual respiratory enzymes. To a lesser extent studies have been made on individual enzymes in fluoride treated plant tissue. The comparison of enzyme activities of individual enzymes between fluoride treated and control plant tissues may provide a new approach to the understanding of the actual effect of fluoride on plant intermediary metabolism. In this paper the enzyme activities of five terminal oxidases namely: cytochrome oxidase, catalase, peroxidase, polyphenol oxidase and ascorbic acid oxidase, in addition to glucose-6-phosphate dehydrogenase were compared in hydrogen fluoride-fumigated and control soybean leaves, to determine whether any relationship existed between the change of enzyme activities and the accelerated oxygen uptake. Experiments were conducted with glucose-6-phosphate dehydrogenase, a connecting enzyme to the hexose monophosphate shunt, to measure changes in activity associated with fluoride injury.

EXPERIMENTAL METHODS

Materials

Soybeans (Glycine max, Merr, Variety Hawkeye) were germinated in vermiculite and transplanted to pots containing Hoagland's solution. After the plants had developed three to four trifoliate leaves, they were placed into chamber for hydrogen fluoride fumigation. The fumigation chambers were constructed as described by Ross, Wiebe and Miller (1962). The concentration of hydrogen fluoride passed through the fumigation chamber was controlled at about 100 ppb. The second youngest trifoliate leaves were marked with paper strips before fumigation.

Enzyme Preparation

About four to seven of the marked trifoliate leaves from both the fluoride fumigation and control chamber were harvested at 24, 96 and 144 hours after the initiation of the experiment. Harvested leaves were ground with about 1/5 volume of cold isotonic 0.05 M Tris buffer at pH 7.0, per weight of tissue with acid washed sand with a precooled mortar and pestle. Severely injured leaves were ground with 1/3 volume of isotonic Tris buffer at pH 7.0 per leaf weight. The extract was passed through cheese cloth, and centrifuged at 1.085 X g for 3 minutes to remove cellular debris (Smillie, Adams and Ferchau, 1955). The supernatant solutions were centrifuged again at 34,800 X g for 20 minutes to separate mitochondria from cytoplasmic

materials. In order to make the cytoplasmic solution as clear as possible, the high speed centrifugation on the supernatant was repeated once more. The final volume of the supernatant was about 1/5 volume per leaf weight. All procedures of enzyme preparation were carried out in the cold room (0° to 5° C). Supernatants were used as extracts for assaying catalase, peroxidase, polyphenol oxidase, ascorbic acid oxidase and glucose-6-phosphate dehydrogenase activities. The pellet from each high speed centrifugation was combined and suspended in about 1/5 volume per leaf weight of isotonic Tris buffer at P_H 7.0. This suspension was used for cytochrome oxidase assay.

Enzyme Assay

The enzyme activities of these systems were spectrophotometrically measured with a Hitachi-Perkin Elmer UV-Visible spectrophotometer with a 3 ml silica cuvette (1 cm light path) at 25° C. All reagents and buffer solutions were prepared with deionized water. The enzyme concentration of the enzyme extract solutions were measured by Folin's phenol reagent method, and expressed as protein based on a casein standard (Lowry, Rosebrough, Farr and Randall, 1951).

Cytochrome oxidase

A stock solution of 12 mg/ml of cytochrome C (Sigma Chemical Company of St. Louis, Missouri) in 0.05 M Tris buffer at pH 7.0 was reduced by sodium bisulfite, thoroughly aerated and allowed to stand for 30 minutes.

In a 3 ml cuvette, 0.25 ml of reduced cytochrome C stock solution

and 3.0 ml of 0.05 M Tris buffer at pH 7.0 were mixed. After the addition of 0.02 ml of mitochondrial suspension, the optical densities at 550 mμ were taken at 15 and 75 seconds. The enzyme activities of cytochrome oxidase were expressed in terms of optical density decrease per second per mg protein (Cooperstein and Lazarow, 1951).

Catalase

A hydrogen peroxide buffer solution was used as substrate solution. Hydrogen peroxide (0.16 ml of 30 percent, W/V) was diluted to 100 ml with 0.05 M Tris buffer at pH 7.0. The optical density of this solution was about 0.500 at 240 mμ with a 1 cm light path.

Enzyme solution was added to 3 ml of hydrogen peroxide-buffer solution. The optical densities at 240 mμ were recorded at the first 30 and 90 seconds after the addition of 0.02 ml of enzyme extract (Lück 1963).

The enzyme activities were expressed as the decrease of optical density at 240 mμ per minute per mg protein.

Peroxidase

The substrate solution was prepared with a modification of Willstatter and Stoll's formula (1917). Three ml of 5 percent pyrogallol solution and 1.5 ml of 0.5 percent (W/V) hydrogen peroxide solution were mixed with 25.5 ml of 0.05 M Tris buffer at pH 7.0. Three ml of the mixture were used as substrate solution. Enzyme (0.02 ml) was added to the substrate solution. Activity was determined by measuring the increase in optical density of orange purpurogallin at 425 mμ, between 15 and 75 seconds after the addition of

enzyme solution to the substrate.

Polyphenol oxidase

Chlorogenic acid (Nutritional Biochemical Corporation of Cleveland, Ohio) was used as substrate (Sisler and Evans, 1958). In the reduced form, chlorogenic acid has a maximum absorption at 300 to 330 mμ, and a minimum at 260 mμ. With oxidation, the 300 to 330 mμ band diminishes and the 260 mμ absorption increases. The enzyme activity can be measured either by the decrease of optical density at 330 mμ or the increase at 260 mμ.

In this study a final concentration of 5.7 mM chlorogenic acid in 0.05 M Tris buffer at pH 7.0 was used as substrate solution. Change in optical density at 330 mμ between one and six minutes after the addition of 0.02 ml of enzyme extract were recorded. The enzyme activity was expressed in terms of optical density decrease per 5 minutes per mg protein.

Ascorbic acid oxidase

A stock solution of 0.025 M ascorbic acid in meta phosphate (1 mg/ml) solution was prepared fresh before each assay. Enzyme extract (0.02 ml) and 0.02 ml of ascorbic acid solution were added to 3 ml of 0.05 M Tris buffer at pH 7.0. The optical density decrease at 265 mμ between the first four and six minutes was used to determine the enzyme activity (Oberbacher and Vines, 1963). Autoxidation of the substrate in an enzyme free blank assay was subtracted from the crude activity. The enzyme activity was expressed as the corrected optical density decrease per two minutes per mg protein.

Glucose-6-phosphate dehydrogenase

Enzyme activity was measured spectrophotometrically at 340 mμ in terms of NADPH formation as follows (Warburg, Christian and Griese, 1963):

Glucose-6-phosphate + $NADP^+$ ==== 6-phosphogluconate + NADPH + H^+

The assay mixture contained 0.05 ml of 3×10^{-2} M $NADP^+$ (β form), 0.1 ml of 0.3 M $mgCl_2$, 2.8 ml of 0.05 M Tris buffer at pH 7.0 and 0.02 ml of enzyme extract. After incubation for 5 minutes at room temperature 0.05 ml of 4×10^{-2} M glucose-6-phosphate solution was added. The change in the optical densities at 340 mμ between 30 and 210 seconds after the addition of substrate were recorded. The enzyme activity was expressed as the optical density increase per 3 minutes per mg protein.

Determination of Time Intervals in Enzyme Assay

The time intervals mentioned in each enzyme assay were obtained from the preliminary tests in which the reaction conditions were the same as those described previously. The activity of cytochrome oxidase, catalase and peroxidase was linear for the first 1.5 minutes. Ascorbic acid oxidase activity was linear between the first 4 and 6 minutes. Since polyphenol oxidase and glucose-6-phosphate dehydrogenase activities were very low in control leaf tissues, the assaying intervals were extended to 5 and 3 minutes respectively.

Effect of KF on Terminal Oxidases In Vitro

In vitro tests for determining fluoride inhibition of each

terminal oxidase was also carried out. Enzyme extracts from the normal second trifoliate leaves were prepared as described above. Three different concentrations of inhibitor, namely: 10^{-2} M, 3×10^{-3} M and 10^{-5} M KF were used for each terminal oxidase assay. Enzyme extract (0.02 ml) was incubated with 0.05 M Tris buffer at pH 7.0, containing KF, for 30 minutes at 25°C. Concentrated substrate solution was added to make a final concentration equal to that described in *in vivo* tests, to start the enzyme reaction. The enzyme activities were measured as previously described.

Manometric Determination of Oxygen Uptake

Manometric determination of oxygen uptake on leaf tissue was performed in parallel with the enzyme assays. Three leaves similar in age and treatment to those used for enzyme assays were selected at random. From each leaf, ten discs of 9 mm diameter were put into a Warburg flask, containing Hoagland's nutrient solution.

Determination of HF Concentration in the Fumigation Chambers

The fluoride concentrations of the air passed through the fumigation chambers were determined by means of resin column chromatography, as described by Nielsen (1958). Dowex 1-8 of the Cl^- form, 200 to 400 mesh (Baker Chemical Company, Phillipsburg, New Jersey) was used to pack the column (9 x 50 mm). The resin was converted to the acetate form by passing through 20 volumes of 1 M sodium acetate per resin weight. Excessive acetate was washed out with distilled water. Aliquots of the water, through which a measured volume of air had been passed, were applied to the column. The adsorbed fluoride ion was

elutriated by stepwise application of 0.1 M, 0.2 M and 0.3 M sodium acetate. To 5.0 ml aliquots of each effluent fraction, 0.5 ml of 0.1 percent erichrome cyanine R solution and 0.5 ml of 0.93 mM Zirconium nitrate in 6 N HCl solution were added. The solution was thoroughly mixed and allowed to stand for at least 10 minutes at room temperature. The optical densities were measured with the Hitachi-Perkin Elmer UV-Visible spectrophotometer at 527.5 mμ. The fluoride content of each effluent fraction was determined by comparing with a standard curve prepared by plotting the absorption of known fluoride solutions.

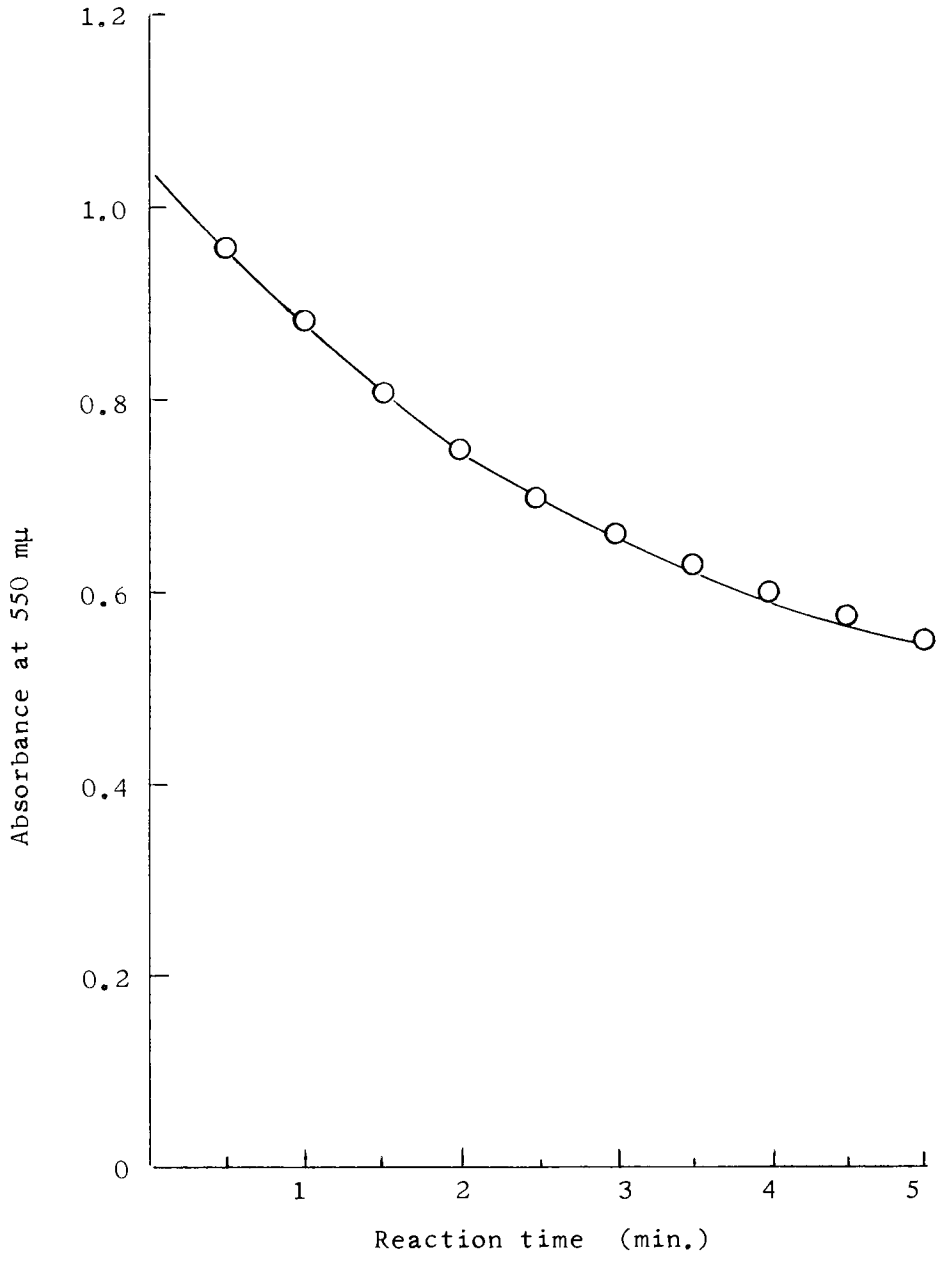

Figure 1. Reaction curve of cytochrome oxidase extracted from the second trifoliate leaves of soybean. The reaction system contained 3 ml of 0.05 M Tris buffer at pH 7.0, 0.25 ml of reduced cytochrome C stock solution and 0.02 ml of mitochondrial suspension.

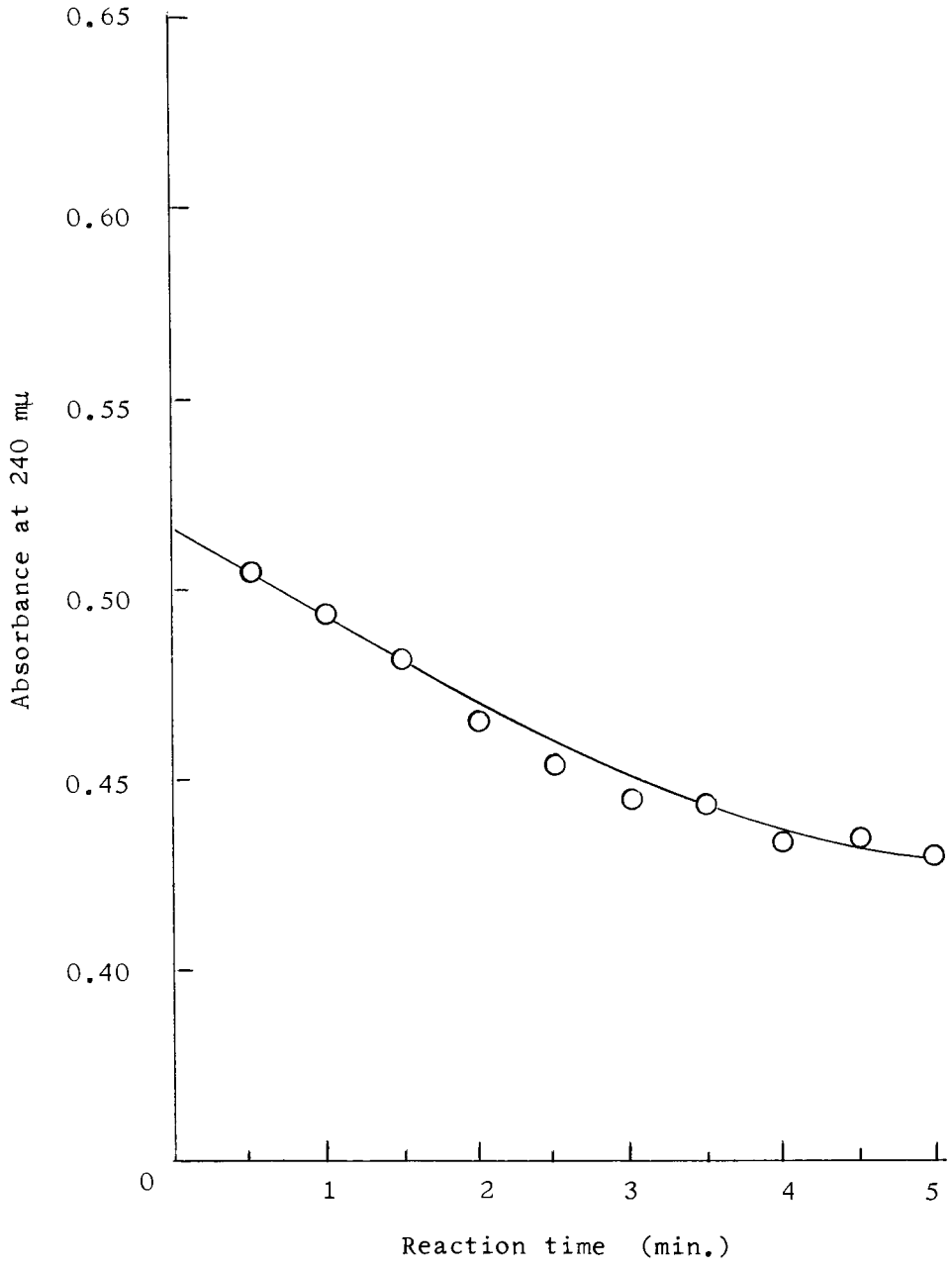

Figure 2. Reaction curve of catalase extracted from the second trifoliate leaves of soybean. The reaction system contained 3 ml of hydrogen peroxide-buffer solution and 0.02 ml of enzyme extract.

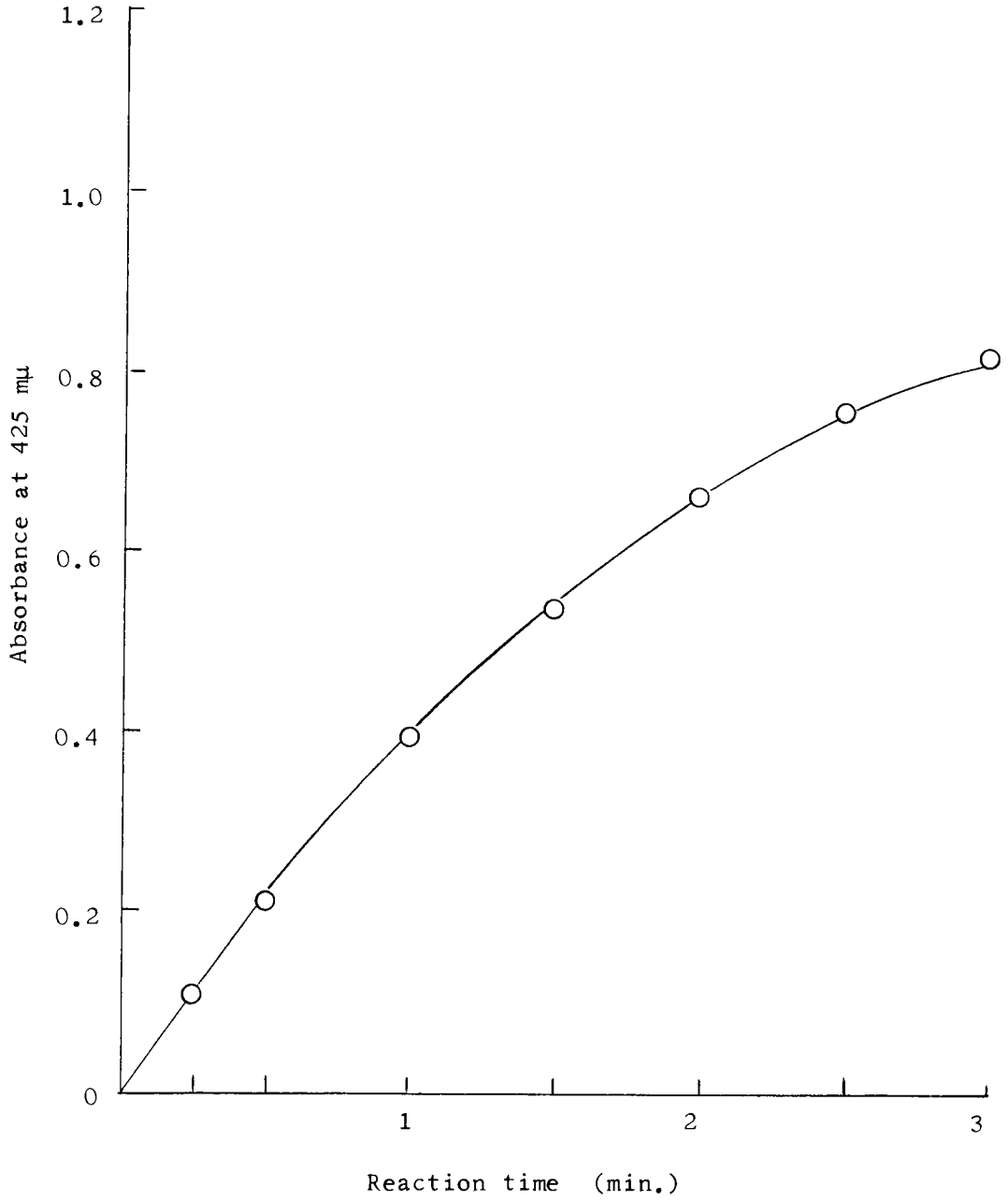

Figure 3. Reaction curve of peroxidase extracted from the second trifoliate leaves of soybean. The reaction system contained 3 ml of substrate solution and 0.002 ml of enzyme extract.

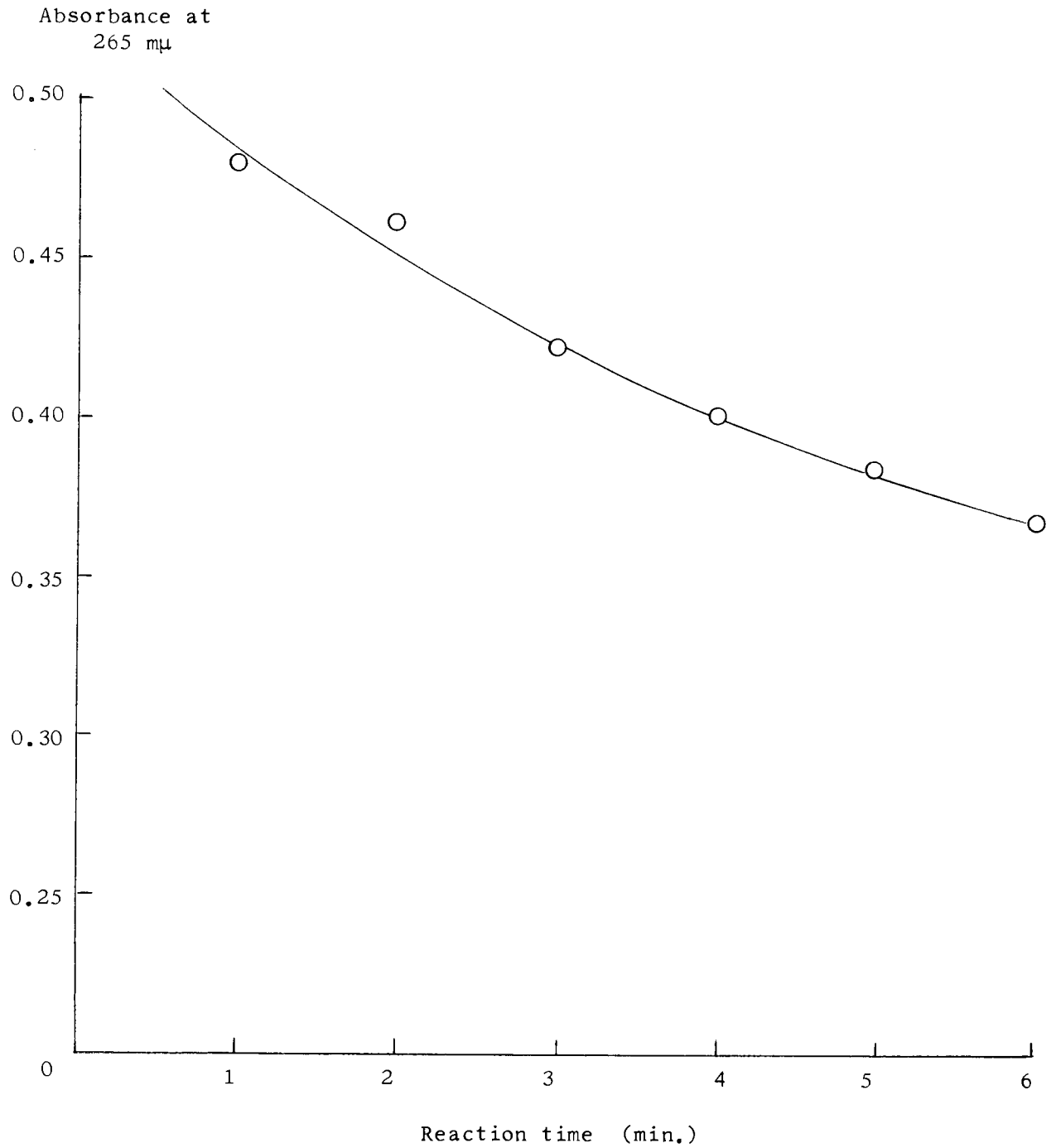

Figure 4. Reaction curve of ascorbic acid oxidase extracted from the second trifoliate leaves of soybean. The reaction system contained 3 ml of 0.05 M Tris buffer, 0.02 ml of 0.025 M ascorbic acid in meta phosphate solution and 0.02 ml of enzyme extract.

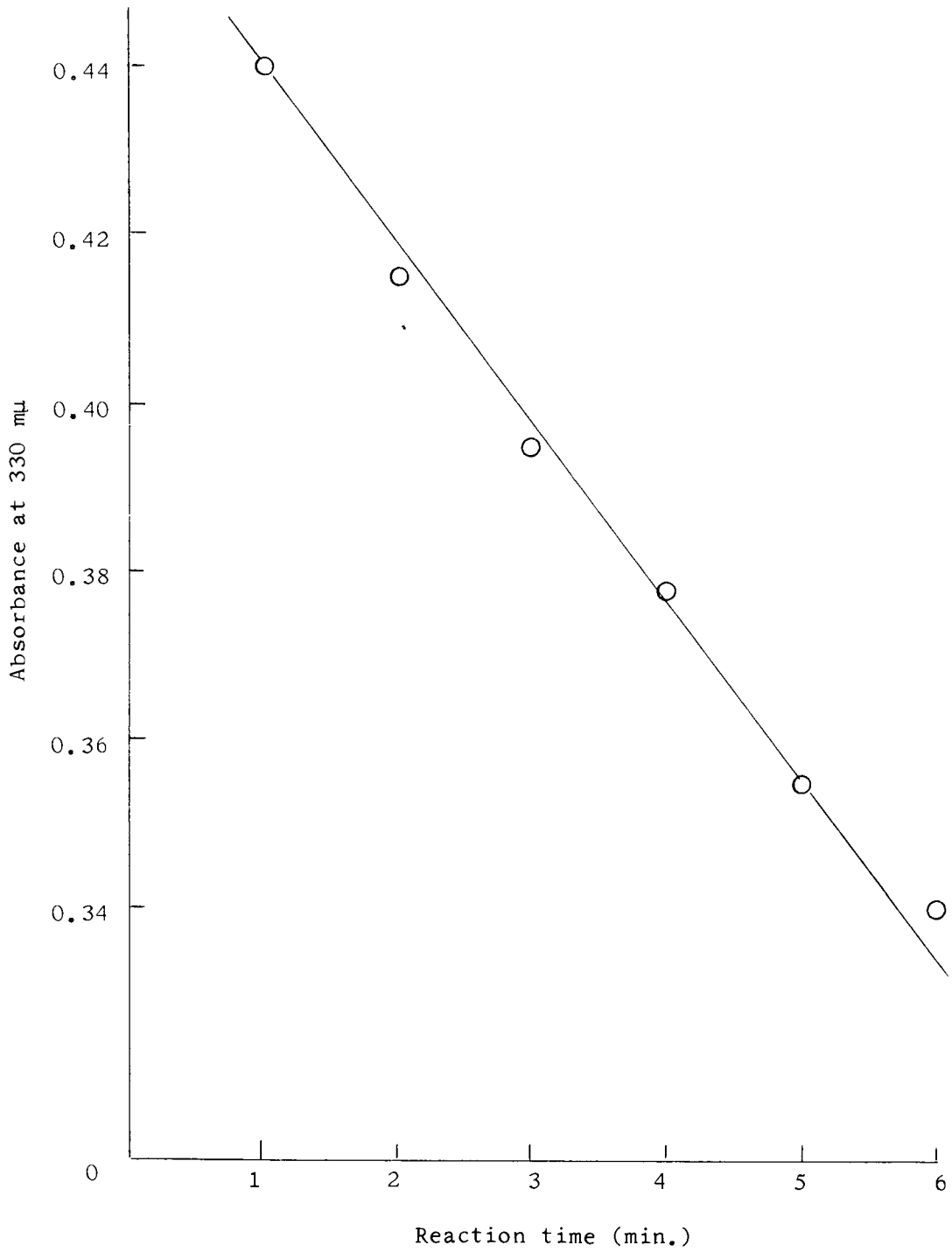

Figure 5. Reaction curve of polyphenol oxidase extracted from the trifoliate leaves of soybean. The reaction system contained 3 ml of 5.7 mM Chlorogenic acid in 0.05 M Tris-Cl buffer solution, and 0.02 ml of enzyme extract.

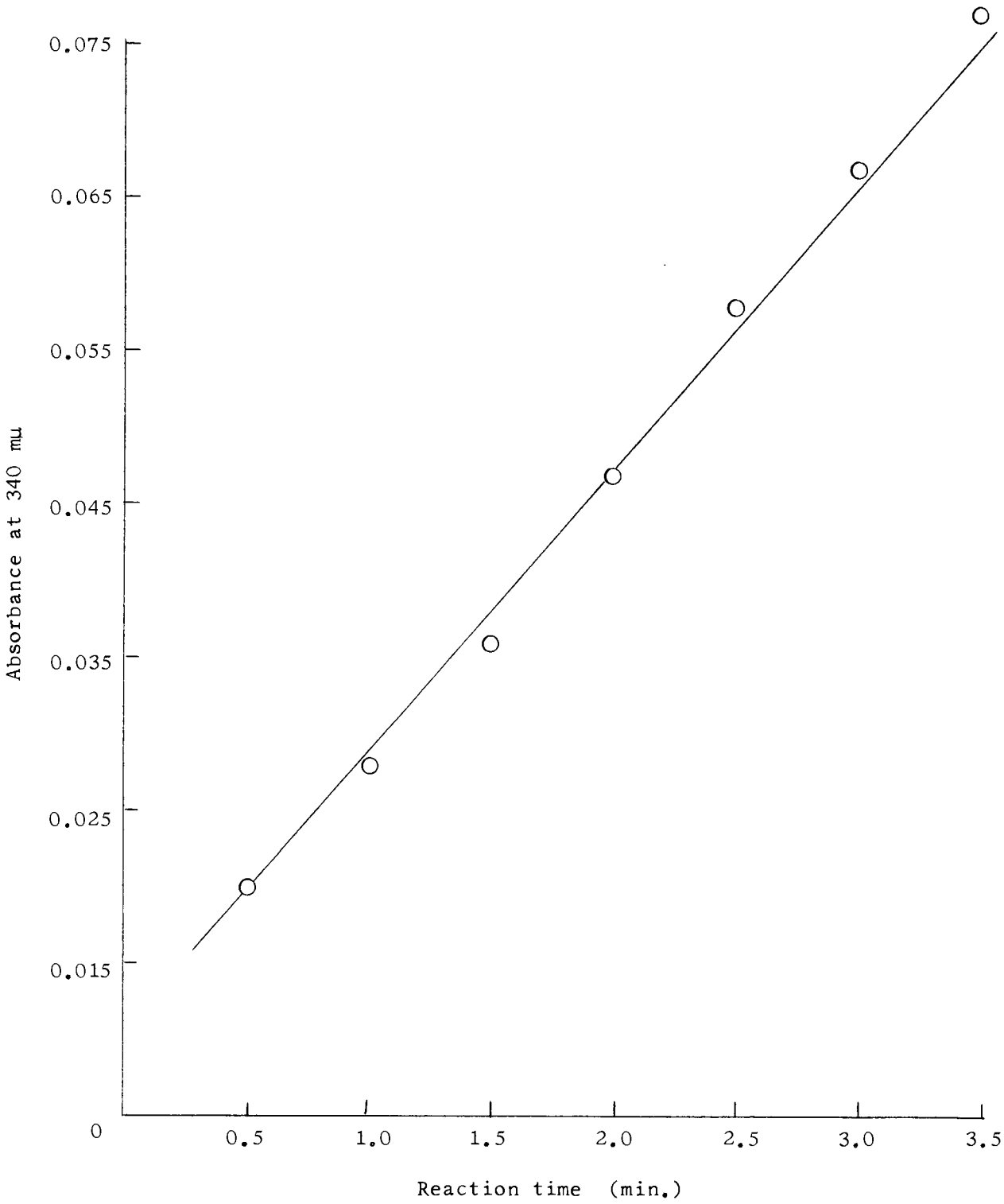

Figure 6. Reaction curve of glucose-6-phosphate dehydrogenase extracted from the second trifoliate leaves of soybean.

RESULTS

Effect of Atmospheric Fluoride on Enzyme Activities

The enzyme extracts of five terminal oxidases and glucose-6-phosphate dehydrogenase were prepared from soybean leaves in three separate experiments. For each experiment the assays were replicated three times. Results are shown in Tables 1 through 6. Leaves fumigated with hydrogen fluoride for 24 hours showed slight injury. Dark green spots were evident. Leaves harvested after 96 hours treatment were slightly wilted, with mild chlorosis, but no necrosis. After 144 hours treatment, severe necrosis was evident.

Cytochrome oxidase

Leaf tissue fumigated with hydrogen fluoride for 24 hours showed (average of three experiments) a 14 percent increase in cytochrome oxidase activity, compared to control tissue of the same age (Table I). After 96 hours fumigation, activity increased about 16 percent over the control. An increase of 62 percent over the control was found after 144 hours of treatment. In all experiments cytochrome oxidase activity increased with increase in fluoride injury.

Peroxidase

In general, the peroxidase activity in control leaves increased with tissue maturity. Hydrogen fluoride fumigation, as shown in Table 2, increased the activity of peroxidase in tissues exposed to it. Average of three experiments showed a 54 to 87 percent increase

in activity. Enzyme activity was markedly increased after 24 hours of treatment and apparently was not directly related to the degree of injury.

Catalase

Catalase activity from control leaves changed little with leaf maturity. The enzyme activity of catalase from fumigated tissues was 2 to 5 times greater than that found in control tissues (Table 3). . For each experiment, maximum activity appeared in leaves that had been fumigated for 96 hours, and declined with longer periods of treatment. The average increases in activity in the three experiments after 24, 96 and 144 hours of fumigation were 217, 322 and 221 percent of the controls respectively.

Polyphenol oxidase

In general polyphenol oxidase activity was inhibited in extracts from hydrogen fluoride fumigated tissue. Polyphenol oxidase activity decreased with an increase in fluoride injury. In experiment 1 and 3, there were slight increases in activity after 24 hours of treatment, but the increases were too small to be conclusive. The average of three experiments showed 8, 22 and 37 percent inhibition in extracts from leaves treated for 24, 96 and 144 hours respectively, when compared to their corresponding controls.

Ascorbic acid oxidase

Ascorbic acid oxidase activity in fumigated soybean leaves was markedly increased in two of the three experiments after 24 hours. It then showed steady inhibition with prolonged treatment. The effect

of hydrogen fluoride on ascorbic acid oxidase activity (average of three experiments) showed an increase of 15 percent after 24 hours, and 26 and 56 percent inhibition after 96 and 144 hours of treatment, respectively.

Glucose-6-phosphate dehydrogenase

The activity of glucose-6-phosphate dehydrogenase was very low in normal soybean leaves, but was increased markedly in hydrogen fluoride fumigated leaves. The largest increase of activity in fumigated leaves was nearly 60 times that found in the control tissue. Average stimulation of this enzyme in fumigated leaves of three experiments was 4.7, 21.7 and 19.6 times the corresponding control after 24, 96 and 144 hours of treatment, respectively. Enzyme activity showed a steady increase up to 96 hours treatment.

Effect of Atmospheric Fluoride on Total Oxygen Uptake

An increase in total oxygen uptake was found in the fumigated leaves, throughout the experiment. The respiration increased 40 percent after 96 hours, but declined with longer periods of treatment. Comparison of total respiration to activity of the various oxidases is shown in Table 7.

Effect of KF on Terminal Oxidases in Vitro

As shown in Tables 8 and 9 and Figures 7 and 8, ascorbic acid oxidase and peroxidase were very sensitive *in vitro*, to fluoride ions. The average of two experiments, showed 33 percent inhibition of ascorbic acid oxidase by 10^{-5} M KF, almost complete inhibition was observed in

the presence of 10^{-2} M KF. The inhibition of peroxidase activity by KF was less than that found with ascorbic acid oxidase. Peroxidase was inhibited 13 percent (average of two experiments) by 10^{-5} M KF. Polyphenol oxidase was only slightly affected by KF in <u>in vitro</u> studies (Table 10 and Figure 9). At 10^{-5} M KF, there was no change in enzyme activity, a slight inhibition at 3×10^{-3} M KF and about 16 percent inhibition at 10^{-2} M KF. Catalase activity as shown in Table 11 was not affected by fluoride at 10^{-5} to 10^{-2} M. Cytochrome oxidase activity (Table 12) was also unaffected by KF up to 10^{-2} M.

Table 1. Effect of hydrogen fluoride fumigation on cytochrome oxidase activity in extracts from soybean leaves.

Experiment	Treatment	Time of treatment (Hours)		
		24	96	144
		O. D. decrease/minute/mg protein at 550 mμ [a]		
1	Control	0.126	0.209	0.165
	HF (107 ppb)	0.178	0.301	0.286
	Activity percent (HF/control)	141	144	173
2	Control	0.339	0.235	0.286
	HF (62 ppb)	0.323	0.248	0.353
	Activity percent (HF/control)	95	106	123
3	Control	0.297	0.382	0.279
	HF (115 ppb)	0.366	0.420	0.472
	Activity percent (HF/control)	123	110	169
Average	Control	0.254	0.279	0.228
	HF (95 ppb)	0.289	0.323	0.370
	Activity percent (HF/control)	114	116	162

[a] Results are the average of three replications for each experiment.

Table 2. Effect of hydrogen fluoride fumigation on peroxidase activity in extract from soybean leaves.

Experiment	Treatment	Time of treatment (Hours)		
		24	96	144
		O. D. increase/minute/mg protein at 425 mμ [a]		
1	Control	1.031	1.604	2.789
	HF (107 ppb)	1.472	2.118	3.895
	Activity percent (HF/control)	143	132	139
2	Control	1.674	1.494	1.364
	HF (62 ppb)	2.529	2.289	2.185
	Activity percent (HF/control)	151	153	160
3	Control	1.061	1.070	1.391
	HF (115 ppb)	1.805	3.392	3.130
	Activity percent (HF/control)	170	317	225
Average	Control	1.255	1.389	1.848
	HF (95 ppb)	1.935	2.600	3.070
	Activity percent (HF/control)	154	187	166

[a] Results are averages of three replications for each experiment.

Table 3. Effect of hydrogen fluoride fumigation on catalase activity in extracts from soybean leaves.

Experiment	Treatment	Time of treatment (Hours) 24	96	144
		O. D. decrease/minute/mg protein at 240 mμ [a]		
1	Control	0.043	0.039	0.056
	HF (62 ppb)	0.092	0.124	0.088
	Activity percent (HF/control)	214	318	157
2	Control	0.061	0.055	0.051
	HF (62 ppb)	0.124	0.185	0.103
	Activity percent (HF/control)	203	336	202
3	Control	0.041	0.039	0.019
	HF (115 ppb)	0.096	0.196	0.089
	Activity percent (HF/control)	234	516	468
Average	Control	0.048	0.044	0.042
	HF (80 ppb)	0.104	0.168	0.093
	Activity percent (HF/control)	217	382	221

[a] Results are averages of three replications for each experiment.

Table 4. Effect of hydrogen fluoride fumigation on polyphenol oxidase activity in extracts from soybean leaves.

Experiment	Treatment	Time of treatment (Hours) 24	96	144
		O. D. decrease/5 minutes/mg protein at 330 mμ [a]		
1	Control	0.059	0.058	0.117
	HF (107 ppb)	0.064	0.049	0.080
	Activity percent (HF/control)	109	85	68
2	Control	0.170	0.145	0.138
	HF (62 ppb)	0.127	0.083	0.071
	Activity percent (HF/control)	75	57	51
3	Control	0.111	0.069	0.098
	HF (115 ppb)	0.122	0.070	0.074
	Activity percent (HF/control)	110	105	76
Average	Control	0.113	0.091	0.118
	HF (95 ppb)	0.104	0.070	0.074
	Activity percent (HF/control)	92	77	63

[a] Results are averages of three replications for each experiment.

Table 5. Effect of hydrogen fluoride fumigation on ascorbic acid oxidase activity in extracts from soybean leaves.

Experiment	Treatment	Time of treatment (Hours) 24	96	144
		O. D. decrease/2 minutes/mg protein at 265 mμ [a]		
1	Control	0.063	0.045	0.030
	HF (107 ppb)	0.105	0.029	0.015
	Activity percent (HF/control)	167	64	50
2	Control	0.079	0.052	0.026
	HF (62 ppb)	0.059	0.028	0.011
	Activity percent (HF/control)	75	54	42
3	Control	0.116	0.042	0.027
	HF (115 ppb)	0.132	0.045	0.011
	Activity percent (HF/control)	114	107	46
Average	Control	0.086	0.046	0.027
	HF (95 ppb)	0.099	0.034	0.012
	Activity percent (HF/control)	115	74	44

[a] Results are the average of three replications.

Table 6. Effect of hydrogen fluoride fumigation on glucose-6-phosphate dehydrogenase activity in extracts from soybean leaves.

Experiment	Treatment	Time of treatment (Hours)		
		24	96	144
		O. D. increase/3 minutes/mg protein at 340 mμ [a]		
1	Control	0.005	0.006	0.011
	HF (107 ppb)	0.052	0.073	0.195
	Activity percent (HF/control)	1,040	1,217	1,773
2	Control	0.015	0.007	0.018
	HF (62 ppb)	0.028	0.085	0.213
	Activity percent (HF/control)	187	1,214	1,183
3	Control	0.008	0.004	0.005
	HF (115 ppb)	0.046	0.233	0.238
	Activity percent (HF/control)	575	5,825	4,560
Average	Control	0.009	0.006	0.011
	HF (95 ppb)	0.042	0.130	0.215
	Activity percent (HF/control)	467	2,167	1,955

[a] Results are the averages of three replications.

Table 7. The effect of hydrogen fluoride fumigation on total respiration in soybean leaf discs compared to some respiratory enzymes in soybean leaves.

	Time of treatment (Hours)		
	24	96	144
O_2 uptake as percentage of control[a]	102	140	114
	Enzyme activity percent[b]		
Cytochrome oxidase	112.8	115.8	162.3
Peroxidase	154.2	187.2	166.1
Catalase	216.7	381.8	221.4
Polyphenol oxidase	92.0	77.8	62.7
Ascorbic acid oxidase	115.1	73.9	44.4
Glucose-6-phosphate dehydrogenase	466.7	2,166.7	1,954.5

[a] Results are the averages of two experiments.

[b] Each enzyme activity percent is an average of three experiments.

Table 8. Inhibitory effect of KF on ascorbic acid oxidase activity in extracts from soybean leaves.

Experiment	KF solution: Control	10^{-5} M	3×10^{-3} M	10^{-2} M
	O. D. decrease/ 2 minutes/mg protein at 265 mμ [a]			
1	0.0244	0.0155	0.0097	0.0049
2	0.0181	0.0130	0.0097	0.0014
Average	0.0213	0.0143	0.0097	0.0032

[a] Results are averages of three replications in each experiment.

Table 9. Inhibitory effect of KF on peroxidase activity in extracts from soybean leaves.

Experiment	KF solution: Control	10^{-5} M	3×10^{-3} M	10^{-2} M
	O. D. increase/minute/mg protein at 425 mμ [a]			
1	2.208	1.458	0.979	0.750
2	4.374	4.260	2.569	1.984
Average	3.291	2.859	1.774	1.367

[a] Results are averages of three replications in each experiment.

Table 10. Inhibitory effect of KF on polyphenol oxidase activity in extracts from soybean leaves.

Experiment	KF solution: Control	10^{-5} M	3×10^{-3} M	10^{-2} M
	O. D. decrease/5 minutes/mg protein at 330 mμ [a]			
1	0.082	0.083	0.075	0.070
2	0.120	0.124	0.116	0.100
Average	0.101	0.103	0.096	0.085

[a] Results are the averages of three replications in each experiment.

Table 11. Inhibitory effect of KF on catalase activity in extracts from soybean leaves.

Experiment	KF solution: Control	10^{-5} M	3×10^{-3} M	10^{-2} M
	O. D. decrease/minute/mg protein at 240 mμ [a]			
1	0.041	0.041	0.040	0.042
2	0.040	0.038	0.037	0.039
Average	0.041	0.040	0.039	0.041

[a] Results are the averages of three replications in each experiment.

Table 12. Inhibitory effect of KF on cytochrome oxidase activity in extracts from soybean leaves.

Experiment	KF solution			
	Control	10^{-5} M	3 x 10^{-3} M	10^{-2} M
	O. D. decrease/minute/mg protein at 550 mμ [a]			
1	0.126	0.122	0.129	0.129
2	0.135	0.131	0.132	0.138
Average	0.131	0.127	0.131	0.134

[a] Results are averages of three replications in each experiment.

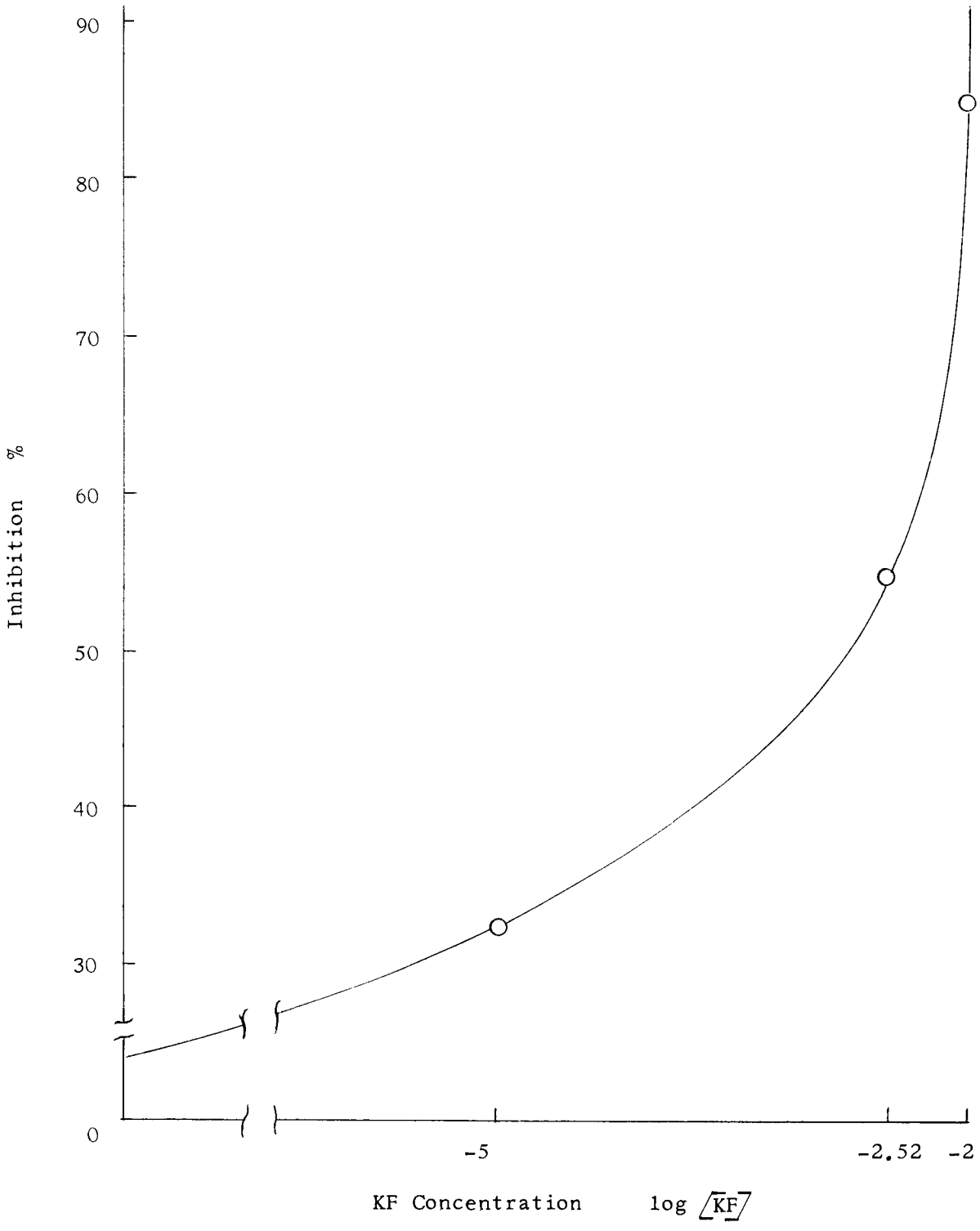

Figure 7. The inhibitory effect of KF on ascorbic acid oxidase extracted from the second trifoliate leaves of soybean.

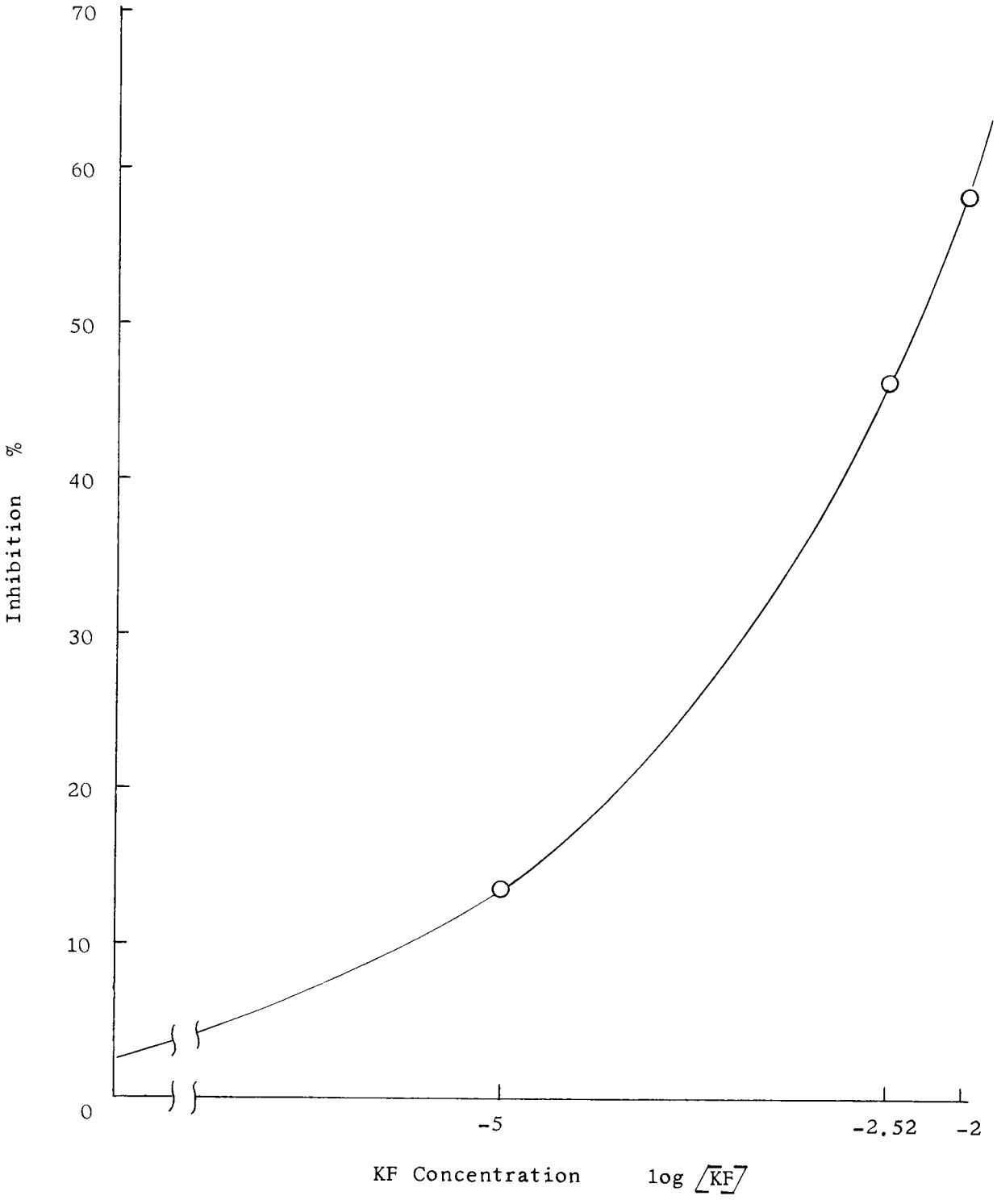

Figure 8. The inhibitory effect of KF on peroxidase extracted from the second trifoliate leaves of soybean.

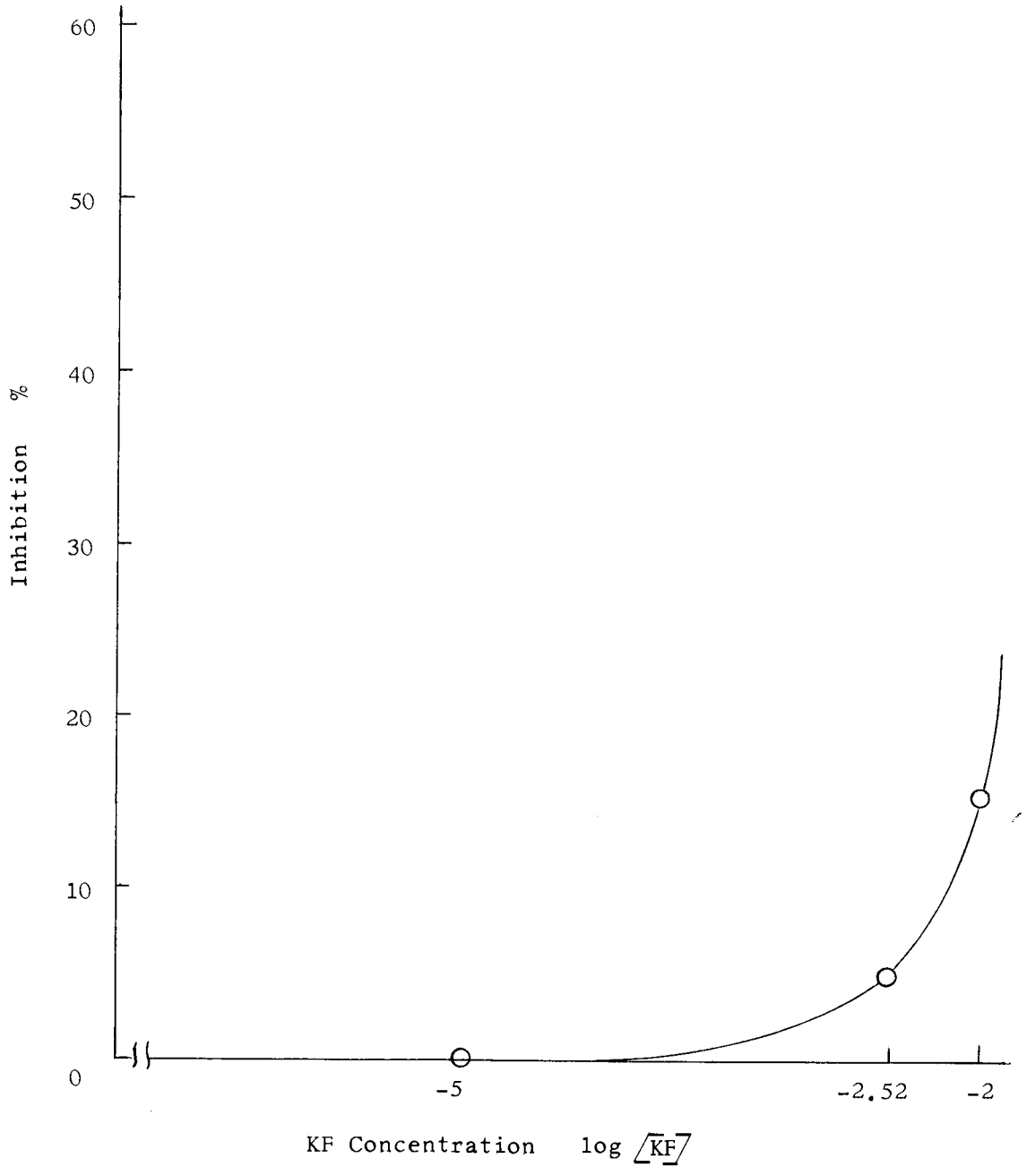

Figure 9. The inhibitory effect of KF on polyphenol oxidase extracted from the secon trifoliate leaves of soybean.

DISCUSSION

Spurr et al. (1961) studied the activity of ascorbic acid oxidase in normal, wounded, and crown gall tomato stem tissue. They reported that wounding increased ascorbic acid oxidase activity and returned to normal 16 days after wounding. In gall developing tissue, the increased activity of ascorbic acid oxidase was directly correlated with the gall development. In fluoride injured soybean leaves, as stated previously, ascorbic acid oxidase activity was at first stimulated and then was steadily suppressed. This enzyme was reported by Mandels (1953), and Honda (1955) to be a cell wall bound enzyme. Dunn and Dawson (1951) found that this enzyme contains 0.26 percent of copper. In vitro studies of this enzyme showed marked inhibition at 10^{-5} M KF. In fumigated tissue, according to Yang and Miller (1962) the fluoride content was about 200 µg per gm of fresh leaf, this would approximate 10^{-2} M fluoride. The initial stimulation of this enzyme might be analogous to ordinary wounding. With the increase of fluoride accumulation in the tissue, the enzyme activity was markedly reduced. The suppressed activity of this enzyme might be due to a complex formation between enzyme and fluoride ion.

Thimann and Hackett (1954) on studying the terminal oxidases in potato tuber tissue, observed increased polyphenol oxidase and cytochrome oxidase activities in sliced and washed discs. Hampton and Fulton (1961) reported elevated polyphenol oxidase activity in cucumber tissue infected with prune dwarf virus or necrotic ring spot virus.

They were able to demonstrate that the increased polyphenol oxidase activity was a kind of defense mechanism of host tissue to limit the invading virus. However, in fluoride fumigated soybean leaves, polyphenol oxidase activity was consistently inhibited throughout the treatment period. Slight inhibition occured before visible injury was evident. According to Mallette et al. (1948) and Keilin and Mann (1938), polyphenol oxidase contains 0.2 to 0.3 percent of copper in the purified enzyme. Five percent of the activity of this enzyme was inhibited by 3×10^{-3} M KF *in vitro*. As shown in Table 4, polyphenol oxidase activity from fumigated soybean leaves was 8 percent lower than the control after 24 hours of treatment. According to Yang and Miller (unpublished data 1962) in soybean leaves fumigated with 30 ppb hydrogen fluoride for 3 days, the accumulated fluoride ion concentration was approximately 6×10^{-3} M. In this study the hydrogen fluoride concentration of the air passed through the fumigation chamber was raised to 100 ppb. With this concentration of HF, it is reasonable to estimate that the accumulated fluoride in leaves fumigated for 24 hours can reach 6×10^{-3} M. Therefore, the inhibitory effect of atmospheric fluoride on this enzyme was probably, as with ascorbic acid oxidase, due to complex formation between enzyme and fluoride ion.

Cytochrome oxidase, peroxidase and catalase activities increased markedly throughout the fumigation period. The increased activity correlated with the increase of total oxygen uptake. Peroxidase and catalase were maximum in leaves fumigated with hydrogen fluoride for 96 hours, where the oxygen uptake was also maximum (40 percent increase). These results in general may suggest that the increased oxygen uptake

of plant tissue exposed to fluoride is a result of the increased activities of certain terminal oxidases. Whether the accelerated enzyme activities were induced by increased substrate concentrations or other physiological conditions is still unknown. In wounded tissue increased activities of these three enzymes have been reported by Keirmeier (1947), Thimann et al. (1954), Hermann (1954) and Prokoshev et al. (1947). In virus infected plants increased cytochrome oxidase activity was reported by Solymosy (1963). With regard to the changed activity of peroxidase and catalase in virus infected plants several investigators reported stimulated peroxidase activity accompanied by suppressed activity of catalase (Vager, 1955; Orlob et al. 1961) but others observed divergent results (Wynd, 1958).

Fluoride injured leaves of many plants show a marked increase in oxygen uptake. Hill and Pack (1959) in gladioli resistant to fluoride injury, observed that the respiration rate was not affected in spite of the accumulation of very high concentration of fluoride, and concluded that the increased oxygen uptake was associated with injury. Yu and Miller (1964) showed a marked increase of oxygen uptake in fluoride fumigated soybean leaves before visual injury symptoms were manifested. Reiner (1947) cultured Saccharomyces carlabergensis in media containing different concentrations of NaF. It was observed that fluoride stimulated respiration at low concentrations and inhibited at high concentrations. By applying a mathematical treatment on the known fact that fluoride inhibited enolase and adenosintriphosphatase, he inferred that it is possible for fluoride to stimulate respiration at low concentrations. He called this mechanism multiple enzyme inhibition. McNulty and Lord (1960), in a study with

Chlorella pyrenoidosa, found a significant increase in phosphorylated nucleotides accompanied by accelerated oxygen uptake. They also explained the results on the basis of multiple enzyme inhibition. In this study activities of three terminal oxidases: cytochrome oxidase, peroxidase and catalase in fluoride fumigated soybean leaves were markedly stimulated, while ascorbic acid oxidase and polyphenol oxidase were in general suppressed by atmospheric fluoride. On the other hand, none of these oxidases were markedly stimulated by fluoride in vitro. The result may suggest that the accelerated oxygen uptake induced by atmospheric fluoride is not simply due to physical injury, but a combination of chemical and mechanical effects.

The enzyme activity of glucose-6-phosphate dehydrogenase was very low in normal soybean leaves, but was greatly increased in hydrogen fluoride fumigated leaves. Enzymes in the Emden Meyerhoff-Parnas pathway and TCA cycle, principally phosphorylase (Rapp and Sliwinski, 1956), phosphatase (Lammer and Hafer, 1953; Pierpoint, 1958), hexokinase (Melchior and Melchior, 1956), adenosintriphosphatase (Reiner, 1947), enolase (Warburg and Christian, 1942; Miller, 1958) and succinic dehydrogenase (Slater and Bonner, 1952) have been reported to be fluoride sensitive. The oxidative break down of carbohydrates in these fluoride sensitive plants may follow the fluoride resistant hexose monophosphate shunt mechanism. The increased operation of this pathway has also been observed in rust infected safflower (Daly, Sayer and Pazur, 1957) mature plant tissues (Gibbs and Beevers, 1955) and TMV infected tobacco leaves (Solymosy and Farkas, 1963). The altered respiratory pathway does not seem to be specific for fluoride injured plant tissues. However, the increased activity of

glucose-6-phosphate dehydrogenase in TMV infected necrotic tobacco leaves was only three times that found in control tissue, while the increase in this enzyme activity in fluoride fumigated soybean leaves was about 20 times that of the control. This great augmentation of glucose-6-phosphate dehydrogenase activity which catalyzes the first reaction in the hexose monophosphate shunt, indicates that the effect of fluoride on plant respiration may be different from that caused by disease or wounding.

SUMMARY

Soybean (Glycine max, Merr, Var. Hawkeye) was cultured in Hoagland's solution. Plants were fumigated with hydrogen fluoride (ca. 100 ppb). After 24, 96 and 144 hours of fumigation, the enzyme activities of cytochrome oxidase, peroxidase, catalase, polyphenol oxidase, ascorbic acid oxidase and glucose-6-phosphate dehydrogenase, both in leaves from fumigated and control plants were studied. The total oxygen uptake after each time of treatment was also measured. In vitro tests with KF solutions on each terminal oxidases extracted from normal young soybean leaves was also carried out.

Glucose-6-phosphate dehydrogenase activity from fumigated leaves showed an average increase of 5 to 22 times that of the control. Cytochrome oxidase, peroxidase and catalase activities were markedly stimulated by fluoride fumigation. Polyphenol oxidase activity was suppressed throughout the fumigation period. Ascorbic acid oxidase was stimulated at the initial state, then showed a steady decrease in activity. In vitro tests revealed that ascorbic acid oxidase and peroxidase were very sensitive to fluoride ion. Polyphenol oxidase was only slightly inhibited by 10^{-2} M KF solution. Cytochrome oxidase and catalase were not affected by KF up to 10^{-2} M. Total respiration throughout the treatment period showed an accelerated rate.

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