A STUDY OF THE TERMINAL OXIDATIVE MECHANISMS IN HIGHER PLANTS.

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By

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INTRODUCTION

This is a study of the terminal oxidative systems in a number of higher plants, namely, sweet potato, Swiss chard, soybean, wheat, and tomato. The enzyme systems investigated were polyphenol oxidase, ascorbic acid oxidase, and alpha-hydroxy acid oxidase. The effect of copper deficiency in tomato plants was studied in connection with these enzyme systems, and especially in connection with polyphenol oxidase.

HISTORICAL

The oxidative mechanisms in higher plants have not been as extensively investigated as have the metabolic pathways in animal tissue. Potato tubers and such other storage tissues as squash, cauliflower, and cucumbers have been most used as experimental material. Leaf tissue has been used less extensively, and such studies as have been made have, with few exceptions, dealt with monocotyledenous plants, specifically the oat coleoptile.

Szent-Gyorgyi (1) used both minced cabbage leaf suspended in phosphate buffer and juice from the cabbage for studies on oxidative mechanisms. Both showed a great increase in oxygen consumption when ascorbic acid was added. He found the oxygen uptake was not inhibited by small amounts of cyanide, urethane, or chloroform, and proposed the name of hexoxidase for this enzyme. He believed that ascorbic acid played an important part in respiration of the cabbage leaf and stated that "it (ascorbic acid) connects as a hydrogen carrier, the system in which molecular oxygen enters into the reaction with the system which supplies hydrogen and is involved in the oxidation of foodstuff."

Powerful extracts of ascorbic acid oxidase, as this enzyme was later designated, were prepared from Hubbard squash by Tauber, Kleiner, and Mishkind, (2). The catalyzing activity on the oxidation of L-ascorbic acid was tested by measuring the rate of oxidation of ascorbic acid using the usual titrimetric procedure with 2,6-dichlorophenolindophenol. The enzyme was shown to be specific for ascorbic acid and did not catalyze the oxidation of phenols, glutathione, cysteine, or adrenalin. The oxidation of ascorbic acid was not inhibited by CO or small amounts of KCN.

Stotz, Carter, and King (19) studied ascorbic acid oxidase in relation to its copper content. They used press juice of cabbage, cauliflower, and squash oxidase, Cu, copper albumin, and copper gelatin and tested the action

of sodium diethyldithiocarbamate, 8-hydroxyquinoline, pyridine, NaCN, potassium ferrocyanide, potassium ethylxanthate, and sodium sulfide on them as inhibitors. All the above inhibitors were found to poison the enzyme completely. They believe the catalytic activity of squash and cauliflower juices on the oxidation of ascorbic acid is due to the presence of copper in combination with a protein.

Extracts from cabbage, cauliflower, cucumber, and marrow were shown by Johnson and Zilva (3) to contain an enzyme capable of oxidizing L-ascorbic acid and D-gluco-ascorbic acid. Subsequent to the complete destruction of ascorbic acid, catechol was then oxidized to the corresponding quinone. Extracts from potato and apple tissue did not catalyze the oxidation of ascorbic acid, but in the presence of catechol the former was oxidized.

James and Cragg (7) used juice expressed from week-old etiolated barley shoots. The addition of ascorbic acid to this caused a marked increase in the oxygen uptake, which was inhibited completely by .002 M KCN. Catechol was not oxidized by these preparations. The addition of glycollic, lactic and tartaric acids to the extract caused an increase in respiration. From these results they conclude that ascorbic acid oxidase is active as a terminal oxidase in the barley plant.

An active ascorbic acid oxidative mechanism was also found in potato tissue by Mommaerts (13). Catechol increased the oxygen uptake even more, and this was inhibited by cyanide, sulfide, hydroxylamine, fluoride, and azide. The inhibition by cyanide was reversed by Cu. Fe, Mn, Zn, Co, nor Mg reversed the inhibition by cyanide. This is characteristic of phenolase (tyrosinase, polyphenol oxidase). Therefore, the oxidation enzyme of potato tissue is a copper protein, probably identical with phenolase.

Keilin and Mann (18) prepared polyphenol oxidase from mushrooms, and

found it to be a copper-proteid that was dependent for its activity on the presence of copper; however, in opposition to previous work done by Kubowitz, the activity was not in direct proportionality to the amount of copper present. The enzyme was found to be specific for catalyzing the oxidation of ortho-dihydroxy phenols such as catechol, pyrogallol, and dihydroxyphenylalanine. This reaction was inhibited by KCN, H_2S, NaN_3, and CO. Polyphenol oxidase was found to catalyze the oxidation of ascorbic acid only in the presence of catechol. On purification the enzyme gradually lost its ability to aid in the oxidation of monophenols.

Boswell and Whiting (4) studied the polyphenol oxidase system in potato tubers using catechol and measuring the increase in oxygen uptake when this substrate was added to tuber slices in Warburg flasks. They concluded that 2/3 of the total respiratory gaseous exchanges of the potato tuber was carried on through this polyphenol oxidase system. The remaining 1/3 was mediated by another mechanism.

Boswell (12) examined potato tubers for oxidation systems involving polyphenols, ascorbic acid, dihydroxymaleic acid, and the four-carbon organic acids. In trying to find a suitable polyphenol he found that dihydroxyphenylalanine increased the oxygen consumption by this tissue.

Tyrosinase (polyphenol oxidase) is responsible for 85% of the oxygen uptake by potato tissue according to Baker and Nelson (8). Catechol was used as a substrate and the course of the oxidation was followed over several hours. At the beginning the rate of oxygen consumption increased but as time progressed the rate began to decrease till it fell below the endogenous level. Also used as substrates were p-tert.-butylcatechol, protocatechuic acid, and p-cresol, and it was found that the oxidation of p-tert.-butylcatechol was similar to that of catechol. Protocatechuic acid as the substrate caused no diminution even at 250 minutes. They found that 4-nitrocatechol and KCN both inhibited the

endogenous respiration.

Robinson and Nelson (9) added L-tyrosine and ascorbic acid to potato tuber slices previously washed in running tap water for 24 hours, and found that no L-tyrosine was oxidized till all of the ascorbic acid had been oxidized. Tyrosinase acted more rapidly on dihydroxyphenylalanine than on tyrosine. Ascorbic acid reduces all of the corresponding quinone to DOPA. They suggested that DOPA is the hydrogen carrier that functions adjacent to the terminal oxidase in the potato tuber.

Bonner and Wildman (21) used both a brei prepared from whole leaves of the spinach and the whole cytoplasm of spinach leaves and showed that these leaves contained a highly active polyphenol oxidase. The addition of catechol or dihydroxyphenylalanine to either of these caused a marked increase in the oxygen consumption. The oxidation of catechol was inhibited by p-nitrophenol, and to a less extent by o-nitrophenol. The endogenous respiration was inhibited by HCN.

Li and Bonner (14) used Chinese tea leaves to investigate the tea oxidase that is responsible for the conversion of green tea to black tea. Catechol added to tea oxidase caused an increased oxygen consumption. KCN did not inhibit the action of catechol, but weak concentrations of p-nitrophenol did inhibit this oxidation. However, using minced leaf tissue in phosphate buffer they were able to show no increased oxygen uptake by the addition of catechol. KCN slightly inhibited normal tea-leaf respiration. p-nitrophenol was less inhibitory than on catechol and the tea oxidase. They concluded therefore, that "tea oxidase does not appear to participate in normal leaf respiration of the tea plant."

The oxygen uptake by carrot roots was found to be reversibly inhibited by

carbon monoxide, and low concentrations of cyanide and azide in work done by Marsh and Goddard (5). These inhibitors are known to markedly affect cytochrome oxidase. 100 mg. samples of immature carrot leaves were floated on phosphate buffer and the inhibitory effect of HCN, NaN_3, and CO was noted. The O_2 consumption by mature leaves was not inhibited by these.

The presence of cytochrome oxidase in wheat embryos was demonstrated by Brown and Goddard (6). They found that HCN, NaN_3, and CO inhibited the oxygen uptake by embryos from intact wheat seeds. The addition of cytochrome and hydroquinone to cytochrome oxidase extracted from the wheat embryo stimulated oxygen consumption. Dihydroxyphenylalanine in combination with cytochrome and the oxidase extract caused an increased O_2 consumption. The inhibitors affecting the intact tissue also decreased the oxygen uptake of the systems, cytochrome + cytochrome oxidase + either hydroquinone, p-phenylenediamine, or dihydroxyphenylalanine.

Henderson and Stauffer (10) investigated the influence of some respiratory inhibitors on the respiration of excised tomato roots. An enzyme system inhibited by azide and cyanide was found. No malonate-sensitive or fluoride-sensitive systems were demonstrated. This has similar characteristics to the cytochrome system. Iodoacetate at 0.001 M concentration also inhibited rather strongly.

Goddard (11) has isolated cytochrome C from wheat germ and found that it has the same absorption spectra as heart cytochrome C, and could be catalytically oxidized by wheat and heart cytochrome oxidase.

EXPERIMENTAL

Material: Swiss chard, Beta Vulgaris Var. Cicla, L. var. unknown, was obtained from a well-fertilized field plot. The center leaves unmolested by insects were used. The wheat, Triticum aestivum, L., var. Pawnee, and soybean, Glycine max, Merr., var. Ogden, leaves were taken from plants grown in soil in the greenhouse. The sweet potato, Ipomoea Batatas, Lam., var. Porto Rican, leaves were obtained from plants grown in low-nitrogen nutrient solution in the greenhouse. The tomato, Lycopersicon esculentum, Mill., var. Rutgers, plants were germinated in soil and when 21 days old were transplanted to nutrient solution which contained the following concentrations of reagents made up in water redistilled from an all glass still:

.002 M K_2HPO_4

.006 M $Ca(NO_3)_2$

.003 M $MgSO_4$

.003 M $(NH_4)_2SO_4$

4.62×10^{-5} M H_3BO_3

1.85×10^{-6} M $MnCl_2.4H_2O$

7.73×10^{-7} M $ZnSO_4.7H_2O$

6.46×10^{-7} M $H_2MoO_4.H_2O$

1 M stock solutions of the first four salts were purified according to the method given by Stout and Arnon (16) to remove Cu, Fe, and Mn. The latter four salts were combined and at a concentration such that 1 ml. could be added to each liter of culture solution with the final concentration obtained as indicated.

This solution was changed about every 10 days and at this time 1 ml. of a 0.05% iron tartrate solution was added. In the intervening time the 2-liter beakers were kept full with redistilled water. Each beaker contained six

tomato plants. There were 10 beakers, 4 controls, and 6 copper free. 1 ml. of .008% copper sulfate solution was added to the control beakers with each change in nutrient solution.

The plants in the copper-free medium grew as rapidly as those in the control culture until about 43 days after transplanting. At this time the leaves of the plants in the copper-free medium began to curl up and yellow spots appeared on the leaves. The control plants continued to grow and the leaves developed normally. However, after this time the leaves of the copper-deficient plants failed to grow larger. It was noted that even within 23 days after transplanting the root system of the plants in the copper-free solution were not as extensive as those in the control solution, and also a fungus growth covered the roots of all the copper-deficient plants, and this growth was absent from all of the control plants.

Copper was added to one set of plants in which the deficiency had become marked. Within one day the leaves of this plant began to straighten out and look healthier in general. Within 10 days this plant looked normal as compared to the controls. In the course of the experiment it outlived all of the other plants with the exception of one set of controls.

Substrates and Inhibitors: The sodium diethyldithiocarbamate and o-nitrophenol inhibitors, and catechol, lactic acid, glycollic acid, and dihydroxyphenylalanine[1] substrates were made up in phosphate buffer of 0.1 M and the pH adjusted to 6 using bromthymol blue indicator. The ascorbic acid substrate was prepared fresh each time it was used.

Collection and Treatment of Samples: Swiss chard was collected in the fall

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[1] Acknowledgment is made of the gift of the Dow Chemical Corp., Midland, Michigan, for the material used.

and in the spring from well-fertilized field culture. Other plants were grown under normal greenhouse conditions during the winter and spring. The petiole and mid-rib were removed and leaf-blade tissue only used. The leaves were never harvested earlier than 1/2 hour before use.

The cell-free extract was usually obtained by grinding about 15 grams of the actively metabolizing leaf tissue with sand and 30 ml. of 0.1 M phosphate buffer, pH 6.0, in a mortar and straining through a muslin cloth. In the case of sweet potato, and Swiss chard later on the cell-free extract was obtained by homogenizing about 25 grams of leaf tissue with 50 ml. of the phosphate buffer in the Waring blender and straining the homogenate through muslin.

Measurement of Oxygen Consumption: The amount of oxygen consumed by the cell-free extracts was measured using the Warburg constant-volume respirometer, at 30.0°C. The total volume of the contents of the flasks was always kept at 3.15 ml. with contents as follows: 0.15 ml. saturated KOH plus a filter-paper wick in the center well to absorb the CO_2 evolved, 1 ml. of substrate in the sidearm, 1 or 2 ml. of cell-free extract in the main portion, 1 ml. inhibitor in the main portion, and when necessary, 1 ml. of 0.1 M, pH6 phosphate buffer in the main portion. The flasks were always equilibrated 15 minutes, after which the contents of the sidearm was added to the cell-free extract, and the decrease in pressure in each flask was noted for each 10 minute interval for a minimum of 60 minutes. The cubic microliters of oxygen per mg. nitrogen per hour ($Q_{O_2}(N)$) was calculated using the 60 minute value.

Chemical Methods: The nitrogen in the leaf tissue preparations was determined by a semi-micro modification of the Kjeldahl procedure. NH_3 liberated by excess alkali was distilled into boric acid, and titrated with N/56 H_2SO_4.

RESULTS

Studies on Polyphenoloxidase: The general importance which has been attached to this enzyme as a principal terminal oxidase in plant organs prompted investigation of its role in leaf tissue as a preliminary step. Initial studies were made using catechol as a substrate; later dihydroxyphenylalanine became available and was substituted. Since it had been shown that sodium diethyldithiocarbamate was a powerful inhibitor of copper-containing enzymes, its effect on the endogenous rate of O_2 consumption was studied and compared with that in the presence of added substrate. Table I presents the results of a number of such experiments using various species of plants, mostly Dicotyledonaceae, with catechol as substrate. Figure 1 presents a portion of this data in graphic form.

It was found that the addition of catechol to cell-free extracts of Swiss chard, sweet potato and tomato caused a great increase in O_2 consumption over the endogenous level. In soybeans and wheat, however, there was a reduced polyphenol oxidase activity. Endogenous O_2 consumption was variable, but in no instance was a large endogenous rate observed in the preparations used. Anomalous results were observed in the employment of sodium diethyldithiocarbamate as an inhibitor in that, with few exceptions, little or no reduction in O_2 consumption either in the presence or absence of substrate was observed. Swiss chard leaf preparations, for example, showed variable results when this inhibitor was added in the presence of catechol as substrate. In different runs, 0.01 M inhibitor showed no inhibition while 0.003 M reduced O_2 consumption by 3%. In several cases, actual increases in O_2 consumption were observed; the significance of these observations is questionable because in all such cases the total O_2 consumed was quite small, thus increasing the possibility of procedural error.

Table I

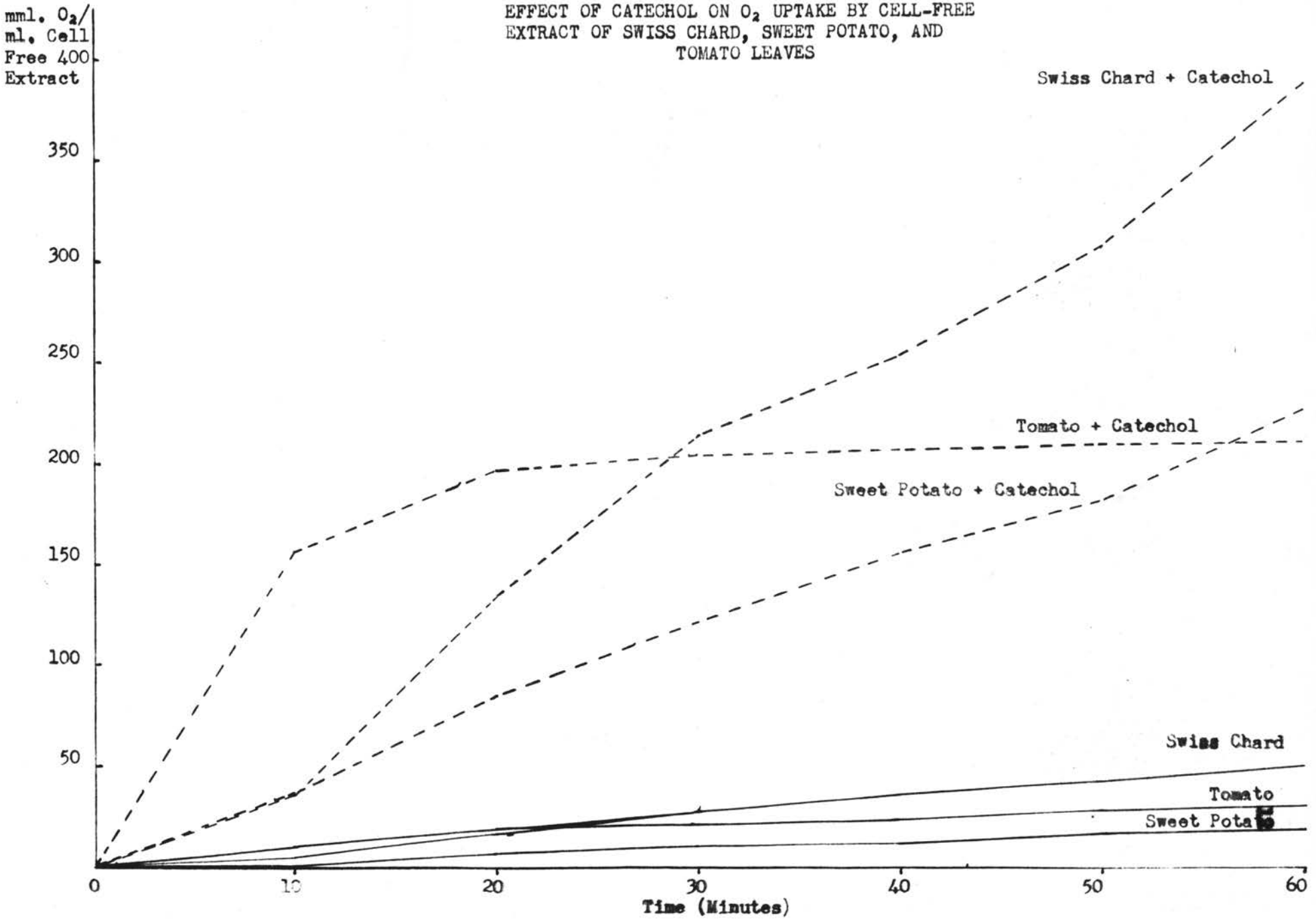
EFFECT OF SODIUM DIETHYLDITHIOCARBAMATE ON CATECHOL OXIDATION

Cell-Free Extract of Leaf Tissue		Final Conc. Na diethyldithiocarbamate	Q_{O_2}(N)	% Inhibition	Q_{O_2}(N)	% Inhibition	Q_{O_2}(N)	% Inhibition
Swiss Chard	Endogenous	none	21		11*		16*	
		.01 M	18	14	13	—	12	—
		.001	20	—	7	—	13	—
	.02 M Catechol	none	244		112		141	
		.01	265	—	86	23	129	9
		.001	250	—	103	7	125	11
Sweet Potato	Endogenous	none	8		24		45	
		.01	0	—	37	—	17	63
		.001	0	—	22	—	45	—
	.02 M Catechol	none	425		298		345	
		.01	394	7	264	11	189	45
		.001	486	—	287	3	297	13
Tomato	Endogenous	none	19					
	.02 M Catechol	none	189					
Soybean	Endogenous	none	15		22			
		.01	28	—	25	—		
		.001	18	—	18	—		
	.02 M Catechol	none	34		24			
		.01	45	—	13	47		
		.001	—	—	16	33		
Wheat	Endogenous	none	7		7		3	
		.01	6	—				
		.001	11	—				
	.02 M Catechol	none	3		46	10		
		.01	1	—				
		.001	6	—				

*Concentrations of inhibitor used were .003 M and .0003 M respectively.

Figure 1

EFFECT OF CATECHOL ON O_2 UPTAKE BY CELL-FREE EXTRACT OF SWISS CHARD, SWEET POTATO, AND TOMATO LEAVES



The addition of dihydroxyphenylalanine to the cell-free extracts of sweet potato and tomato likewise caused a marked increase in the consumption of oxygen over the endogenous level. This is shown graphically in Figure 2. In soybean, the increase in O_2 consumption was much less noticeable in the presence of added substrate. Polyphenol oxidase action on DOPA could not be demonstrated in wheat leaf tissue. It had been shown that o-nitrophenol (21) is an inhibitor of polyphenol oxidase. Table II shows that the use of this inhibitor under our conditions had practically no effect on the rate of O_2 consumption either in the presence or absence of DOPA as a substrate. The effect of sodium diethyldithiocarbamate is shown in Table III. Considerable inhibition was demonstrable in only one instance.

Representative determinations showing the effect of these two inhibitors with tomato preparations are presented graphically in Figure 3.

Ascorbic Acid Oxidase: This enzyme has also been suggested as a metabolically important terminal oxidase in higher plants. A series of studies were made, therefore, using ascorbic acid as a substrate. The action of sodium diethyldithiocarbamate as an inhibitor is shown by data presented in Table IV.

The addition of ascorbic acid to the cell-free extract of soybeans caused a marked increase in oxygen consumption over the endogenous level. In sweet potato and Swiss chard ascorbic acid oxidase was present in reduced amounts, and only a low level of activity could be demonstrated in wheat.

Sodium diethyldithiocarbamate inhibited ascorbic acid oxidation in Swiss chard and soybeans in proportion to the concentration of carbamate used. This is shown graphically in the case of Swiss chard in Figure 4. This effect was also noted in the case of wheat in two out of three runs. The endogenous respiration of these plants was not inhibited except in one or two isolated instances by the concentrations of inhibitor used. In contrast to the effect

Table II

EFFECT OF o-NITROPHENOL ON OXIDATION OF DIHYDROXYPHENYLALANINE

Cell-Free Extract of Leaf Tissue		Final Conc. of o-nitrophenol	Q_{O_2}(N)	% Inhibition	Q_{O_2}(N)	% Inhibition
Sweet Potato	Endogenous	none	19		61	
		.003 M	18	—	60	—
		.0003	19	—	61	—
	.02 M DOPA	none	304		397	
		.003	288	5	450	—
		.0003	290	5	431	—
Tomato Soil Culture	Endogenous	none	15		17	
		.003	11	27	20	—
		.0003	9	37	19	—
	.02 M DOPA	none	329		414	
		.003	336	—	443	—
		.0003	323	—	453	—
Soybean	Endogenous	none	12		7	
		.003	15	—	15	—
		.0003	14	—	17	—
	.02 M DOPA	none	33		42	
		.003	48	—	61	—
		.0003	26	—	68	—
Wheat	Endogenous	none	8			
	.02 M DOPA	none	7			

Table III

EFFECT OF SODIUM DIETHYLDITHIOCARBAMATE ON OXIDATION OF DOPA

Cell-Free Extract of leaf tissue		Final conc. Na diethyldithio-carbamate	Q_{O_2}(N)	% Inhibition
Tomato Soil Culture	Endogenous	none	1	
		.01	2	—
		.001	1	—
	.02 M DOPA	none	67	
		.01	67	—
		.001	67	—
Tomato Solution Culture	Endogenous	none	5	
		.01	11	—
		.001	5	—
	.02 M DOPA	none	153	
		.01	116	24
		.001	166	—

Figure 2

EFFECT OF DIHYDROXYPHENYLALANINE ON OXYGEN UPTAKE BY CELL-FREE EXTRACT OF SOYBEAN, SWEET POTATO, AND TOMATO LEAVES

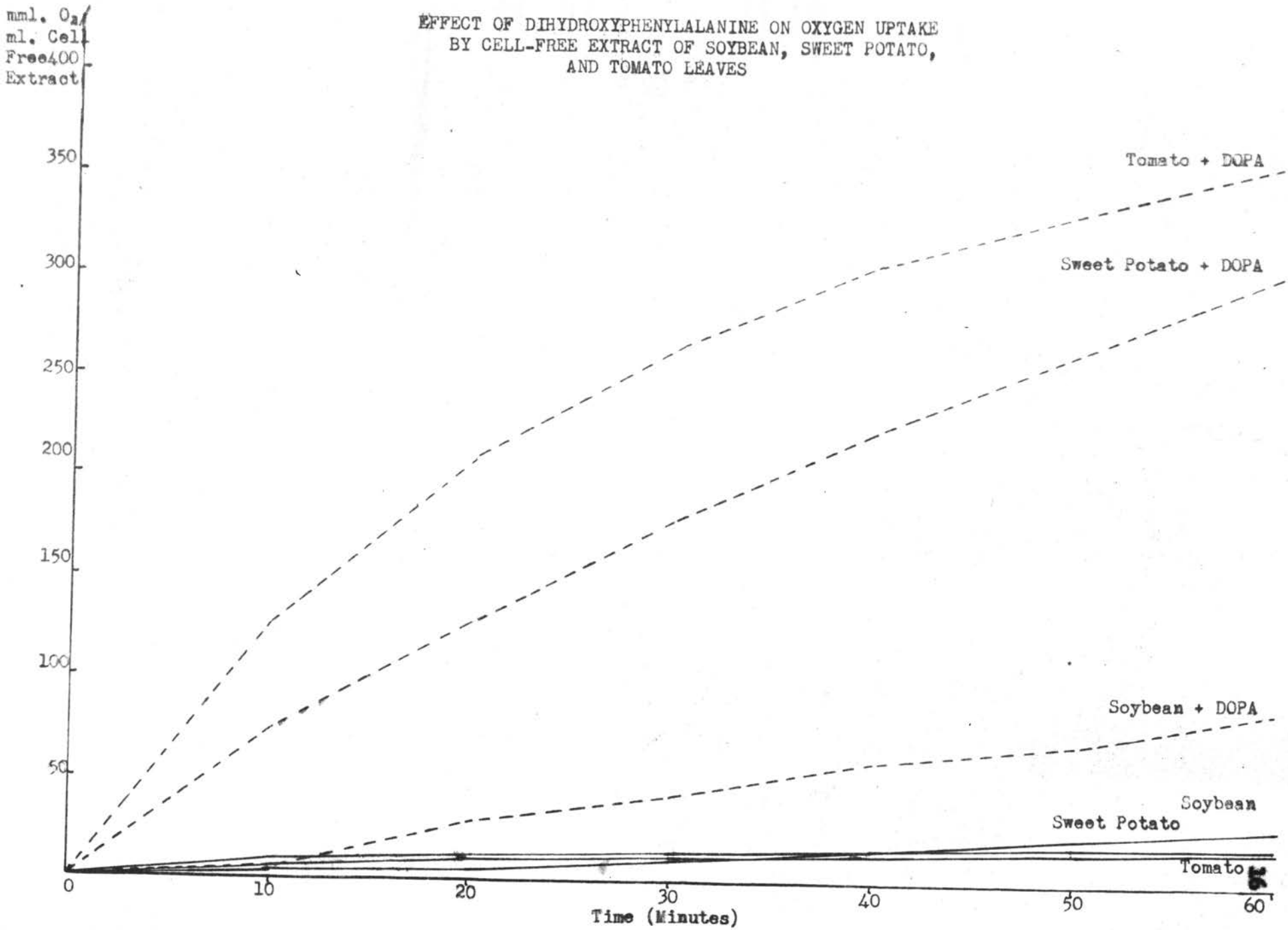


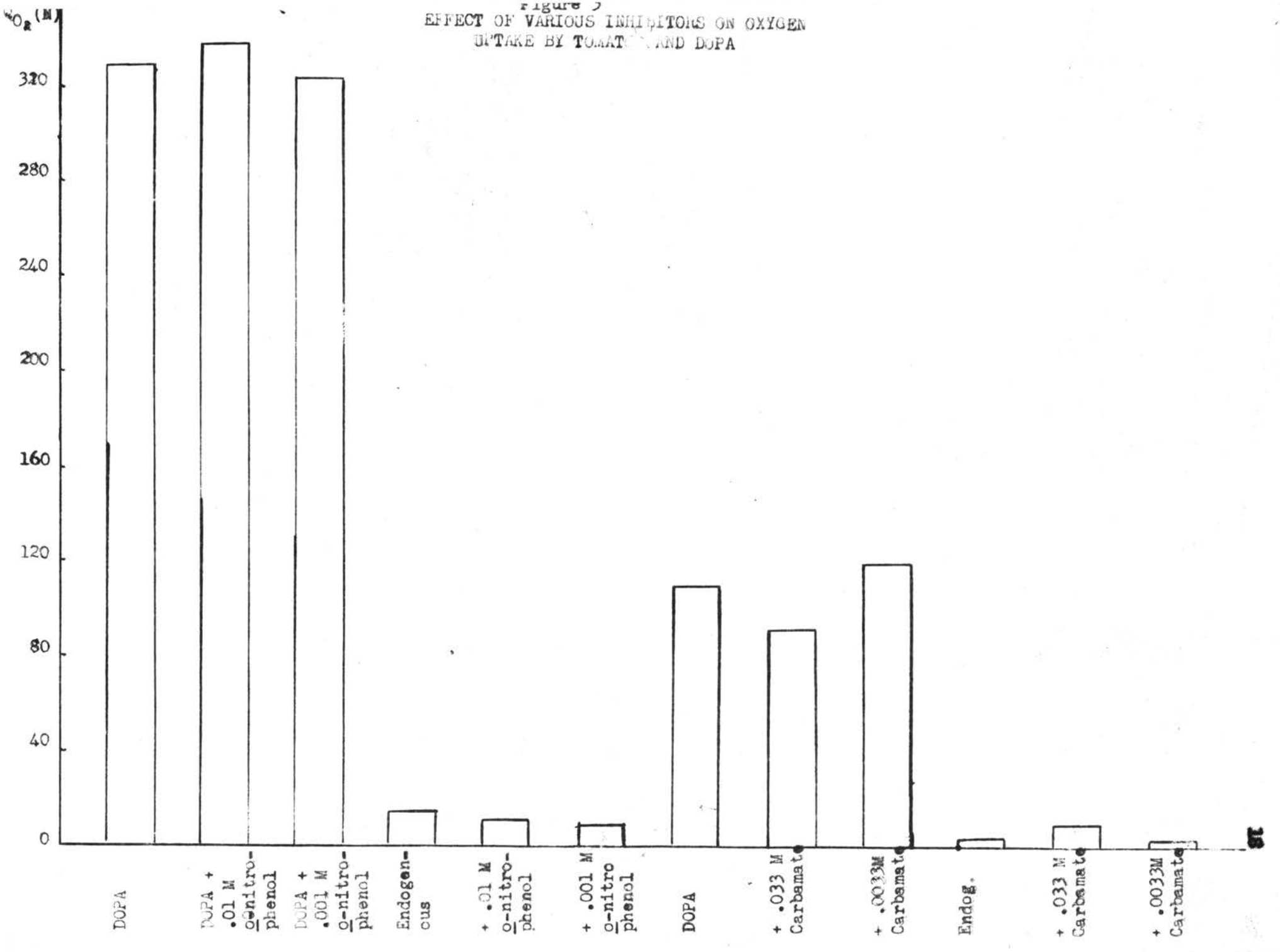
Table IV

EFFECT OF SODIUM DIETHYLDITHIOCARBAMATE ON ASCORBIC ACID OXIDATION

Cell-Free Extract Leaf Tissue		Final conc. Na diethyldithiocarbamate	$Q_{O_2}(N)$	% Inhibition	$Q_{O_2}(N)$	% Inhibition	$Q_{O_2}(N)$	% Inhibition
Swiss Chard	Endogenous	none	14		13		24	
		.01 M	16	—	8	—	24	—
		.001	14	—	17	—	25	—
	.02 M Ascorbic	none	38		90		149	
	acid	.01	20	40	21	37	54	64
		.001	33	14	75	16	111	26
Sweet Potato	Endogenous	none	—		10		22	
		.01	—	—	9	—	15	—
		.001	—	—	18	—	33	—
	.02 M ascorbic	none	44		89		128	
	acid	.01	50	—	196	—	263	—
		.001	49	—	124	—	156	—
Soybean	Endogenous	none	15		15		15	
		.01	32	—	19	—	23	—
		.001	13	—	15	—	17	—
	.02 M ascorbic	none	153		94		177	
	acid	.01	12	92	35	63	36	80
		.001	110	23	91	3	20	89
Wheat	Endogenous	none	11		4		10	
		.01	9	—	11	—	12	—
		.001	11	—	5	—	5	—
	.02 M ascorbic	none	27		23		21	
	acid	.01	15	43	16	31	26	
		.001	32	—	22	5	29	—

Figure 5

EFFECT OF VARIOUS INHIBITORS ON OXYGEN UPTAKE BY TOMATO AND DOPA



in these plants, carbamate inhibitor had no effect on the ascorbic acid oxidation by cell-free extract of sweet potato leaves. In fact a 0.001 M concentration stimulated O_2 consumption markedly in two out of three cases. One of these instances is presented graphically in Figure 5.

Alpha-hydroxy Acid Oxidase: This enzyme, first described by James and Cragg (7) is, aside from the two enzymes previously studied, one of the few terminal oxidative enzymes which have been demonstrated in plant tissues. The details of the mechanism of oxidation has not been thoroughly studied, but it appears from the work of Clagett (17) that lactic acid is oxidized to pyruvic acid; the oxidation product of glycollic acid, the oxidation of which is relatively much more rapid than Lactate, has not been characterized. Preliminary studies with this enzyme were made to assess its relative activity in comparison with polyphenol oxidase and ascorbic acid oxidase. Data from these studies is presented in Table V. Lactic acid caused a slight increase in the oxygen consumption over the endogenous level when added to the cell-free extracts of soybeans, and tomato grown in both soil and solution culture. Wheat leaf preparations did not oxidize either lactate or glycollate. Tomato leaf preparations from plants in both soil and solution culture oxidized glycollate more rapidly than lactate. Representative examples are presented in graphic form in Figure 6.

Effect of Copper Deficiency on Plant Oxidases: The known presence of copper in some of the terminal plant oxidases made it of interest to determine the effect of copper deficiency on the oxidases previously studied. Copper deficient tomato plants were grown as previously described and the relative concentration of enzyme in normal and copper deficient plants determined. These data are presented in Table VI.

The addition of dihydroxyphenylalanine to the cell-free extract from the

Figure 4

EFFECT OF SODIUM DIETHYLDITHIOCARBAMATE ON OXYGEN UPTAKE BY SWISS CHARD AND ASCORBIC ACID

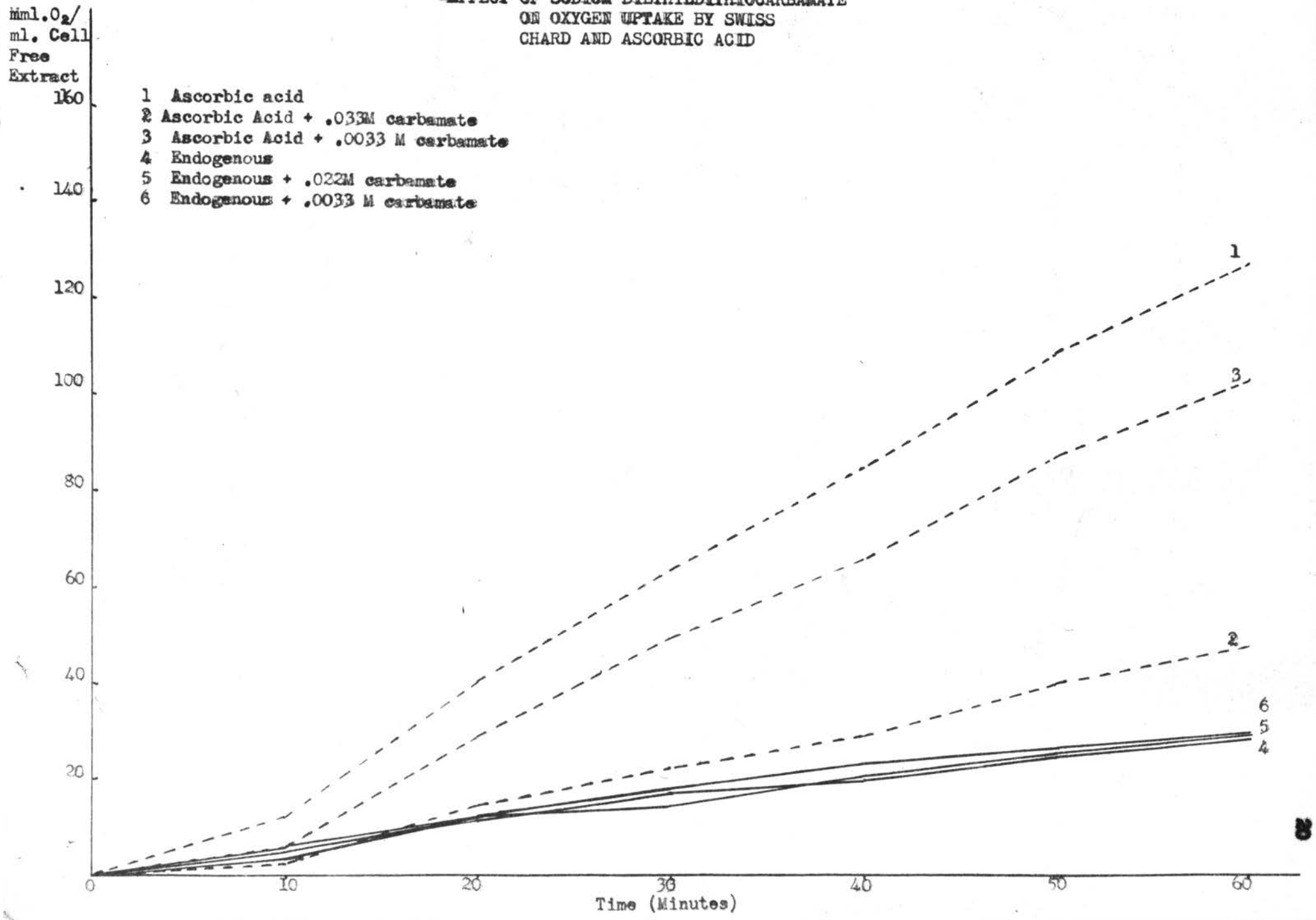


Table V

EFFECT OF GLYCOLLIC AND LACTIC ACIDS ON OXYGEN CONSUMPTION

$Q_{O_2}(N)$

	WHEAT	TOMATO SOLUTION CULTURE	TOMATO SOIL CULTURE	SOYBEAN
Endogenous	7	11	6	17
Lactic Acid	7	43	30	28
Endogenous	13	16	6	
Glycollic Acid	15	50	61	

Figure 5
EFFECT OF SODIUM DIETHYLDITHIOCARBAMATE ON OXYGEN UPTAKE BY SWEET POTATO AND ASCORBIC ACID

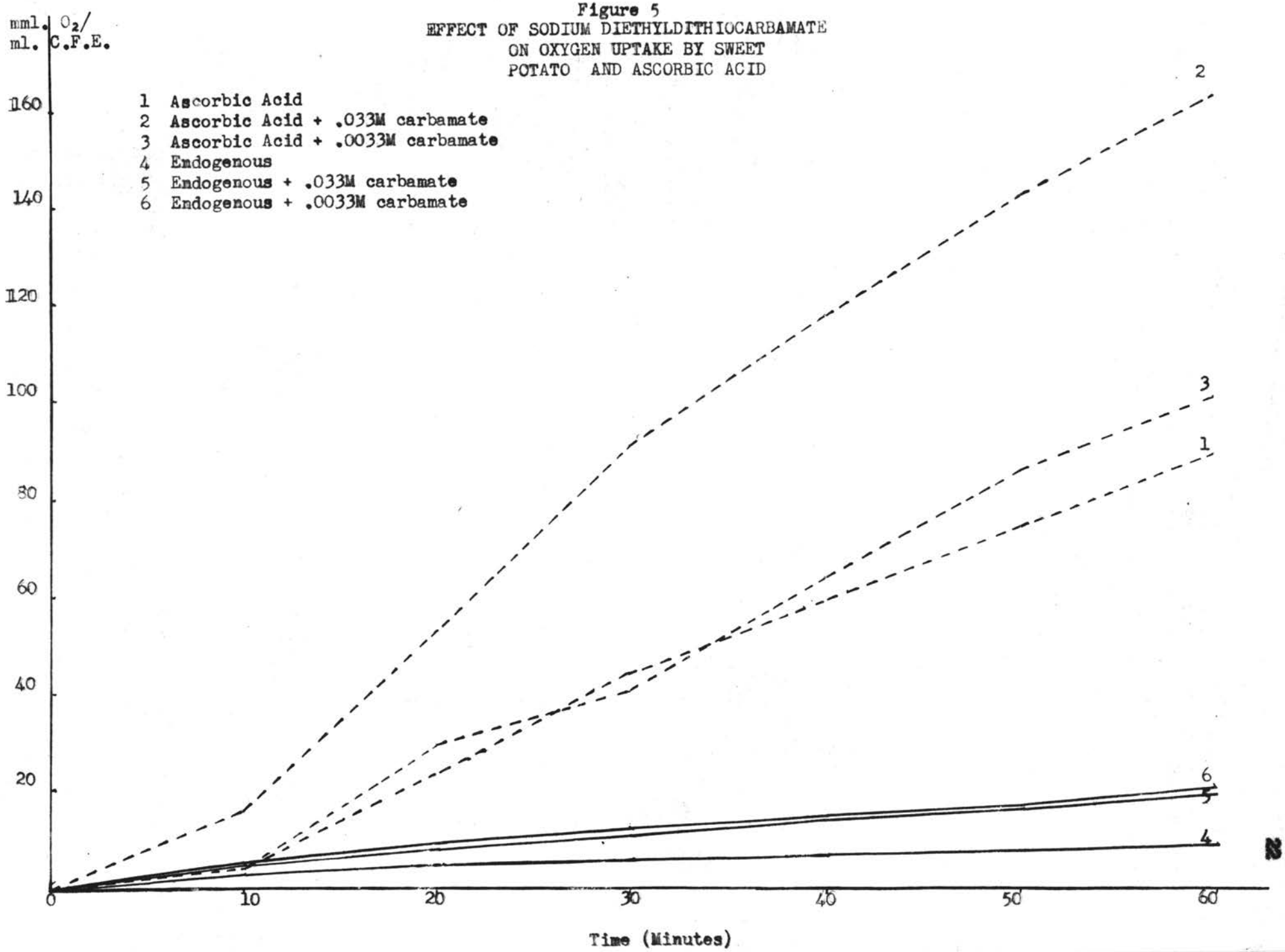


Table VI

EFFECT OF DOPA ON OXYGEN UPTAKE BY TOMATO LEAVES

Description of Tomato Plant Used.	Days after Transplanting	$Q_{O_2}(N)$ Endogenous	DOPA
Soil Control		8	159
	28	6	98
	31	1	68
	45	2	110
	97	15	328
Nutrient Solution	35	5	154
	47	3	214
	50	1	485
	64	11	301
	71	12	439
Copper Deficient	42	9	3
	47	12	14
	50	1	0
	64	14	2
Copper Deficient To Which Copper Was Administered	71 (5)[1]	24	59
	64 (14)[1]	5	270
	97 (32)[1]	9	740
Copper Deficient to Which Copper Was Administered For 15 Days Only.	71 (5)[2]	5	592
	97 (31)[2]	35	483
	104 (38)[2]	14	432

[1]Days after beginning copper treatment.
[2]Days after copper treatment ceased.

leaves of the copper-deficient tomato produced little or no increase in the O_2 consumption above the endogenous level, whereas, this same substrate increased the oxygen consumption of the normal tomato plant many hundred per cent. This is shown in Figure 7.

It was noted that at the end of a run using the normal and deficient tissue extracts + DOPA, that the flasks containing the normal extract + DOPA showed a great amount of oxidation of the substrate to the dark pigment, whereas, no oxidation of DOPA by the abnormal tissue was evident.

The effect of the administration of 2 mg. of copper per culture to the copper-deficient tomato plant appears in the ability of the cell-free extract from the leaves of this plant to oxidize DOPA within five days after the treatment was begun. Figure 8 shows the effect of the length of time of copper administration on the increase in DOPA oxidation by the extract from the once copper-deficient plant.

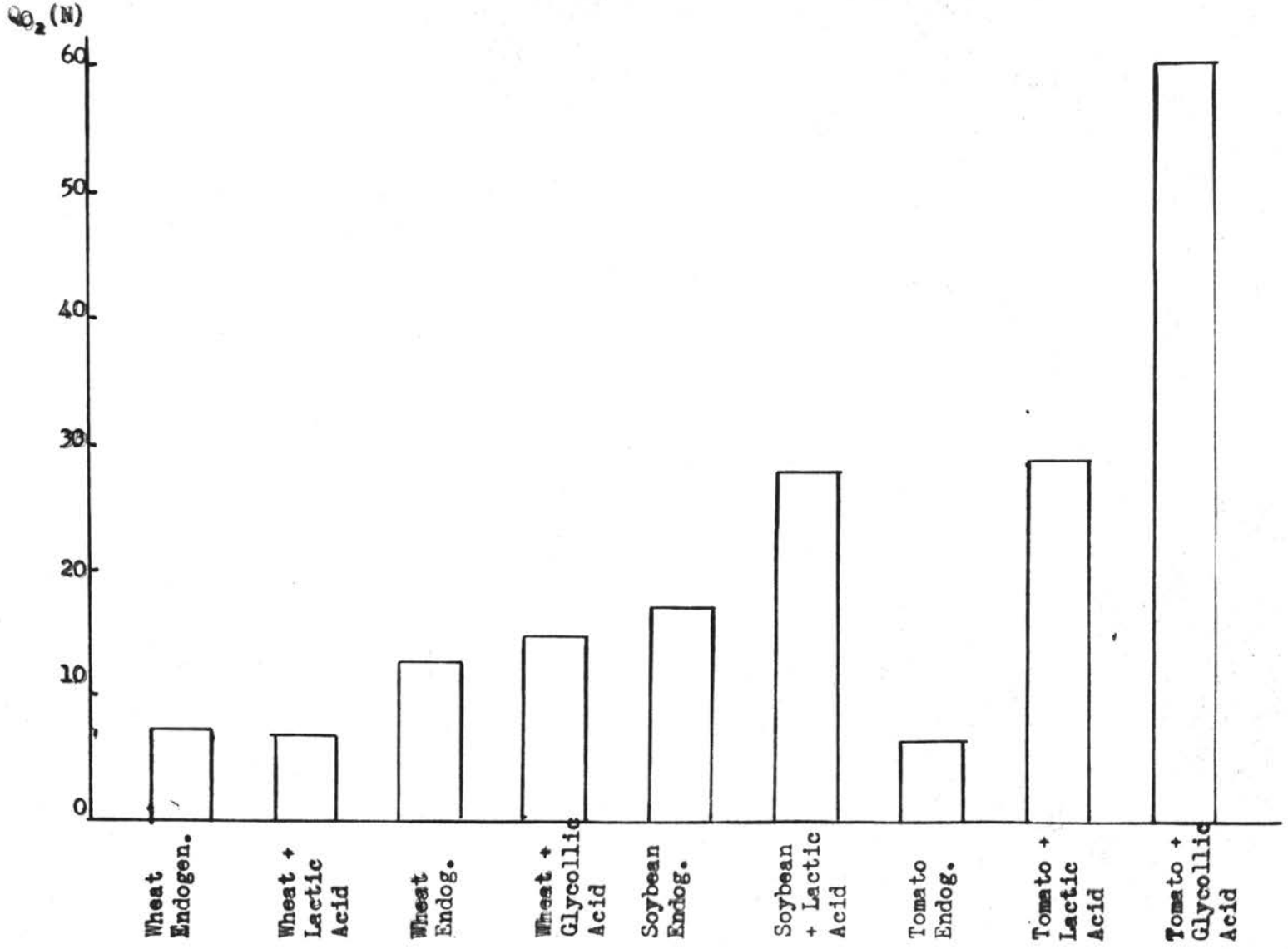
After the administration of 2 mg. of copper over a period of 15 days to one set of copper-deficient plants, the plants were again placed in a copper-free medium. Thirty-eight days after copper was again removed, the appearance of the plants was normal and no reduction in polyphenoloxidase activity was observed.

A run was made in which 3 mg. of copper was added to the cell-free extract + DOPA system. There was a very slight increase in oxygen consumption over the endogenous level, to which no copper had been added.

Determination of the ascorbic acid oxidase activity of cell-free extracts of normal, copper-deficient, and deficient to which Cu had been administered tomato plants was made using ascorbic acid as the substrate. Normal plants from both soil and solution culture showed the presence of an active ascorbic acid oxidase. The extract from the copper-deficient plants did not show any

Figure 6

GLYCOLLIC AND LACTIC ACIDS AS SUBSTRATES



appreciable activity; normal content of the enzyme was restored after 15 days on a solution containing copper.

The addition of lactic acid to the extract from the Cu-deficient tomato produced a $Q_{O_2}(N)$ of 29 against an endogenous $Q_{O_2}(N)$ of 2. This was of the same order of magnitude as that produced in the control tomato in both soil and solution culture in which the respective $Q_{O_2}(N)$, corrected for the endogenous O_2 consumption, were 24 and 32.

Table VII

EFFECT OF ASCORBIC ACID ON $Q_{O_2}(N)$ IN TOMATO

Description of Tomato Plant Used	Days after Transplanting	$Q_{O_2}(N)$ Endogenous	$Q_{O_2}(N)$ Ascorbic Acid
Soil Control		6	76
Nutrient Solution Control	49	0	43
Copper deficient	49	1	0
Copper deficient to which Copper was administered for 48 days	113	0	56
Copper deficient to which Cu was administered for 15 days only 37 days after ceasing to supply Cu.	103	14	26

Figure 7

EFFECT OF COPPER DEFICIENCY ON OXYGEN UPTAKE BY TOMATO AND DOPA

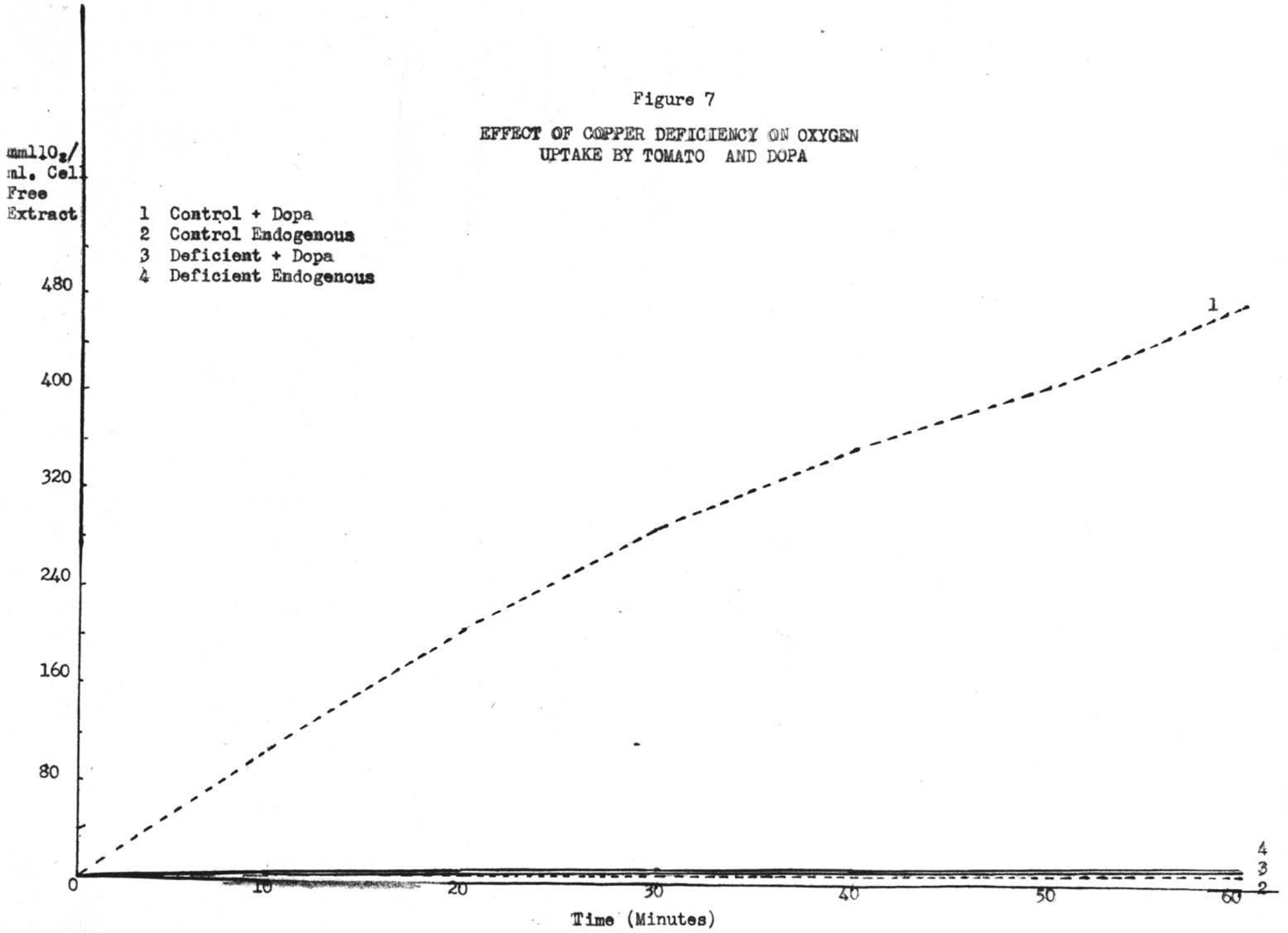
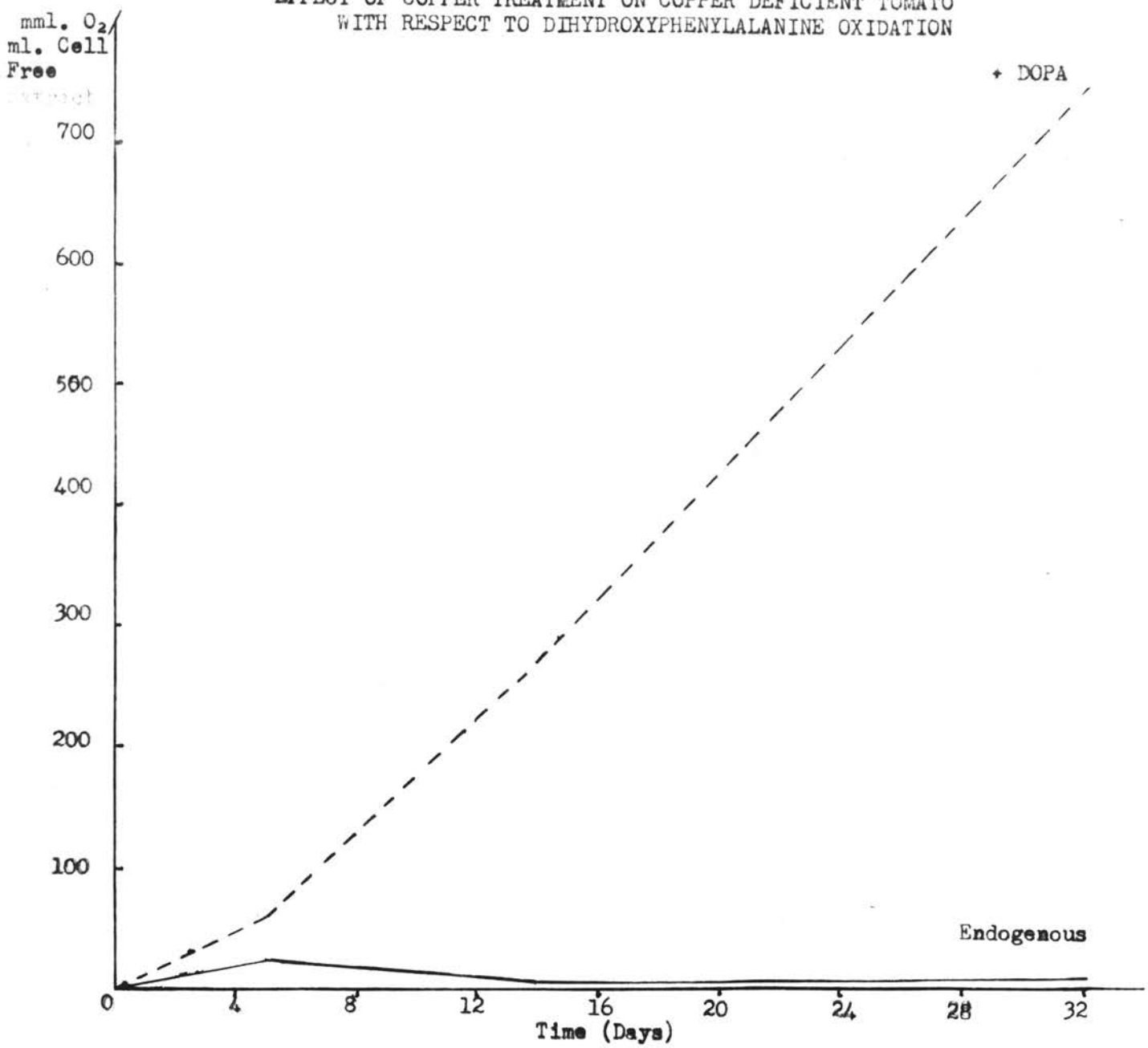


Figure 8

EFFECT OF COPPER TREATMENT ON COPPER DEFICIENT TOMATO
WITH RESPECT TO DIHYDROXYPHENYLALANINE OXIDATION



DISCUSSION

A very powerful polyphenol oxidase was found to be present in tomato and sweet potato, and to a less extent in Swiss chard, as was demonstrated by the very great increase in $Q_{O_2}(N)$ on addition of catechol or dihydroxyphenylalanine to extracts from the leaf tissue of these plants. Soybean has a somewhat less active polyphenol oxidase since the addition of the above mentioned substrates caused only a slight rise in the oxygen consumption over the endogenous level. Polyphenol oxidase is not active in wheat since no constant increase in $Q_{O_2}(N)$ could be produced with the addition of DOPA or catechol.

No inhibition by sodium diethyldithiocarbamate or o-nitrophenol in the concentrations used could be demonstrated for either catechol or DOPA oxidation. Keilin and Mann (18) had shown that polyphenol oxidase was a copper protein complex, and Stotz, Carter, and King (19) had shown that sodium diethyldithiocarbamate is a powerful inhibitor of ascorbic acid oxidase, a copper containing enzyme. Bonner and Wildman (21) showed that o-nitrophenol worked fairly well as an inhibitor for the oxidation of phenols by polyphenol oxidase. The failure to demonstrate an inhibitory action of sodium diethyldithiocarbamate, therefore, presents an anomaly unless one assumes that either the polyphenol oxidase of leaf tissue is not dependent on the presence of copper for its activity or that the copper-protein complex is of such a nature to prevent the action of the inhibitor. It may also be that oxidation of catechol and DOPA can be accomplished by an enzyme system other than the one characterized by Keilin and Mann (18) in mushrooms as a copper proteid. Although Keilin and Mann felt that copper was essential for the action of polyphenol oxidase, they were not able to find a directly proportional relationship between activity and copper content.

In contrast to the findings mentioned above, the addition of DOPA to extracts of the copper-deficient tomato did not increase O_2 consumption. Furthermore, extracts from a copper-deficient plant that had been treated with copper only a short time were shown to oxidize DOPA, the amount of oxidation being in proportion to the length of time of treatment. All of this indicates that copper is necessary for DOPA oxidation. Since administration of only 2 mg. of copper per culture over a period of 15 days was necessary to prevent the diminution of polyphenol oxidase activity up to 38 days after treatment was ceased, only very small amounts of copper are necessary for polyphenol oxidase activity.

Nothing can be said regarding the importance of polyphenol oxidase as a terminal oxidase in the plants investigated since the inhibitors used had no effect on the substrates that are oxidized by polyphenol oxidase.

A powerful ascorbic acid oxidase was found in Swiss chard and soybeans, as shown by the marked increase in oxygen consumption by the cell-free extracts of these plants in the presence of ascorbic acid. The oxidation of ascorbic acid was markedly inhibited by sodium diethyldithiocarbamate. The enzyme, or some other enzyme capable of oxidizing ascorbic acid was found in sweet potato as was demonstrated by the increase in $Q_{O_2}(N)$ by ascorbic acid over the endogenous level, but no inhibition by carbamate was produced. Ascorbic acid oxidase appears to be weakly active in wheat since the extract from wheat was shown to oxidize ascorbic acid, and this oxidation could be inhibited by the concentrations of carbamate used.

Ascorbic acid oxidase, however, does not appear to be an important terminal oxidase in the above mentioned plants, since no inhibition by carbamate was demonstrable in the endogenous oxidation.

In the case of this enzyme as well copper deficiency in tomato resulted

in an inability to oxidize ascorbic acid. This is sustaining evidence for the other that copper is an integral part of ascorbic acid oxidase.

A weakly active alpha-hydroxy acid oxidase was found in tomato and soybean as was shown by the increased oxygen consumption by the cell-free extracts of these plants in the presence of lactate and glycollate. This enzyme was not found to be active in wheat, since the addition of lactate or glycollate had no effect on increasing the $Q_{O_2}(N)$. Alpha-hydroxy acid oxidase appears to be as active in the copper-deficient tomato as in normal as was shown by increases in $Q_{O_2}(N)$ of comparable magnitude in these plants on the addition of lactic acid. This suggests that in contrast to the suggestion of James & Cragg (7), ascorbic acid oxidase is not involved in the hydrogen transport mechanism since these copper-deficient plants were essentially devoid of the ability to oxidize ascorbic acid.

From these data it seems clear that copper-deficient tomato plants are essentially incapable of oxidizing either DOPA or ascorbic acid. The ability to oxidize these substrates is returned by adding copper to the nutrient solution. This strongly suggests that copper plays an essential role in the formation or action of both of these enzymes. The fact that these copper-deficient tomato plants survived as long as they did might be attributable to the fact that the important terminal oxidase in the tomato is not a Cu containing enzyme, or that enough copper that was obtained when the seeds were germinated in soil was retained. Both explanations may, in fact, offer a partial explanation of the continued life of the plant. The almost complete absence of either polyphenol oxidase or ascorbic acid oxidase activity, however, favors the former.

SUMMARY

1. A very powerful polyphenol oxidase is present in tomato and sweet potato. Moderate polyphenol oxidase activity is present in Swiss chard. Polyphenol oxidase is not active in wheat and only weakly active in soybean.

2. A strongly active ascorbic acid oxidase is present in Swiss chard, soybeans, and tomato, and weakly active in wheat. An enzyme was found in sweet potato that oxidized ascorbic acid but was not inhibited by sodium diethyldithiocarbamate.

3. Ascorbic acid oxidase does not appear to be an important terminal oxidase in sweet potato, Swiss chard, soybean, wheat, or tomato in vivo.

4. A weakly active alpha-hydroxy acid oxidase is present in soybeans and tomato, and absent from wheat.

5. In copper deficient tomato plants it was observed that enzymatic oxidation of dihydroxyphenylalanine and ascorbic acid by cell-free extracts of leaf tissue was absent.

6. Alpha-hydroxy acid oxidase is not decreased by the copper deficiency as measured by lactic acid oxidation.

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Mary-Louise Rothschild was born in Minneapolis, Minnesota on January 3, 1923. She attended grammar school, high school, and Wright Jr. College in Chicago, majoring in accounting at the latter. She received a B. S. in Chemistry from Northwestern University in June 1945.

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She began her graduate work at the University of Illinois in Sept. 1946.

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