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Activation of Polyphenol Oxidase of Chloroplasts[1]

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ABSTRACT

Polyphenol oxidase of leaves is located mainly in chloroplasts isolated by differential or sucrose density gradient centrifugation. This activity is part of the lamellar structure that is not lost on repeated washing of the plastids. The oxidase activity was stable during prolonged storage of the particles at 4 C or −18 C. The Km (dihydroxyphenylalanine) for spinach leaf polyphenol oxidase was 7 mM by a spectrophotometric assay and 2 mM by the manometric assay. Polyphenol oxidase activity in the leaf peroxisomal fraction, after isopycnic centrifugation on a linear sucrose gradient, did not coincide with the peroxisomal enzymes but was attributed to proplastids at nearly the same specific density.

Plants were grouped by the latency properties for polyphenol oxidase in their isolated chloroplasts. In a group including spinach, Swiss chard, and beet leaves the plastids immediately after preparation from fresh leaves required a small amount of light for maximal rates of oxidation of dihydroxyphenylalanine. Polyphenol oxidase activity in the dark or light increased many fold during aging of these chloroplasts for 1 to 5 days. Soluble polyphenol oxidase of the cytoplasm was not so stimulated. Chloroplasts prepared from stored leaves were also much more active than from fresh leaves. Maximum rates of dihydroxyphenylalanine oxidation were 2 to 6 mmoles × mg^{-1} chlorophyll × hr^{-1}. Equal stimulation of latent polyphenol oxidase in fresh or aged chloroplasts in this group was obtained by either light, an aged trypsin digest, 3-(4-chlorophenyl)-1,1-dimethylurea, or antimycin A. A variety of other treatments did not activate or had little effect on the oxidase, including various peptides, salts, detergents, and other proteolytic enzymes.

Activation of latent polyphenol oxidase in spinach chloroplasts by trypsin amounted to as much as 30-fold. The trypsin activation occurred even after the trypsin had been treated with 10% trichloroacetic acid, 1.0 N HCl or boiled for 30 minutes. No single peptide from the digested trypsin was found to be the sole activating factor. About 0.25 μg of trypsin activated 50% the polyphenol oxidase activity in a standard chloroplast assay containing 2.1 μg of chlorophyll. Treatment of spinach chloroplasts with tris buffer or ethylenediamine tetraacetate extracted the ATPase activity, but the polyphenol oxidase activity remained with the broken plastids. However these treatments increased the latent polyphenol oxidase activity 50- to 100-fold.

Chloroplasts from a second group of plants, including alfalfa, wheat, oats, peas, and sugarcane leaves, oxidized dihydroxyphenylalanine at a rate of 11 to 120 μmoles × mg^{-1} chlorophyll × hr^{-1}. Polyphenol oxidase in these chloroplasts required a low intensity of red light for activity. Fifty or 75% activation of the oxidase in wheat chloroplasts required 4 to 6 foot candles of light and more light was required for alfalfa chloroplasts. Blue or far red light were ineffective. Trypsin was inhibitory. Upon aging chloroplasts from wheat leaves, but not alfalfa or peas, for 5 to 7 days at 4 C the total polyphenol oxidase activity did not increase, but the activation characteristics changed to those of chloroplasts from the spinach group. Chloroplasts from a third group of plants, including bean, tomato, and corn leaves, slowly oxidized dihydroxyphenylalanine in the dark and exhibited no latency.

In leaves of plants some polyphenol oxidase is present in the soluble fraction, and as Li and Bonner (10) and Arnon (1) demonstrated earlier, it is also in the chloroplast fraction. The chloroplastic location of this oxidase has been confirmed by other investigations (3, 7, 11, 13, 17). Because chloroplast prepared by differential centrifugation are not homogenous particles, but contain mitochondria and peroxisomes, we further separated these particles by isopycnic sucrose density gradient centrifugation to confirm that most of the polyphenol oxidase activity is in the chloroplasts and to establish that none is in the peroxisomes.

Polyphenol oxidase is present in most tissue in substantial amounts and catalyzes the oxidation with molecular oxygen of mono and dihydroxy phenolic compounds of the cell. Regulation or suppression *in vivo* of this oxidase must occur; upon crushing or aging of a cell, uncontrolled polyphenol oxidation results in browning. Latent phenolase activity was investigated in extracts of broad bean leaves (8). Activation was achieved by aging, alkali treatment, or repeated ammonium sulfate treatment. Other activating agents have been reported to be anionic detergents (9, 13), some denaturing agents (17), and certain proteolytic digestions (12). Catechol oxidase in chloroplasts from apple epidermis is extractable by Triton X-100 (4). More recently trypsin has been used to activate the polyphenol oxidase of chloroplasts from sugar beet leaves (7, 11, 12). These results have been variously interpreted as indicative of a protein precursor or a phenolase inhibitor. In this investigation it is concluded that a very active polyphenol oxidase is present, generally in a latent form, in chloroplasts of all plants. Aging or treatment with peptides from autolysized trypsin releases the latent oxidase in chloroplasts of some plants, but catalytically active trypsin is not the component for this activation. Chloroplasts from other plants, which previously had been reported to contain no polyphenol oxidase, will catalyze polyphenol oxidation in the presence of light, and aged trypsin somewhat inhibits this light activation.

MATERIALS AND METHODS

Fresh spinach (*Spinacia oleracea* L.) designates plants which were harvested from the field not longer than 24 hr before

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use, or which were harvested from a plant growth chamber about 1 hr before use. Plants in the growth chamber received 2000 ft-c light for a 10-hr day at 20 C, and the soil was supplemented with Hoagland nutrient solution. Spinach leaves from supermarkets were designated as aged leaves. All the other plants were field grown during the spring and summer months and were harvested only 1 or 2 hr before use.

Preparation of Chloroplasts and Other Fractions. Most of the assays were run with chloroplast preparations obtained by differential centrifugation. All leaf grinding media normally employed for chloroplast isolation from spinach leaves yielded preparations which contained active polyphenol oxidase, and no grinding medium appeared better than the other. The procedure, which utilized a grinding medium of 0.5 M sucrose and 20 mM glycylglycine buffer at pH 7.5, is referred to as the sucrose medium (19). It was used to prepare chloroplasts for all the spectrophotometric assay. Thirty g of washed leaf tissue, without the main veins, were ground in 100 ml of grinding medium for 10 sec by a Waring Blendor at maximum speed. After passage through eight layers of cheesecloth, the chloroplasts were sedimented at about 3600*g* for 2 min. The chloroplasts were resuspended in 10 ml of grinding medium and contained about 3 mg of chlorophyll per ml of suspension. Aliquots of 1 to 50 μl were used in the spectrophotometric assay.

Chloroplasts were also prepared as recommended by Cockburn, *et al.* (2) in a grinding medium of sorbital, pyrophosphate, $MgCl_2$, and isoascorbate. These chloroplasts were washed once in a resuspending medium at pH 7.6 which contained 0.33 M sorbitol, 1mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA, and 50 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonate. These suspensions contained 1.3 to 2.0 mg of chlorophyll per ml and will be referred to as chloroplasts in sorbitol medium.

Separation of chloroplasts, mitochondria, and peroxisomes by isopycnic sucrose density gradient centrifugation, and the assays for the marker enzymes for each particle have been detailed elsewhere (19, 20).

Spectrophotometric Assay (5). Polyphenol oxidase (E.C. 1.10.3) activity was measured at 480 nm with a Gilford recording spectrophotometer to detect the red colored oxidation products from dopa.[2] In a cuvette with a 1-cm light path, the water aliquot necessary to bring the final volume to 1 ml and 0.5 ml of pH 7.0 buffer was added. It consisted of 0.1 M citrate and 0.1 M phosphate. Then, if trypsin were to be used, a 25-μl aliquot was added that contained 8 mg of trypsin per ml, and that had been stored at 4 C for up to 5 days. This provided 200 μg of original trypsin protein per assay and was at least a 10-fold excess. The mixture of buffer, trypsin, and water in the cuvette was gassed with O_2 for 5 min through a disposable dropper. Saturation of the reaction mixture with O_2 gave initial linear reaction rates for longer periods, which generally lasted for 5 to 10 min. A 1- to 100-μl aliquot of enzyme or particle suspension was added to the cuvettes, and the endogenous rates, which were nearly zero, were recorded for 5 min. During this 5 min, trypsin, if used, was present with the particles. The polyphenol oxidase activity was initiated with 0.25 ml of 24 mM L-dihydroxyphenylalanine which provided a final concentration of 6 mM. With aged spinach chloroplast preparations no differences in rates were noted if trypsin were added after the addition of the dopa. With chloroplast preparations from fresh spinach leaves, an initial lag in the rate for about 2 min was observed if the reaction was initiated with trypsin. Data were expressed as ΔE per 5 min, which corresponded with the scale of the graph paper in the recorder.

Manometric Assay. Polyphenol oxidase was measured in the Gilson Warburg respirometer equipped with photoflood lamps providing 750 ft-c of light to the bottom of the flasks. In the main compartment of 15-ml flasks was added 2 ml of 12.5 mM L-dopa prepared in 0.4 strength chloroplast sorbitol suspension medium of Cockburn *et al.* (2) and supplemented with 6 mM phosphate buffer at pH 7.6. Because of the low solubility of dopa, it was necessary to add it in this manner. If trypsin were used, 25 to 100 μl of the stock solution were placed in the main compartment. A 0.1- to 0.5-ml aliquot of the chloroplast preparation was placed in the side arm. Final volume was 3.0 ml. Manometric assays were run at 26 C with KOH and a wick in the center wall. Data are expressed as μl O_2 uptake observed in the assay and calculated for μatoms of oxygen uptake per mg chlorophyll, which is assumed to be equivalent to μmoles of dopa oxidized to dopa quinone.

Reagents. L-β-3,4-Dihydroxyphenylalanine, trypsin type III (2 × crystallized from bovine pancreas), and trypsin inhibitor (type II-0 form ovomucoid) were purchased from Sigma Chemical Co. Chlorophyll was determined after extraction with 80% acetone (1).

RESULTS

Catechol or dopa was a substrate for polyphenol oxidase in the chloroplast fractions. Oxidation of tyrosine by spinach chloroplasts with or without trypsin was negligible or only a few per cent of the rate with dopa. Dopa was used in most of the work, because of its stability in aerobic solution, because enzymatic rates with it were most reproducible, and because its solubility in water was sufficient to avoid the use of organic solvents which could interfere with chloroplast integrity. The activity is designated as polyphenol or dopa oxidase.

The spectrophotometric assay measured the formation of colored melanin-like compounds. Polyphenol oxidase in chloroplasts from spinach, sugar beet, and tomato leaves could be assayed by this procedure. Chloroplasts from other tissues showed good polyphenol oxidase activity when assayed manometrically, but failed to exhibit significant initial rates of activity by the spectrophotometric assay. In general the manometric assay had to be used, and the spectrophotometric assay was used mainly with spinach chloroplasts. Spectrophotometric data, recorded as the ΔE increase in 5 min, represent a chain of reactions that cannot be put on a molarity basis, but a direct comparison between the spectrophotometric rate and the manometric rate with the same chloroplast preparation served to estimate the significance of the ΔE changes. An aliquot of a spinach chloroplast preparation with excess trypsin, which brought about a 1 mm ΔE change, catalyzed 0.65 nmole O_2 uptake in the same time period. In typical assays, 5-μl aliquots of chloroplasts, containing 10 to 15 μg of chlorophyll, catalyzed a ΔE per 5 min of about 100 to 200 mm. Rates of 600,000 to 1,200,000 Δmm × mg^{-1} chlorophyll × hr^{-1} were observed which were equivalent to 80 to 160 μmoles of O_2 uptake × mg^{-1} chlorophyll × hr^{-1} by the manometric assay or 160 to 320 μatoms. Rates of 0.2 to 0.3 mmole of dopa oxidation to dopa quinone × mg^{-1} chlorophyll × hr^{-1} are thus calculated for the spectrophotometric assay. These values for both assays with spinach chloroplasts are subject to enormous variations depending upon the chloroplast treatments to be described.

Addition of trypsin to spinach chloroplasts greatly (3- to 30-fold) stimulated polyphenol oxidase activity, but did not effect the other assays. So many parameters influenced this activation

[2] Abbreviations: dopa: dihydroxyphenylalanine; CMU: 3-(4-chlorophenyl)-1,1-dimethylurea; PVP: polyvinylpyrrolidone.

Table I. *Distribution of Dopa Oxidase Among Particulate Fractions of Spinach Leaves*

Aged spinach leaves (200 g) were ground for 10 sec in 300 ml of sucrose medium. This short time did not rupture all the cells, but was used to reduce the breakage of particles. Therefore, the total activity should be considered on the basis of chlorophyll or protein. Particles were first separated by differential centrifugation. The 600*g* pellet contained mainly whole chloroplasts; the 6,000*g* pellet contained mostly ruptured or broken chloroplasts, as well as most of the peroxisomes and many mitochondria. An aliquot of the 6,000*g* pellet was further separated by isopycnic nonlinear sucrose density gradient fractionation (19). Glycolate oxidase served as the marker for peroxisomes and chlorophyll for chloroplasts.

	Dopa Oxidase					Glycolate Oxidase		Chlorophyll		Soluble Protein
	No Trypsin		+ Trypsin		Trypsin Increase					
	$\Delta E \times 10^3 \times 5\ min^{-1}$	%	$\Delta E \times 10^3 \times 5\ min^{-1}$	%	%	$nmoles \times 10^3 \times min$	%	mg	%	mg
Original	1990		8000		401	426		166.5		3660
600*g* pellet	480	23.6	4520	46.1	943	18	4.3	77.0	46.3	780
6000*g* pellet	682	33.6	4050	41.3	593	85	23.2	79.0	47.4	760
10,000*g* pellet	40	2.0	120	1.2	300	12	2.0	8.5	5.1	18
Supernatant	827	40.8	1123	11.5	136	300	69.6	2.0	0.2	2140
% Original	102	100.0	123	100.1		99	98.1		99.0	101
6000*g* pellet sucrose gradient fractions										
1 (2.5 M sucrose)	0.06	0.1	0.7	0.1	1200	0.01	0.1	0.016	0.1	0.6
2 (2.3 M sucrose)	0.05	0.1	0.8	0.1	1800	0.02	0.1	0.013	0.1	0.7
3 (interface)	0.79	2.1	16.6	2.0	2090	5.09	35.2	0.037	0.3	2.8
4 (1.8 M sucrose)	0.71	1.9	23.2	2.8	3280	3.08	21.2	0.033	0.3	3.1
5 (interface)	6.53	17.2	104.0	12.6	1590	2.24	15.5	0.008	2.7	13.3
6 (1.5 M sucrose)	1.27	3.3	31.8	3.9	2500	0.65	4.5	0.145	1.3	7.4
7 (interface)	14.00	37.0	532.0	64.4	3800	0.23	1.6	7.859	70.0	59.2
8 (1.3 M sucrose)	5.44	14.4	93.9	11.4	1730	0.15	1.1	2.563	22.8	23.5
9 (supernatant)	9.03	23.8	22.8	2.8	250	3.00	20.7	0.240	2.4	16.8

of a latent polyphenol oxidase in chloroplast that nearly any degree of activation could be obtained. Those factors, which have so far been discovered for activation of the oxidase, are described in subsequent sections.

Location of Polyphenol Oxidase in Cell. Polyphenol oxidase has been isolated from the supernatant fraction of leaf homogenates and from the chloroplast fraction obtained by differential centrifugation (1, 10). Its location in both fractions is confirmed by data in the upper half of Table I. Data for glycolate oxidase, a peroxisomal marker enzyme is also included. After differential centrifugation, the supernatant fraction contained 40.8% of the total polyphenol oxidase when assayed without trypsin, but only 11.5% when assayed with trypsin due to stimulation of the particulate activity. The enzyme, when solubilized, as partially occurs during grinding of the leaf, has little latency. In the 600*g* and 6000*g* pellet fractions, containing the bulk of the chlorophyll, were 57.2% of the polyphenol oxidase activity, when assayed without trypsin, but 87.4% when assayed with trypsin. Thus the true distribution of total (latent plus activated) polyphenol oxidase is probably the 11.5% value in the supernatant as obtained by assaying with trypsin to activate the enzyme in the particles. This low percentage of polyphenol oxidase in the supernatant suggests that all of the activity *in situ* is in the chloroplasts.

The 6000*g* pellet fraction was subjected to isopycnic centrifugation on a discontinuous sucrose gradient for partial separation of the subcellular organelles (lower half of Table I). The location of peroxisomal glycolate oxidase in the gradient was highest in fraction 3 as previously reported (19, 20) and distinctly separated from chloroplasts in fraction 7 (chlorophyll analysis, Table I) and from mitochondrial cytochrome *c* oxidase in fraction 5 (data not shown). Polyphenol oxidase was mainly located in the major broken chloroplast band which is fraction 7. However, polyphenol oxidase activity (17%) was also found in fraction 5, which contained mitochondria and whole chloroplasts, and some polyphenol oxidase (4.8%) was in fraction 3 plus 4, but the distribution profile was different from the peroxisomal marker, glycolate oxidase. Fraction 3 plus 4 contained only 0.6% of the chlorophyll. If the data were expressed on a chlorophyll basis, extremely high values for polyphenol oxidase activity per mg chlorophyll would be obtained in fractions 2 to 5. It was concluded that most of the polyphenol oxidase was located in the chloroplasts (fraction 7). Polyphenol oxidase activity was also located in the mitochondrial fraction 5, but because of contaminating chloroplasts this is no proof for its presence in these particles.

Peroxisomes have been characterized as containing flavin oxidases and catalase, and so far, other oxidases, such as polyphenol oxidases, have not been proven to be in them. In order to further examine the particle location of polyphenol oxidase, the impure microbody fraction 3 from the discontinuous sucrose gradient shown in Table I was rerun by isopycnic centrifugation on a linear sucrose gradient from 40 to 60% sucrose. The results, shown in Figure 1, indicate that the peaks for enzymic peroxisomal glycolate oxidase and malate dehydrogenase did not coincide with the polyphenol oxidase activity. Also the cytochrome *c* oxidase activity in the few contaminating mitochondria did not coincide with the polyphenol oxidase activity. Thus the polyphenol oxidase activity in the microbody fraction cannot be attributed to peroxisomes, mitochondria, or chloroplasts. Further investigations have located this polyphenol oxidase with other marker enzymes for proplastids (18).

Properties. *Km* (dihydroxyphenylalanine) values for oxidase

activity in proplastid with trypsin were 7 mM by the spectrophotometric assay and 2 mM by the manometric assay. K*m* values for 4-methylcatechol oxidation by spinach chloroplasts have been reported as 2.1 mM without trypsin and 4.8 mM with trypsin (7).

Many pH optima for polyphenol oxidases are reported in the literature depending upon the tissue and substrate. Generally the enzyme is active between pH 5 to 8 and above pH 8 one must be careful of endogenous nonenzymic oxidation. Repeated investigation on the various fractions, with and without trypsin, confirmed that the pH optimum was broad, but maximal about pH 7.0 to 7.5. One such curve is shown in Figure 2 for the proplastid fraction. There was a significant decrease in activity below pH 6.0 and above 8.0 when corrected for rates with boiled enzyme. pH 7.0 was selected because in other tests (not shown) the rates were sometimes much less below pH 7.0.

Effect of Washing Spinach Chloroplasts. Polyphenoloxidase activity was retained with the chloroplast fraction upon repeated washing of the particles with grinding or resuspending medium (Table II). The source and nature of the soluble polyphenol oxidase in the original homogenate has not been further investigated. If fresh leaves had been used, much more of the oxidase activity would remain with chloroplasts (Table I). Polyphenol oxidase activity appears to be a part of the lamella

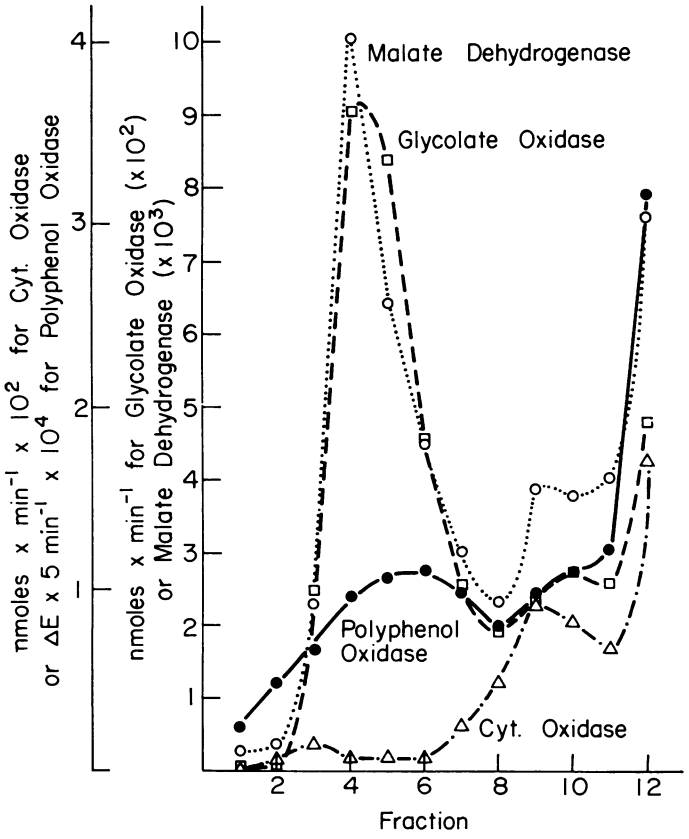


FIG. 1. Distribution of polyphenol oxidase in the microbody fraction. Fraction 3 from a discontinuous isopycnic sucrose gradient (Table I) was recentrifuged isopycnically in a linear sucrose gradient. Fraction 1 was a pad of 2.5 M sucrose, fraction 2 to 11 a linear gradient between 2.1 to 1.7 M sucrose, and fraction 12 is the top fraction containing soluble or nonparticulate activity. Polyphenol oxidase was assayed by the spectrophotometric procedure. Polyphenol oxidase (●), glycolate oxidase (□), NAD-malate dehydrogenase (○), cytochrome *c* oxidase (△).

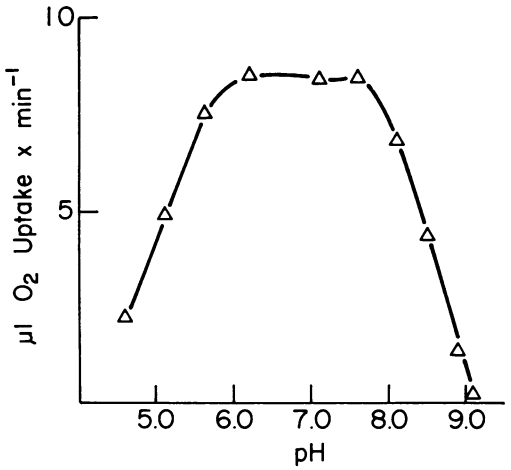


FIG. 2. Effect of pH on polyphenol oxidase activity from the proplastid fraction assayed manometrically with trypsin.

Table II. *Effect of Washing of Spinach Chloroplasts on Polyphenol Oxidase*

Spinach plants from a growth chamber were harvested and stored for 3 days at 4 C before use. The leaf homogenate was prepared in the sucrose medium and centrifuged 20 min at 6000*g* for pellet I and supernatant I. Pellet I was resuspended in a medium of 0.25 M sucrose and 20 mM glycylglycine at pH 7.5 and then recentrifuged, which produced pellet II and supernatant II. This procedure was repeated twice.

Fraction	Total Protein	Polyphenol Oxidase		
		−Trypsin	+Trypsin	Increase
		$\Delta E \times 10^3 \times 5\ min^{-1}$		%
Pellet				
I	476	35	168	480
II	392	35	189	540
III	364	31	241	780
IV	311	40	336	840
Supernatant				
I	724	4	114	3155
II	78	1	23	1755
III	30	2	17	730
IV	28	3	25	984

structure that is not readily removable by repeated washing of the chloroplasts. Consequently, the immense amount of oxidase activity in chloroplasts is not likely to be a soluble protein which normally may have other enzymic activity such as ribulose diphosphate carboxylase. However, a ribulose diphosphate carboxylase preparation (an acetone precipitate from Sigma) had about twice the specific activity for polyphenol oxidase activity as it did for the carboxylase activity (data not shown). This may be attributed to the very large amount of polyphenol oxidase activity in spinach leaves, which would be a contaminate in enzyme preparations.

Washing the chloroplasts a number of times, activated the polyphenol oxidase to varying degrees. Unwashed chloroplasts always had minimal polyphenol oxidase activity, and washing them with grinding medium increased the oxidase activity when assayed with or without trypsin. In the experiment shown in Table II with spinach leaves aged only 3 days, the oxidase of the chloroplasts without trypsin increased only little from 35,000 to 40,000 $\Delta E \times 5\ min^{-1}$, whereas a total of 6,000 $\Delta E \times 5\ min^{-1}$ was washed off the particles by the

Table III. *Effect of Age of Chloroplast Preparation on Polyphenol Oxidase*

Spinach leaves were freshly harvested, and chloroplasts were prepared in sucrose medium and stored at 4 C.

Age of Preparation	Polyphenol Oxidase Activity	
	−Trypsin	+Trypsin
hr	$\Delta E \times 5\ min^{-1} \times \mu g^{-1}\ chl$	
<1	8	99
5	29	238
29	22	271
53	29	382
125	102	642

second, third, and fourth centrifugations. When assayed with trypsin, the effect of washing was a doubling of the activity of the plastids. In some experiments with spinach leaves stored for several weeks (grocery store purchases), repeated washing of the chloroplasts also doubled the oxidase activity without trypsin.

With washed chloroplasts trypsin activation of the latent oxidase increased several fold after repeated washing (Table II). This effect of washing on the chloroplasts was somewhat similar to aging of the unwashed chloroplasts for a couple of days. During each washing of the chloroplast, about 5 to 10% of the polyphenol oxidase activity was not precipitated by 6000*g*. It is not known whether the oxidase was solubilized or whether small chloroplast fragments would account for this activity in the supernatant fraction.

The increase in polyphenol oxidase activity after various treatments of spinach chloroplast has been referred to as latent activity. This latent activity accounted for most of the total polylphenol oxidase activity in the chloroplasts and was increased by aging, by disrupting the particles, or by treatment with trypsin. A combination of aging and trypsin released more activity than either treatment alone. Chloroplasts isolated from spinach leaves, that had been harvested and stored at 4 C for 1 or more weeks, showed more activity for dopa oxidation, and the latent activity, as manifested by trypsin treatment, was much greater than in chloroplasts from freshly harvested leaves.

The soluble polyphenol oxidase activity in the original homogenate (Table I) or that solubilized from isolated chloroplasts generally exhibited little latency but rather appeared fully active (Table I). An exception is indicated in the data in Table II, but in this case the supernatant activity without trypsin was very low. Perhaps the solubilizing process activated the oxidase. The results suggest that in intact chloroplasts polyphenol oxidase is either not active or substrates cannot reach its active site.

Age and Stability of Chloroplast Preparation. Chloroplast preparations from fresh spinach leaves showed increasing levels of polyphenol oxidase activity during the first 2 days after preparation (Table III). Trypsin treatment of these newly prepared chloroplasts increased the polyphenol oxidase to varying degrees. Aging of a spinach chloroplast at 4 C for several days increased the polyphenol oxidase activity in two ways. The total activity without trypsin increased, and the total latent activity, which could be detected by assaying with trypsin, also increased many fold. Although the nature of these increases in activity is unknown, aging of the chloroplasts *in vivo* or *in vitro* may render the polyphenol oxidase activity more sensitive to trypsin activation. In addition, the chloroplasts may lose their integrity so that the polyphenol oxidase active sites become exposed. These results are similar to the reports mentioned in the introduction concerning the activation of polyphenol oxidase in chloroplasts by disruptive procedures.

In general polyphenol oxidase activity in the chloroplasts was stable for a week at 4 C, indefinitely when frozen, but inactivated by boiling for 2 min. Experiments on stability were not definitive because of continued activation of the latent enzyme. Polyphenol oxidase activity in chloroplast that had been washed four times (Table II) were subjected to stability tests when resuspended in 0.25 M sucrose. After freezing the chloroplasts in 0.25 M sucrose at −18 C, 26% of the activity was not sedimentable by 6000*g*. Treatment with 0.01% Triton X-100 solubilized 33% of the polyphenol oxidase activity. Osmotic shock from resuspending the particles in 20 mM glycylglycine buffer at pH 7.5 without sucrose solubilized half of the oxidase activity. In all cases the solubilized activity was probably in large part with small chloroplast fragments which were not sedimented by 6000*g*.

Trypsin Concentration. The amount of trypsin required to activate polyphenol oxidase in the chloroplast was evaluated as if it were a substrate in the 1-ml spectrophotometric assay (Fig. 3). A 50% activation of the polyphenol oxidase in chloroplasts containing 2.1 μg of chlorophyll occurred with 0.25 μg of trypsin (a relative *Km* concentration). This relative value is cited to emphasize the vary small amount of trypsin needed to activate the oxidase in chloroplasts. When studying other variables, an excess, 10 to 200 μg, trypsin was used. No inhibition of polyphenol oxidase activity in the chloroplasts by large amounts of trypsin was noted during the period of the assays. After prolonged incubation of the chloroplasts for 24 hr at room temperature with the trypsin, polyphenol oxidase activity was significantly reduced, but this loss may be attributed to general oxidase inactivation. Activation by trypsin was very rapid, but it has not been studied in detail. When trypsin was added to a spinach chloroplast suspension already containing dopa, full activation occurred in less than 30 sec.

A residual trypsin effect in the assay cuvettes and Warburg flasks was very apparent after normal washing and rinsing of the glassware. It was necessary to rinse them in 2 N HCl to prevent residual amounts of trypsin activation in subsequent experiments.

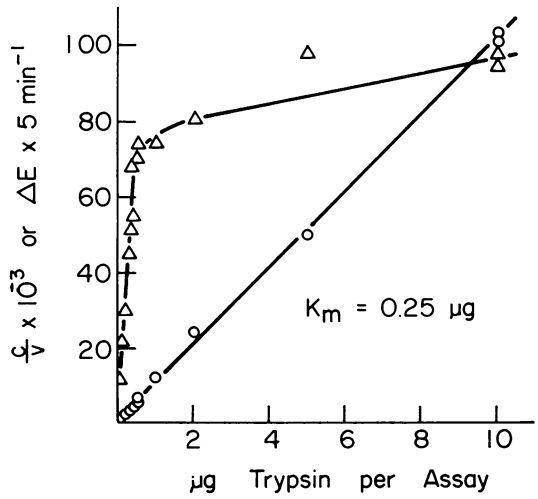


FIG. 3. Effective trypsin concentration for polyphenol oxidase activation in chloroplasts. Three-μl aliquots of chloroplasts prepared in sucrose medium containing 2.1 μg of chlorophyll were assayed spectrophotometrically. △: $\Delta E \times 5\ min^{-1}$; ○: reciprocal rate $C/V \times 10^{-3}$, where C is concentration and V, velocity.

Stability of Trypsin and Type of Activation. Chloroplasts after being treated at 100 C for 2 min exhibited no polyphenol oxidase activity with or without added trypsin. Trypsin had no polyphenol oxidase activity. Addition of boiled chloroplasts did not affect the rate of oxidation by untreated chloroplasts. Thus peptides in boiled chloroplasts will not activate the latent oxidase when added to unboiled chloroplasts.

It appears to have been assumed in previous literature citations (7, 11, 12) that trypsin activation of polyphenol oxidase in sugar beet chloroplasts was the result of tryptic action. However, in my experiments it was observed that trypsin activation of polyphenol oxidase in chloroplasts was not destroyed by boiling the trypsin for 30 min and only 23% of the activation effect was lost after 60 min at 100 C (Table IV). Treatment of the trypsin with 10% trichloroacetic acid or 1.0 N HCl did not inhibit polyphenol oxidase activation in spinach or sugar beet chloroplasts by the trypsin protein precipitate that had been neutralized and redissolved. In other assays with limiting amounts of trypsin, treatment of trypsin at room temperature for 1 day or for 3 hr at 37 C did not inactivate the trypsin effect. After 2 days at room temperature the trypsin preparation was only one-half as active toward polyphenol oxidase activation. It was stable for up to 5 days at 4 C. Trypsin in solution autodigests itself in a short time so that the trypsin effect on the chloroplasts does not require the intact trypsin protein. It is concluded that the effect from trypsin on activating polyphenol oxidase is not due to tryptic digestion of the chloroplast. Nevertheless, 600 μg of trypsin inhibitor, type II-0 from ovomucoid, prevented 200 μg of fresh or aged trypsin from activating the polyphenol oxidase in chloroplasts. This trypsin inhibitor reduced by about 60% the rate of dopa oxidation by chloroplasts untreated by trypsin (Table IV).

In this manuscript the term "trypsin activation" has also been used because of its specificity, although a trypsin solution capable of proteolytic attack is not necessarily the active component. That trypsin does exert a profound effect upon some chloroplasts is evident by polyphenol oxidase activation and disruption of the grana and lamellar structure (7). However trypsin did not inhibit the Hill reaction by spinach chloroplasts, when experiments were run with 30 mM $K_3Fe(CN)_6$ as electron acceptor, and 0_2 evolution was measured polarographically at 15 C (data not shown).

Specificity of Trypsin Activation. Substances are listed in Table V which did not activate the latent polyphenol oxidase of chloroplasts. All of this work was done with spinach chloroplasts. Triton X-100 was ineffective, although it has been often used to solubilize chloroplast fragments. Sodium dodecyl sulfate at 10 μM had no effect, but at 10 mM it completely inhibited polyphenol oxidase activity, even in the presence of trypsin. Bovine serum albumin, boiled or not heated, did not substitute for trypsin. About 75-fold more papain was needed to achieve the same activation as trypsin. Carboxypeptidase was also much less active (about 1/100) than trypsin. Since such small amounts of trypsin were used, the papain and carboxypeptidase appeared reasonably active and full activation of latent polyphenol oxidase could be achieved with them.

Because of the heat stability of this trypsin effect the activation of polyphenol oxidase in spinach chloroplasts cannot be due to a classical trypsin cleavage of peptide bonds containing L-lysine or L-arginine with a free ϵ-amino group. Many compounds listed in Table V, particularly peptides, were not able to substitute for trypsin in activating polyphenol oxidase. None of these compounds inhibited trypsin activation of polyphenol oxidase in spinach chloroplasts.

Exploratory experiments were run to attempt to isolate the active factor in aged trypsin solutions. A trypsin solution (10 mg/ml) was incubated at 37 C for 2 hr and then passed through a Sephadex G25 or G50 column. At pH 3.0 the material capable of activating latent polyphenol oxidase in spinach chloroplasts passed through the columns in the first void volume without loss of total activation ability. At pH 7 activity was in each of the first three void volumes.

In another experiment trypsin (4 mg/ml) was incubated for 2 hr at 37 C and then subjected to paper electrophoresis for 75 min at 250 v in an acetic acid-pyridine buffer at pH 3.0. Ten bands were visualized by ninhydrin. These bands plus areas between them were eluted with 25% pyridine, evaporated to dryness, and redissolved in water. Low levels of activity were recovered in several peptide bands, but no highly active fractions were recovered. Pyridine inhibited the polyphenol oxidase, but controls were run to assure that it had been removed below inhibitory amounts. A recombination of the fractions did not activate the polyphenol oxidase. When the electrophoresis was run at pH 6.4, the peptides all ran as one band which, when eluted, partially activated the polyphenol oxidase of the chloroplast.

Association of Latent Polyphenol Oxidase Activity with Lamellar Structure. Chloroplast contains a Mg^{2+}-dependent ATPase and treatment with tris buffer or EDTA solubilizes

Table IV. *Stability Of Trypsin for Polyphenol Oxidase Activation in Chloroplasts*

Chloroplasts from market spinach were prepared in sucrose medium and stored 1 day before use. Aliquots of 5 μl containing 7 μg of chlorophyll/μl were assayed by the spectrophotometric method with 200 μg of trypsin.

Trypsin Treatment	Polyphenol Oxidase Activity
	$\Delta E \times 5\ min^{-1} \times ml^{-1}$
No trypsin	1,000
Untreated trypsin	33,200
Stored 1 to 5 days at 4 C	33,600
Heated at 100 C for 30 min	33,200
Heated at 100 C for 60 min	26,400
1 N HCl for 10 min, neutralized	33,000
Trichloroacetic acid precipitate, redissolved and neutralized	25,500
No trypsin, 600 μg of trypsin inhibitor	400
Trypsin + 600 μg of trypsin inhibitor	5,600

Table V. *Compounds Ineffective for Activating Polyphenol Oxidase in Spinach Chloroplasts*

Between 20 and 100 μg of each compound was used in the spectrophotometric assay in which 1 μg of trypsin gave full activation.

L-Arginine	Phe-glu-ser-asn-phe-asn-thr-his-ala-thr-asn-arg[1]
L-Lysine	Poly-L-lysine hydrobromide
DL-Leu-glyc-DL-phenylalanine	Bovine albumin
Glyc-phenylalanine	Carnosine
L-Leu-L-alanine	AMP
L-Leu-glyc-phenylalanine	Cyclic AMP
Glycylglycine	Benzyladenine
Glycylglycine amide	Triton X-100
L-Tryp-L-methi-L-aspa-L-phenylalanine amide	Sodium dodecyl sulfate
DL-Leu-glyc-DL-phenylalanine	Ethanol
Gly-try-ser-leu-glyc-asn-try-val-CMC-ala-ala-lys[1]	$CuSO_2$ (0.1 mM final)
	$MgSO_2$ (0.1 mM final)

[1] Gift from J. LaRue and J. C. Speck.

this CF_1 component, after which trypsin treatment converts it into a Ca^{2+}-dependent ATPase (14, 15). These treatments while solubilizing ATPase, also activated polyphenol oxidase, but the oxidase remained bound to the lamella membranes. For these experiments chloroplasts were prepared in buffered sucrose by the procedures used for ATPase assays (14, 15) and were washed once. Different aliquots were then treated for 10 min as described in Table VI, centrifuged to separate the particles from the solubilized enzymes, and each fraction assayed for polyphenol oxidase and Ca^{2+}-ATPase activity.

Regardless of the treatment of chloroplasts, polyphenol oxidase activity remained mainly with the particles. The results are consistent with the concept that polyphenol oxidase is a part of the lamellar membrane (7, 11, 12). Ca^{2+}-ATPase or CF_1 was solubilized by tris and EDTA treatments. If one uses the conversion factor cited in the methods (1 ΔE change equivalent to about 0.65 nmole or 1.3 natoms of O_2 uptake), the activity of the polyphenol oxidase was comparable to, or greater than that ATPase activity. Treatment with tris or EDTA in the presence of trypsin (treatment C and D, Table VI) did not solubilize the polyphenol oxidase. Nothing in these data supports any direct relationship between polyphenol oxidase and ATPase of the chloroplast, although both are activated by trypsin. Speculation is, that when the CF_1 is removed or uncoupled by trypsin, there is exposed in the chloroplast membrane a quinone oxidase which is normally inactive, covered up, or tightly coupled in membrane electron transport. The large amount of the polyphenol oxidase activity exposed by trypsin indicates a major role *in vivo* for this enzyme.

Light Activation of Polyphenol Oxidase in Alfalfa Chloroplasts. Older reviews on polyphenol oxidase (16) lists plants without this activity. Considering the immense activity of this enzyme in chloroplasts from spinach and sugar beet leaves we first reinvestigated activity in chloroplasts from field-grown alfalfa plants, which was a plant reported to have no polyphenol oxidase. The alfalfa chloroplasts were prepared in the sorbitol medium (with or without PVP), washed once, and were assayed for polyphenol oxidase in the manometric assay. Indeed no activity with or without trypsin was detected in the dark or at the low light intensity in the Warburg apparatus. However, in light of 750 ft-c the rate of polyphenol oxidation ranged between 300 and 800 μl O_2 uptake hr^{-1} mg^{-1} chlorophyll for alfalfa chloroplasts prepared in sorbitol medium (Fig. 4). The oxidation was strictly light-dependent. If dopa was added to the chloroplasts in the dark there was no oxidation until light was used. After oxidation was initiated in the light, there was a slow rate in a subsequent dark period, but the rapid rate would return again only in the light.

The light dependent polyphenol oxidase activity was severely impared by repeated washing of the alfalfa chloroplasts, which inactivation had not occurred with spinach chloroplasts. The presence of 10 mM EDTA had no effect upon the light-dependent rate of polyphenol oxidase. Trypsin was never stimulatory to light-dependent polyphenol oxidation by alfalfa chloroplasts, but rather trypsin generally inhibited the rate

Table VI. *Activation and Distribution of Polyphenol Oxidase and ATPase*

Fifty g of freshly harvested spinach leaves were ground for 15 sec in 100 ml of grinding medium (0.4 M sucrose, 10 mM NaCl, 20 mM Tricine-NaOH, pH 8.0). The homogenate was filtered through cheesecloth, centrifuged for 20 min at 6000*g*, and the chloroplasts resuspended in 40 ml of grinding medium. The chloroplasts were sedimented again by centrifugation and resuspended in 10 ml of grinding medium. This suspension contained 3.6 mg of chlorophyll/ml. One-ml aliquots were treated and then recentrifuged, and both the supernatant and the chloroplasts resuspended in 2.5 ml of 10 mM NaCl were assayed. Polyphenol oxidase assay was spectrophotometric, and the ATPase followed Racker's procedure (15, 16). Treatments were: A: 1 ml of chloroplasts + 29 ml 0.75 M tris, pH 7, for 10 min at 25 C; B: 1 ml of chloroplasts + 29 ml 0.75 M EDTA, pH 7, for 10 min at 25 C; C: 1 ml of chloroplasts + 29 ml 0.1 M tris, pH 8.0, + 1 ml of trypsin (3 mg) + 6 ml water for 5 min. Nine ml of trypsin inhibitor were added before centrifugation; D: Same as C with 1 ml 20 mM EDTA in addition and only 5 ml of water. Data are expressed as the total from the 1-ml aliquot of chloroplasts. Chloroplast preparation, treatments, and assay were all run on the same day. Percentage of increase is based upon the original activity before treatments.

Treatment	Polyphenol Oxidase						Ca^{2+}-ATPase		
	No trypsin			Plus trypsin			No trypsin		
	Total ΔE/5 min	Distribution	Increase	ΔE/5 min	Distribution	Increase	Total nmoles Pi	Distribution	Increase
		%			%			%	
Original Chloroplasts	1,500			19,000			3,100		
A. Tris									
Total	6,180		412	85,110		5,680	29,170		940
Pellet	4,000	65		82,500	97		4,170	14	
Supernatant	2,180	35		2,610	3		25,000	86	
B. EDTA									
Total	23,300		1,555	147,000		9,800	19,780		640
Pellet	9,500	41		128,500	87		2,780	14	
Supernatant	13,800	59		18,500	13		17,000	86	
C. Tris plus trypsin									
Total	167,500		11,180	152,500		10,180	23,900		770
Pellet	157,500	94		142,500	93		18,000	75	
Supernatant	10,000	6		10,000	4		5,900	25	
D. EDTA plus trypsin									
Total	154,000		10,280	150,800		10,050	12,490		400
Pellet	145,000	94		142,500	94		6,490	52	
Supernatant	9,000	6		8,300	6		6,000	48	

about 20 to 30%. Trypsin also inhibited or inactivated an endogenous rate of O_2 uptake.

Effect of Light Upon Polyphenol Oxidase Activity of Chloroplasts from Different Plants. Three main patterns were observed in a limited survey for polyphenol oxidase activity in chloroplasts from various plants (Table VII). (*a*) In chloroplasts from one group of plants, typified by spinach, the rate of dopa oxidation in the dark varied from nil (Fig. 5) from fresh leaves, to the maximum rates which were found in chloroplast preparations from aged or store-bought leaves. In the newly prepared chloroplasts, light stimulated the rate of

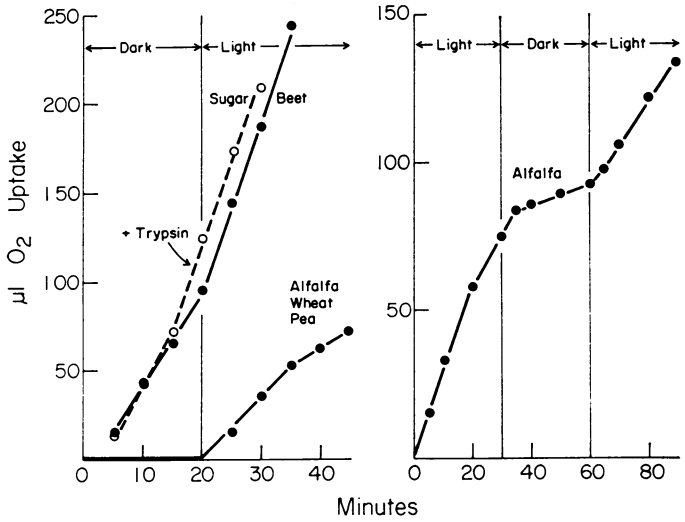


FIG. 4. Effect of light upon polyphenol oxidase activity of chloroplasts. The chloroplasts were freshly prepared in sorbitol medium. An amount of each chloroplast preparation was selected to catalyze a significant rate of dopa oxidation as μl O_2 uptake. Specific activities on a chlorophyll basis are calculated in Table VII.

Table VII. *Grouping of Plants by Conditions that Stimulate Polyphenol Oxidase in Isolated Chloroplasts*

Plants	Maximum Rates of Dopa Oxidation[1]
	μatoms O_2/mg chlorophyll·hr
Group A: Chloroplasts stimulated by aging, trypsin, uncouplers, and light	
Spinach	300–2000
Sugar beet	450
Swiss chard	800–6500
Group B: Chloroplasts requiring light, inhibited by trypsin, no stimulation on aging	
Alfalfa	68
Wheat	60
Pea	120
Oat	40
Sugarcane	11
Group C: Chloroplasts equally active in light or dark and insensitive to stimulation	
Bean	54
Tomato	13
Corn	20

[1] Assayed by manometric procedure. Values are the maximum observed after stimulation, if it occurred. All values are subject to variations as discussed in text.

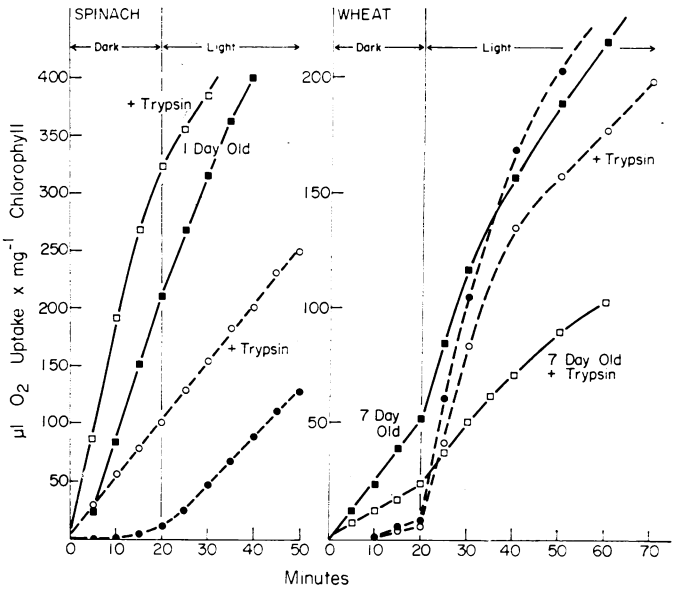


FIG. 5. Effect of aging of isolated chloroplasts on rate of dopa oxidation. Chloroplasts were isolated in sorbitol medium from field-grown leaves shortly after harvest. Genesee wheat leaves were from plants before heading. Manometric assays were run immediately and at designated days later after storage at 2 C. Experiments were initiated in the dark, and after 20 min 750 ft-c of white light was added. Assayed immediate without trypsin (●), or with trypsin (○). Assayed on later days without trypsin (■) or with trypsin (□). On the 5th day (data not shown) the wheat chloroplasts without trypsin still required light to oxidize dopa as on the day of preparation, but with trypsin the rate of dopa oxidation was similar to the curve shown for the 7th day with trypsin.

polyphenol oxidase to the same extent as did trypsin. Chloroplasts prepared from leaves of sugar beets or Swiss chard were similar to those from spinach leaves, but the dark rate was always high, (Fig. 4) and stimulation by trypsin or light was not more than 1-fold and often the dark rate immediately after preparation was the same as the rate in light. After aging of any of these chloroplast preparations the rate in the dark increased, while light stimulation became less marked or nil. Most activity in freshly prepared chloroplasts was observed with plastids from this group of plants (Table VII). For Swiss chard chloroplasts dopa oxidation rates as high as 6 matoms $O_2 \times hr^{-1} \times mg^{-1}$ chlorophyll were observed and for spinach chloroplasts, 2 matoms. These results confirm the presence of polyphenol oxidase activity in sugar beet chloroplasts, when assayed in the dark, as previously reported (7, 11, 12).

(*b*) Chloroplast from leaves of alfalfa, pea, wheat, or oat leaves did not oxidize dopa in the dark regardless of the age of the leaves after harvest and before preparation of the chloroplasts. In the presence of light chloroplasts from these plants readily oxidized dopa (Figs. 4 and 5 and Table VII). Chloroplasts from sugarcane leaves were similar to those from alfalfa, but less active in the light. Trypsin did not initiate or stimulate dopa oxidation by this type of chloroplasts. Rather in this group of chloroplasts, aged trypsin inhibited 10 to 80% the light-stimulated rate of dopa oxidation. This inhibition is in contrast to trypsin stimulation in spinach chloroplasts. The rate of dopa oxidation by these chloroplasts in the light was generally less (10–50%) than the rate by chloroplasts from spinach or other plants in group A. Chloroplasts from plants in group B when broken by ultrasonication still oxidized dopa at the same rate in the light only, but trypsin inhibition was more severe.

Chloroplasts from plants of group B, which had in the beginning a zero rate of oxidation of dopa in the dark, after oxidizing dopa in the light for 20 to 30 min, were then returned to darkness. The rapid rate of oxidation continued for about 3 to 5 min before leveling off at a low dark rate (Fig. 4). This slow change suggests a protein configurational modification by light rather than a photoconversion. The new dark rate was now not zero, but about 15 to 20% of the light rate. Exposure of the chloroplasts to light without dopa did not partially activate the oxidase for dark oxidation. The change from the initial zero dark rate to the light rate in the presence of substrate was as immediate as could be measured by the manometric assay.

Some plants (group C, Table VII) yielded chloroplasts that catalyzed the oxidation of dopa but which were not stimulated or significantly inhibited by light or trypsin (data not shown). Chloroplasts from corn or soybean leaves were active in both light and darkness for dopa oxidation and activity was inhibited about 10% by trypsin. Corn chloroplasts were stored at 4 C for up to 7 days with no change in the rate of oxidation of dopa or response to light, darkness, or trypsin. The effect of other compounds upon stimulation of dopa oxidation by chloroplasts of group C has not been explored.

Chloroplasts from pea, wheat and alfalfa leaves of group B were particularly attractive to work with because of complete inactivity in the dark and high activity in the light. Further these chloroplast preparations acted similarly for 2 to 3 days after preparation and storage at 2 to 4 C. After storage of wheat chloroplasts for 5 days, they acted then like fresh spinach chloroplasts in that trypsin now stimulated dopa oxidation in the dark up to a rate equal to that in the light. After 7 or 9 days of storage, chloroplasts from wheat leaves oxidized dopa in the dark but were still somewhat stimulated by light (Fig. 5). Thus, isolated chloroplasts from wheat leaves appeared to have changed slowly, over a period of days, from a tightly coupled system requiring light for dopa oxidation to a more degenerative one that oxidized dopa in the dark. The significance of these results are unknown. Stored chloroplasts from pea leaves (up to 6 days) and alfalfa behaved similarly as freshly prepared particles toward dopa oxidation, and did not become sensitive to trypsin activation or develop a dark rate.

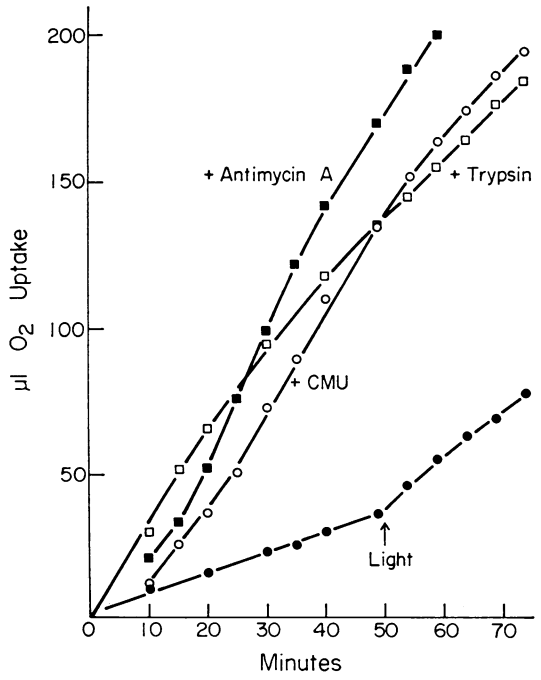


FIG. 6. Effect of light, trypsin, CMU, and antimycin A on polyphenol oxidase activity of spinach chloroplasts. Chloroplasts were isolated in sorbitol medium from field-grown spinach leaves shortly after harvest and assayed immediately by the manometric procedure. All values are corrected for low endogenous rates, as determined separately for each system. Each Warburg flask contained 0.1 ml of the standard chloroplast preparation. Experiments were run in the dark except light was added, as indicated to the flasks with dopa only.

Light Requirement for Activating Polyphenol Oxidase in Chloroplasts. Low intensity white light from photoflood lamps activated the polyphenol oxidase activity. With chloroplasts from wheat leaves that did not oxidize dopa in the dark, 4 to 6 ft-c of white light provided nearly (50–75%) as much activation as 200 or 2600 ft-c. Little activity was observed with 3 ft-c. Activation by these very low light intensities necessitated careful attention to light leaks, and reading of the manometers had to be done with a small flashlight, while manometers were covered with a dark cloth. Ordinary light conditions in the laboratory provided enough light for partial activation of wheat plastids. Chloroplasts from alfalfa leaves required a higher light intensity for activation and were inactive at about 100 ft-c. Certainly a more careful evaluation of this phenomenon is necessary. The low light intensity suggests that a configurational change rather than electron transport is involved in light activation of polyphenol oxidase of the chloroplast.

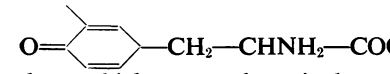
Red light passing through a filter (Carolina Biological Supply No. 650) transmitting above 650 nm activated polyphenol oxidase in pea or wheat chloroplasts nearly equal to the rate from white light. Blue light between 400 to 490 nm from a Carolina Biological Supply filter No. 450 did not stimulate dopa oxidation. In these experiments removal of the blue filter, thus introducing white light, immediately induced activity. These results indicate the activation phenomenon is not likely to be from chlorophyll, carotenoid, or flavin absorption.

Exploratory experiments were run with far red light passed by a Corning No. 2600 filter transmitting light of 720 nm or longer wavelength. This far red light did not significantly stimulate dopa oxidation by chloroplasts from wheat leaves.

Effect of Antimycin A and CMU. With freshly prepared spinach chloroplasts, in which the rate of oxidation of dopa in the dark was stimulated nearly 3-fold by trypsin or light, CMU or antimycin A, both at 50 μM final concentration, were equally stimulatory (Fig. 6). The rate of dopa oxidation in the presence of CMU or antimycin A was not significantly faster when trypsin was also added with these compounds. Light stimulated dopa oxidation by the chloroplasts to a rate about equal to that with trypsin, CMU, or antimycin A. Light did not further stimulate the rate with trypsin, CMU or antimycin A (data not shown). In view of the often studied effect of these inhibitors of electron transport, it appears as if uncouplers or inhibitors of electron transport also stimulate the particle bound polyphenol oxidase activity. However, neither trypsin, CMU, antimycin A or ferredoxin stimulated the dark oxidation, nor inhibited the light-stimulated oxidation of chloroplasts from pea, oat, or alfalfa leaves, typical of the type that only oxidized dopa in the light. The absence of CMU and antimycin A inhibition of the light requirement by this second type of chloroplast suggests that complete electron flow in the chloroplasts is not required for dopa oxidation.

Inhibition of Reaction with Time. Polyphenol oxidation by the chloroplasts in a typical assay slowed down after the oxidation of about 2 to 3 μmoles of dopa (24 μmoles had been added). Chloroplasts from tomato leaves were particularly sensitive in this respect. Even the rate of oxidation of dopa by chloroplasts from spinach leaves often decreased severely after the reaction mixture turned dark from dopa oxidation. This

occurred in the presence or absence of trypsin after about the same total oxidation. The behavior were as if quinone end products of the reaction were inhibitory; the solutions were nearly black from the oxidation products. Chloroplasts from sugar beets were an exception in that the rate of dopa oxidation did not slow down with time. Naphthoquinone sulfate did not inhibit dopa oxidation. This quinone did serve as an electron acceptor for a Hill fraction in the light, so that in the light gas evolution was observed with the quinone alone, or with combinations of dopa and naphthoquinone sulfate initial O_2 uptake rates were reduced. If dopa quinone was serving as a Hill acceptor simultaneously with its oxidation by the polyphenol oxidase, observed initial rates of dopa oxidation were minimal.

Effect of Mimosine. Mimosine (Calbiochem) or leucinol,  , is a structural analog of dopa which accumulates in leguminous plants. Since chloroplasts in group B which could only oxidize dopa in the light, were primarily from leguminous plants, the effect of mimosine on dopa oxidation by isolated chloroplasts was investigated. It had no inhibitor or stimulatory effect upon dopa oxidation by isolated chloroplasts from spinach, wheat, oats, or alfalfa leaves.

Magnitude of Polyphenol Oxidase Activity in Chloroplasts. With manipulation of the various treatments of the chloroplasts and from selection of different plants for chloroplast preparation a wide range of polyphenol oxidase activity may be observed. Values up to 2 matoms O_2 uptake or mmoles dopa oxidized $\times$ mg^{-1} chlorophyll $\times$ hr^{-1} were observed in aged spinach chloroplasts and chloroplasts from Swiss chard leaves were even more active (Table VII).

Polyphenol oxidase activity in chloroplasts of group B and C ranged from 10 to 100 μatoms O_2 uptake $\times$ mg^{-1} chlorophyll $\times$ hr^{-1}, and these values were not increased by any of the stimulants that were effective on chloroplasts in group A. The magnitude of polyphenol oxidase activity in chloroplasts of plants in group A were equal to or greater than the rate of ATP synthesis or NADP reduction by chloroplasts, which generally range from 0.1 to 1 mmole $\times$ mg^{-1} chlorophyll $\times$ hr^{-1}. Photosynthetic activity in isolated chloroplasts from other plants has not been extensively investigated due to inactivity in the isolate plastid fractions. In general the CO_2 fixation rate in leaves is in the order of 250 μmoles $\times$ mg^{-1} chlorophyll $\times$ hr^{-1}.

DISCUSSION

The existence of an immense latent activity for polyphenol oxidase tightly bound to the chloroplast lamellar structure, is a major unexplained component of the plastids. The natural substrate is unknown. The latent nature of the activity implies that *in vivo* the enzyme does not couple to oxygen. Polyphenol oxidase activity of the chloroplasts in plants of groups A and B was stimulated by very low levels of red light and this stimulation was not inhibited by CMU, antimycin A, ferredoxin, or naphthoquinone sulfate. The requirement for a small quantity of red light suggests some degree of photoregulation by other than chlorophyll, carotenes, or flavin absorption. These facts almost certainly exclude electron flow between the photosystems as a prerequisite for polyphenol oxidase activity. Rather light may be specifically effecting the enzyme involved in dopa oxidation.

Because of the very large amount of dopa oxidase in chloroplasts, it is doubtful that its function is only for synthesis of phenolic compounds or pigments. Chloroplasts normally do not exhibit a terminal respiration, as engendered by polyphenol oxidase. The latency of the chloroplast polyphenol oxidase, its location in the lamellar structure, its activity, which may be as great as the rate of photosynthetic phosphorylation, all implicate a major protein catalyst whose true function in the chloroplast might be involved with quinone oxidation-reduction during electron transport.

Isolation and further characterization of chloroplast polyphenol oxidase has not yet been done. By analogy with this oxidase from many other sources, the enzyme should be a copper-containing protein. Arnon (1) found it was inhibited by cyanide and diethyldithiocarbamate. Chloroplasts contain other copper enzymes, such as plastocyanin and ascorbate oxidase. Polyphenol oxidase is not part of the soluble protein fraction I, because it remains tightly bound to the lamellae by procedures that completely solubilize ribulose diphosphate carboxylase. In investigations for the content of plastocyanin in chloroplasts, only about half of the total copper in the chloroplast lamellae was accounted for (6). Thus there is probably enough copper unaccounted for in the chloroplast for a polyphenol oxidase protein. Alternatively plastocyanin itself may participate in this oxidation. In the future the red absorption spectra of plastocyanin, should be compared with the red light activation of polyphenol oxidase.

Oxidation of a monophenol, such as tyrosine by phenol oxidases, involves oxygen activation and insertion onto the phenyl ring. Since the path of oxygen in photosynthesis must also involve activated oxygen, it is possible that the manifestation of polyphenol oxidation by chloroplasts involves proteins associated with O_2 evolution. However, spinach chloroplasts that rapidly oxidized dopa did not oxidize tyrosine. In this respect the polyphenol oxidase of chloroplasts appears different from the oxidase from other sources such as mushrooms or potato tubers.

In pseudocyclic photophosphorylation by chloroplasts, catalytic amounts of a quinone probably serve as a hydrogen acceptor in the Hill reaction, and then the reduced quinone could be reoxidized by a chloroplast polyphenol oxidase (21). Phosphorylation then occurs although net oxygen exchange is nil. The potential of this phenomenon is evident by the amount of latent polyphenol oxidase activity in spinach chloroplasts. The magnitude of pseudocylic photophosphorylation *in vivo* is unknown, but the amount of polyphenol oxidase suggests that the latent oxidase, if it were to function in this manner, would have to be regulated.

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