AN ABSTRACT OF THE THESIS OF

FRANK FARNHAM TAYLOR for the M. S.
(Name of student) (Degree)

in Chemistry presented on October 4, 1968
(Major) (Date)

Title: SOME ASPECTS OF AN OXIDATIVE TRYPTOPHAN DECARBOXYLATING ENZYME IN *PISUM SATIVUM*

Redacted for privacy

Abstract approved: Donald J. Reed

Three types of experiments were designed to further characterize the tryptophan decarboxylating enzyme of Reed and Crecelius (1967).

1) Light inhibition studies were made with etiolated pea seedlings to determine the *in vitro* response of the tryptophan decarboxylating enzyme. Assays of indoleacetic acid oxidase (IAA oxidase) and guaiacol-active pea seedling peroxidase were included for comparison. The studies showed that IAA oxidase and the tryptophan decarboxylating enzyme had essentially the same inhibition response caused by constant exposure of etiolated pea seedlings to light. The guaiacol-active peroxidase was not inhibited.

2) Sephadex chromatography was performed to ascertain any differences in the elution of the three enzymes. A large portion of the tryptophan decarboxylating enzyme activity was retarded by the

column. The activity peaks of the other enzymes assayed, IAA oxidase and pea seedling peroxidase, coincided with the main protein peak.

3) A study was performed with catechol to determine the type of inhibition caused by polyphenolic compounds. The results indicate that the inhibition was competitive. A survey of polyphenolic compounds was conducted to determine their relative efficiency of inhibition of the tryptophan decarboxylating enzyme. All of the compounds tested were found to be very potent inhibitors of the tryptophan decarboxylating enzyme.

Some Aspects of an Oxidative Tryptophan Decarboxylating Enzyme in *Pisum Sativum*

by

Frank Farnham Taylor

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Master of Science

June 1969

APPROVED:

Redacted for privacy

Associate Professor of Biochemistry
in charge of major

Redacted for privacy

Chairman of Department of Chemistry

Redacted for privacy

Dean of Graduate School

Date thesis is presented October 4, 1968

Typed by Opal Grossnicklaus for Frank Farnham Taylor

ACKNOWLEDGMENTS

I would like to thank Dr. Donald J. Reed for his guidance in the investigation and writing of this thesis.

I wish to express my appreciation to my wife, Mary, for her support and assistance during the preparation of this thesis.

Partial support for this research was supplied by grant USDA 12-14-8052(35).

TABLE OF CONTENTS

INTRODUCTION 1

EXPERIMENTAL PROCEDURE 8

- Materials 8
- Methods 9
  - Buffers 9
  - Seed Germination 9
  - Pea Preparations 9
  - Tryptophan Decarboxylating Enzyme Assays 10
  - IAA Oxidase Assay 12
  - Peroxidase Assay 13
  - Micro-Kjeldahl Protein Determination 13
  - Sephadex Column 14
  - Enzyme Preparation for Sephadex Chromatography 14
  - Light Inhibition 16
  - Inhibitor Study 16

RESULTS 38

- Light Inhibition Studies 38
  - Tryptophan Decarboxylating Enzyme 38
  - Indoleacetic Acid Oxidase 39
  - Peroxidase 39
- Protein 40
- Sephadex Chromatography 40
- Inhibitor Determination 41

DISCUSSION 42

BIBLIOGRAPHY 48

LIST OF FIGURES

Figure		Page
1.	Sections of the epicotyl used for assays.	18
2.	Time sequence of the light inhibition of the tryptophan decarboxylating enzyme in the leaf sections of Little Marvel pea seedlings.	20
3.	Time sequence of the light inhibition of the tryptophan decarboxylating enzyme in the upper stem section of Alaska pea seedlings.	21
4.	Time sequence of the light inhibition of the tryptophan decarboxylating enzyme in the upper stem sections of Little Marvel and Alaska pea seedlings.	22
5.	Time sequence of the light inhibition of IAA oxidase in the leaf sections of Little Marvel and Alaska pea seedlings.	24
6.	Time sequence of the light inhibition of IAA oxidase in the upper stem sections of Little Marvel and Alaska pea seedlings.	25
7.	Time sequence of the light inhibition of IAA oxidase in the lower stem sections of Little Marvel and Alaska pea seedlings.	26
8.	IAA oxidase assay of the eluate fractions from a Sephadex G-25 column.	30
9.	Peroxidase assay of the eluate fractions from a Sephadex G-25 column.	31
10.	Protein determination of the eluate fractions from a Sephadex G-25 column.	32
11.	An assay of the tryptophan decarboxylating enzyme activity of the eluate fractions from a Sephadex G-25 column.	33

LIST OF FIGURES (CONTINUED)

Figure		Page
12.	A comparison of the tryptophan-^{3}H-(G) and tryptophan-1-^{14}C assays for the same fractions of eluate from a Sephadex G-25 column.	34
13.	Lineweaver-Burk plot of the tryptophan decarboxylating enzyme from pea seedling homogenates.	36

LIST OF TABLES

Table		Page
1.	Summary of data on light inhibition of the tryptophan decarboxylating enzyme.	19
2.	Summary of data on the light inhibition of IAA oxidase in the seedlings.	23
3.	Peroxidase activity after constant light exposure of seedlings.	27
4.	Protein content of pea seedlings exposed to continuous light.	28
5.	Enzyme activities of effluent fractions from Sephadex G-25 chromatography.	29
6.	Inhibition of Alaska pea seedling homogenates by catechol.	35
7.	Inhibitor effects on tryptophan decarboxylation.	37

SOME ASPECTS OF AN OXIDATIVE TRYPTOPHAN DECARBOXYLATING ENZYME IN *PISUM SATIVUM*

INTRODUCTION

It has long been assumed that L-tryptophan is a precursor of beta-indoleacetic acid in plants. Until recently very little work had been done to establish the nature and properties of the enzyme systems which catalyze this conversion in higher plants. Wiltshire (1953) demonstrated that in the presence of added hydrogen peroxide, tryptophan was converted to oxidation products by horseradish peroxidase preparations and an enzyme preparation from pea plants. Hinman and Lang (1965) have shown that in the absence of added hydrogen peroxide, tryptophan was not a substrate to horseradish peroxidase.

Recently a tryptophan decarboxylating enzyme has been found in pea seedlings (Reed and Crecelius, 1967). Valdovinos and Ernest (1966) have shown that gibberellin enhances the production of $^{14}CO_2$ from tryptophan-1-^{14}C in Little Marvel peas. Reed and Crecelius (1967) and Reed (1968) have demonstrated that etiolated Alaska pea seedlings have 50 to 100 times more tryptophan decarboxylating activity than light-grown pea seedlings. They found that seedlings exposed to continuous light for 24 hours caused the formation of flavonoid derivatives which almost completely inhibited the

tryptophan decarboxylating activity. This enzyme does not appear to be a typical L-aromatic amino acid decarboxylase (Lovenberg, Weissbach, and Udenfriend, 1962) because oxygen is required and tryptamine cannot be identified as a product. Various methods were tried to identify tryptamine as a product without success. Acid-base extracts of the reaction mixture were chromatographed and an attempt was made to identify the chromatographic spots through their R_f values. Isotope dilution was tried, with negative results. Decarboxylation is certain because of the $^{14}CO_2$ released when tryptophan-1-^{14}C is used in the reaction mixture. If tryptophan-2-^{14}C or tryptophan-3-^{14}C is added, the radioactivity remains in solution and can be partially extracted from acidic and basic reaction mixtures with toluene.

Indications that this is an enzymic reaction include the heat lability and oxygen requirement of pea seedling homogenates for activity, and the inhibition of activity in etiolated pea seedlings by light within 24 hours.

The light inhibition and oxygen requirement characteristics are similar to those of indoleacetic acid oxidase (IAA oxidase), which has prompted a study of these similarities. There is ample evidence that indoleacetic acid oxidase is either a peroxidase or has many peroxidase-like properties (Hinman, Bauman and Lang, 1961; Ray, 1958). IAA oxidase activity has been shown with horse-radish peroxidase preparations (Hinman and Lang, 1965; Siegel and

Galston, 1967). The reaction can be carried out without added peroxide and is one of the few known cases with a peroxidase where oxygen is consumed and peroxide is not required (Mason, 1957; Hinman and Lang, 1965). Experiments have shown that removal of the heme group of horseradish peroxidase destroyed the conventional peroxidase activity of the enzyme but not the IAA oxidase activity, provided the apoenzyme is supplied with the IAA oxidase cofactors, 2,4-dichlorophenol and Mn^{++} ion (Siegel and Galston, 1967). These cofactors were not needed when the heme group was left in the enzyme or reconstituted into the enzyme. However, other workers have been unable to confirm these results (L. Shannon, private communication, 1968).

The *in vitro* degradation of IAA by IAA oxidase involves the conversion of IAA to 3-hydroxymethyloxindole, then 3-methyleneoxindole, probably by a free radical mechanism (Still, Oliver and Moyed, 1965; Siegel and Galston, 1967). Still, Oliver and Moyed (1965) determined that extracts of etiolated pea seedlings can oxidize indoleacetic acid to 3-hydroxymethyloxindole, which can then be enzymically dehydrated to 3-methyleneoxindole. They showed 3-methyleneoxindole to be a cell growth inhibitor and a very reactive sulfhydryl reagent.

Meudt (1967) has proposed that the biological activity of IAA may be due to a transitory oxidation product of IAA. He demonstrated

that an oxidation product starts forming immediately after addition of horseradish peroxidase to an IAA reaction mixture. This initial product is very transitory and a secondary product starts forming within five minutes. Meudt hypothesized that the initial product or products may be biologically active and that the inactive secondary products may be prevented from occurring *in vivo*.

Although it has long been proposed that tryptophan is a precursor of IAA in higher plants, some earlier studies gave results which may have been due to bacterial contamination (Winter, 1966; Libbert *et al.*, 1966). Recently, however, Moore and Shaner (1967) have presented evidence with labeled compounds which they feel is not due to bacterial contamination and shows significant conversion of tryptophan to IAA. Lantican and Muir (1967) used an Avena curvature test to elucidate a pathway from tryptophan to tryptamine to indoleacetic acid. Using isotopes, however, Reed (1967) was unable to show any conversion of tryptophan to tryptamine. Possibly tryptophan and tryptamine are degraded by parallel pathways to IAA or bioactive IAA oxidation products.

Light-grown pea epicotyls have been demonstrated to contain inhibitors of IAA oxidase activity. Hillman and Galston (1957) have shown that they can induce inhibition in etiolated pea seedlings through exposure to weak red light. They also observed that this inhibition could be reversed by the application of far red light

immediately after the exposure to red light, suggesting phytochrome control of the inhibition. This inhibition was found to be due to a heat stable, dialyzable inhibitor. Furuya, Galston and Stowe (1962) isolated the inhibitor fraction and found it contained the flavonols, kaempferol and quercetin, and their glycosides. It was found that etiolated tissues contained only kaempferol glycosides while light-grown tissues contained quercetin glycosides. Later, Bottomley, Smith and Galston (1966) determined that kaempferol glycoside synthesis is not under red-far red control and that quercetin glycoside synthesis is under red-far red control and is the IAA oxidase inhibiting agent.

It has been reported that IAA oxidase is usually activated by monophenols and inhibited by polyphenols (Galston, 1967). In vitro studies show substituted phenols activate IAA oxidase and substances which include caffeic acid, chlorogenic acid, P-benzoquinone and scopoletin inhibit activity (Furyura, Galston and Stowe, 1962). Tomaszewski and Thimann (1966) intimate that phenol-oxidizing enzymes must act as general growth controllers since the introduction of a second, adjacent hydroxyl group into a phenolic compound changes it into an IAA oxidase inhibitor. Rabin and Klein (1956) reported that chlorogenic acid is a competitive inhibitor in vitro while one moiety of chlorogenic acid, caffeic acid, was a noncompetitive inhibitor. Quinic acid, the other moiety of chlorogenic acid, was

found to be inactive. In comparison, Reed (1968) has shown that with the tryptophan decarboxylating enzyme, kaempferol, quercetin, chlorogenic acid, caffeic acid, MK-485, catechol, resorcinol and beta-hydroxy-ethylhydrazine all inhibit this enzyme system, while quinic acid has no effect.

Galston (1967) has noted that while intact etiolated pea plants have little 3-methyleneoxindole, if the plants are supplied with large amounts of IAA, then this compound appears. Also, known inhibitors suppress the appearance of the compound and known cofactors enhance the appearance of this compound.

Morgan (1964) suggested that IAA oxidase is regulated by the inhibitor system in cotton. Perhaps this system could also be functioning on the tryptophan decarboxylating system in peas, as both enzymes are sensitive to the same inhibitors.

The apparent similarities of IAA oxidase and the tryptophan decarboxylating enzyme in their physical properties raises the possibility that they are different functions of the same enzyme or at least of very similar enzymes. IAA oxidase activity has been shown in peroxidases and some studies have shown that IAA oxidase is an isozyme of peroxidase. This thesis considers various aspects of the tryptophan decarboxylating enzyme from etiolated pea seedling homogenates. The time sequence of the inhibition due to light will be examined, as will the type of inhibition. These aspects, and

the activity peaks from Sephadex chromatography, will be compared with IAA oxidase and peroxidase from pea preparations.

EXPERIMENTAL PROCEDURE

Materials

Pisum sativum, var. Alaska, seeds were purchased from Gill Bros. Seed Company. Little Marvel peas were kindly provided by Dr. T. C. Moore. L-tryptophan-1-^{14}C (8.26 mc/mmole), DL-tryptophan-3-^{14}C (4.08 mc/mmole), and tryptophan-^{3}H-(G) (17.6 mc/mg), were purchased from New England Nuclear Corporation. L-tryptophan, indoleacetic acid, horseradish peroxidase, chlorogenic acid, caffeic acid, kaempferol, quercetin, resorcinol, and guaiacol were purchased from Sigma Chemical Company and were used without further purification. Catechol was from Matheson, Coleman and Bell. Sephadex, G-25 coarse, was from Pharmacia. Fluor chemicals, p-terphenyl and POPOP, were from Packard Instrument Corp. Toluene was B & A reagent grade. p-Dimethylaminocinnamaldehyde was purchased from Aldrich Chemical Company and K & K Laboratories, Inc. Ammonium sulfate was a special enzyme grade from Mann Research Laboratories. Nitrogen gas was from National Cylinder Gas division of Chemtron Corp. Dichlorophenol, manganous chloride, and ethanolamine were used from laboratory stock.

Methods

Buffers

Borate-phosphate buffer was prepared by combining boric acid and potassium dihydrogen phosphate in glass-distilled water to obtain a final solution of 0.033 M in each. The solution was then titrated to pH 8.1 with potassium hydroxide.

Phosphate buffer, pH 6.1, was prepared at 0.066 M with potassium dihydrogen phosphate.

Seed Germination

Pea seeds were germinated at 20° on 11 x 14 1/2 inch seed germination paper (Anchor Paper Co.) soaked in glass-distilled water. The towel packets were placed in 9 x 13 inch "Freezette-flat" plastic boxes with air tight lids. The boxes were placed in a growth chamber which excluded all light. The seedlings were harvested for use after six or seven days.

Pea Preparations

Pea seedling homogenates were prepared by taking the desired tissue segments and suspending them in buffer (25 mg fresh/weight/ ml buffer). Usually approximately 250 mg of tissue was used. The tissue was then homogenized at 0° to 4° in 50 ml homogenizing tubes

with a teflon pestle. The ground tissue was placed in Nalgene centrifuge tubes and spun at 0° to 4° at maximum speed, approximately 41,000 × g, for 30 minutes in a Sorvall RC-2 refrigerated centrifuge. The resultant supernatant was used for all enzyme assays involving light inhibition, inhibitors, or relative tissue activity.

Tryptophan Decarboxylating Enzyme Assays

Three different assays were used to determine the radioactive products of the tryptophan decarboxylating enzyme.

$^{14}CO_2$ Formation Method. To facilitate the trapping of $^{14}CO_2$, the reaction mixtures were placed in 10 ml Kimax centrifuge tubes stoppered with a serum stopper. The reaction mixture consisted of 0.1 ml plant homogenate, 0.1 ml tryptophan-1-^{14}C, specific activity approximately 1 μc/0.1 ml/40 mμmoles tryptophan, made up in enough buffer to make a final reaction mixture of 0.4 ml (Reed, 1967). After a one hour incubation the reaction was stopped with a 0.2 ml of 2 N HCl added by syringe through the serum stopper. The $^{14}CO_2$ was then flushed into an ethanol-ethanolamine mixture (v:v 3:1) with nitrogen by means of a long needle (BD #20, 6 inch) which bubbled the nitrogen through the reaction mixture. A smaller needle also penetrated through the serum stopper and was connected by rubber tubing to a needle (BD #20, 6 inch) immersed in 5 ml of the ethanol-ethanolamine mixture.

After bubbling for 15 to 20 minutes, the needle was removed from the ethanolamine mixture and the mixture was poured into 10 ml of toluene scintillator solution and counted on a Packard 3375 or 314EX2 scintillation counter. The samples were counted to a relative standard deviation of 1% except those with a low level of radioactivity which were counted for ten minutes. The toluene scintillator solution contained 3 gms/liter of terphenyl and 30 mg/liter of POPOP.

Extractable ^{14}C Method. Activity from tryptophan-3-^{14}C was assayed by making toluene extractions from a reaction mixture consisting of 0.1 ml plant homogenate, 0.1 ml tryptophan-3-^{14}C, specific activity approximately 1 μc/0.1 ml/40 mμmoles tryptophan, and enough buffer to make up to 0.4 ml total volume. After incubating one hour the reaction was stopped by the addition of 0.2 ml HCl. Two 5 ml portions of toluene were added successively and were shaken thoroughly, the toluene being separated by centrifugation in an International clinical centrifuge at top speed. Four ml of the first portion was added to 10 ml of toluene scintillator solution. After removal of the remaining toluene, the homogenate was made basic by adding 0.4 ml 6N KOH and extracting with 5 ml of toluene, 4 ml of which was added to 10 ml of scintillator solution. The samples were counted on either a Packard 3375 or 314EX2 scintillation counter to 1% standard deviation or for ten minutes.

Extractable ^{3}H Method. Using tryptophan-^{3}H-(G) the assay was performed as with the tryptophan-3-^{14}C initially. However, it was discovered that a base only extraction would give proportional results, although with a somewhat higher background and sample count rate. The base only extraction consisted of adding 0.2 ml 2N HCl to stop the reaction, then 0.4 ml 6N KOH to make the reaction mixture basic, and then proceeding with an extraction with 5 ml of toluene as described previously.

IAA Oxidase Assay

IAA oxidase was assayed using the procedure of Meudt and Gaines (1967). The reaction mixture consisted of 0.2 ml IAA (80 mμmoles/0.1 ml) in phosphate buffer, pH 6.1, 0.2 ml 10^{-4} M 2,4-dichlorophenol in phosphate buffer, and 0.2 ml 10^{-4} M Mn^{++} as the chloride in phosphate buffer. The reaction was allowed to proceed for 20 minutes before being stopped by 0.8 ml (equivolume) of a 1% solution of dimethylaminocinnamaldehyde in 2N HCl. The resultant mixture was placed in the dark for 70 minutes and then read on a Beckman DB spectrophotometer at 562 mμ. It was found that samples had to be made up without IAA, DMACA added and then the IAA added and the sample read to provide the reference blanks because of a difference in O.D. between boiled and unboiled homogenates. This difference is probably due to the precipitation

of protein from the solution during boiling.

Peroxidase Assay

The procedure of Sequeira and Mineo (1966) was used for the peroxidase assay. The reaction mixture consisted of 2.5 ml buffer, 380 μmoles of hydrogen peroxide, 0.1 ml homogenate (75 mg fresh weight/ml buffer), and 20 μmoles guaiacol in a total volume of 3.7 ml. The reference blank did not contain guaiacol. The $O.D._{470}$ was read at six minutes after addition of guaiacol to the reaction mixture.

Micro-Kjeldahl Protein Determination

Total protein in the homogenates was determined according to the method of Lang (1958). To 0.5 ml of pea seedling homogenate in a 18 × 150 mm pyrex tube, catalog number 9820, was added 0.2 ml digestion mixture consisting of 40 gm K_2SO_4 and 2 ml selenium oxychloride diluted to 250 ml with glass distilled water and added to 250 ml conc. H_2SO_4. The tubes were then placed in a sandbath. A transite sheet with holes approximately the diameter of the tubes was used to hold the tubes erect. A marble was placed on the top of each tube to prevent splattering. The bath was then placed on a Chromalox heater for three hours. The tubes were then removed from the sandbath and allowed to cool for at least 20 minutes,

usually an hour. After cooling, 2 ml glass distilled water, 2 ml Nessler's reagent, and 3 ml of 2N NaOH were added and the mixture was allowed to stand 15 to 20 minutes. The tubes were then read in a Beckman DB spectrophotometer at 490 mμ. All tubes were done in duplicate, including the blanks. Ammonium sulfate controls were included in duplicate with every assay.

Sephadex Column

Sephadex G-25 coarse, 24.4 grams dry weight, were soaked for three hours before packing in a column to give final column dimensions of 2.5 cm × 21 cm. After packing, 2 ml of Blue Dextran 2000 was applied and was eluted in 8 to 9 ml, after a 50 ml void volume.

Enzyme Preparation for Sephadex Chromatography

Enough ten-day old etiolated pea seedlings were cut just above the cotyledon to make at least 500 grams of tissue. The epicotyls were then crushed in a Carver laboratory press with a cage adaptor. Ammonium sulfate (0.4417 gm NH_4SO_4/ml) was added to the sap while the sap was kept at 0° to 4° to make a 70% saturated solution. The mixture was stirred with a magnetic stirrer overnight while kept in the cold room. The resultant precipitate was spun in 250 ml Nalgene centrifuge tubes in a Sorvall RC-2 refrigerated centrifuge at 16,300 × g for 15 minutes. To the supernatant was added 0.173 gm of

ammonium sulfate per ml to make a 95% saturated solution and the resultant mixture was again stirred with a magnetic stir bar in the cold room until all the ammonium sulfate was dissolved. The solution was then again centrifuged at 16,000 × g for 15 minutes and the resulting precipitate was resuspended in a minimum volume of buffer, approximately 5 ml. This suspension was layered on top of a Sephadex G-25 column. Five ml samples were collected from the column by a constant volume collector. The buffer used depended upon the enzyme being assayed. Phosphate buffer, pH 6.1, was used to elute samples for the IAA oxidase and peroxidase assays. Borate-phosphate buffer, pH 8.0, was used when the tryptophan decarboxylating enzyme was to be assayed.

The column was washed with a minimum of three void volumes (150 ml) of the proper buffer before the samples were layered on the surface of the sephadex.

Originally, the Sephadex G-25 chromatography was done to desalt the resuspended ammonium sulfate precipitation prior to addition of the protein fraction to a Sephadex G-200 column. However, it was soon determined that part of the activity of the tryptophan decarboxylating enzyme was withheld on the Sephadex G-25 column. This activity was quickly lost, with complete loss of activity occurring within three days. Most probably this loss of activity was due to the very low protein concentration in these later fractions.

Light Inhibition

The light inhibition studies were conducted under constant incandescent and fluorescent light in a growth chamber at 25.5°. Six-day pea seedlings were supported with their roots through a wire screen and placed in distilled water so that their roots and cotyledons were completely covered with water. The peas were then covered with Saran wrap. At intervals of six hours, 18 hours, and 30 hours a minimum of ten seedlings was collected and the various tissue sections excised with a razor blade.

Inhibitor Study

Due to the unavailability of the quercetin-3-p-coumaroyltriglucoside and the poor solubility of kaempferol and quercetin, catechol was used in the inhibitor study. Varying concentrations of catechol dissolved in buffer were added to the tryptophan decarboxylating enzyme assay in place of the buffer. Once inhibitions of approximately 20% and 80% were determined, duplicate assays were performed to determine the velocity of the reaction at the varying substrate concentrations of 40, 100, and 400 mμmoles of tryptophan-^{3}H-(G), with the two inhibitor concentrations and with no inhibitor. These values were used to make a Lineweaver-Burk reciprocal plot.

A survey was made of several phenolic inhibitors to determine

the inhibition characteristics. Each compound, dissolved in buffer, was added to a tryptophan-1-^{14}C assay in place of the buffer and the percent inhibition determined by comparing with a simultaneously assayed reaction mixture containing no inhibitor.

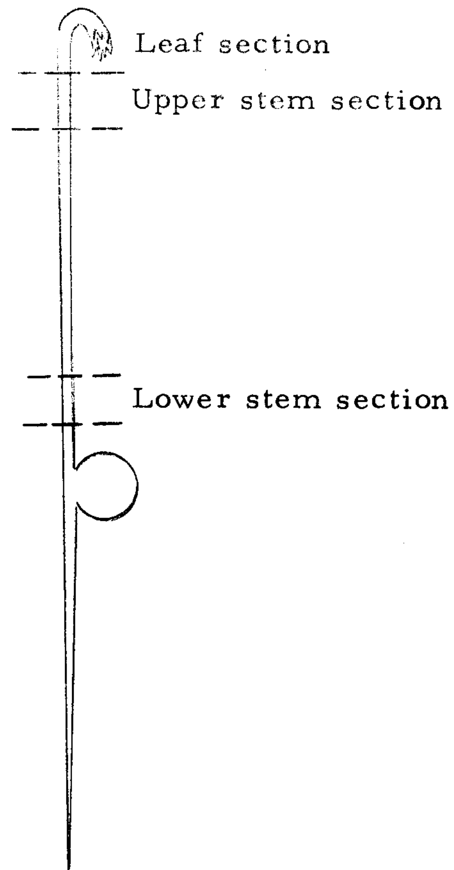

Figure 1. Sections of the epicotyl used for assays.

Table 1. Summary of data on light inhibition of the tryptophan decarboxylating enzyme.

Hours of light exposure	μμmoles of extractable oxidation products		
	Leaf	Stem section	
	Little Marvel	Little Marvel	Alaska
0	61	20	13
6	143		
8		23	43
12	80		
16		36	30
24	8.3	37	5.8
30		34	----
48	0.4		

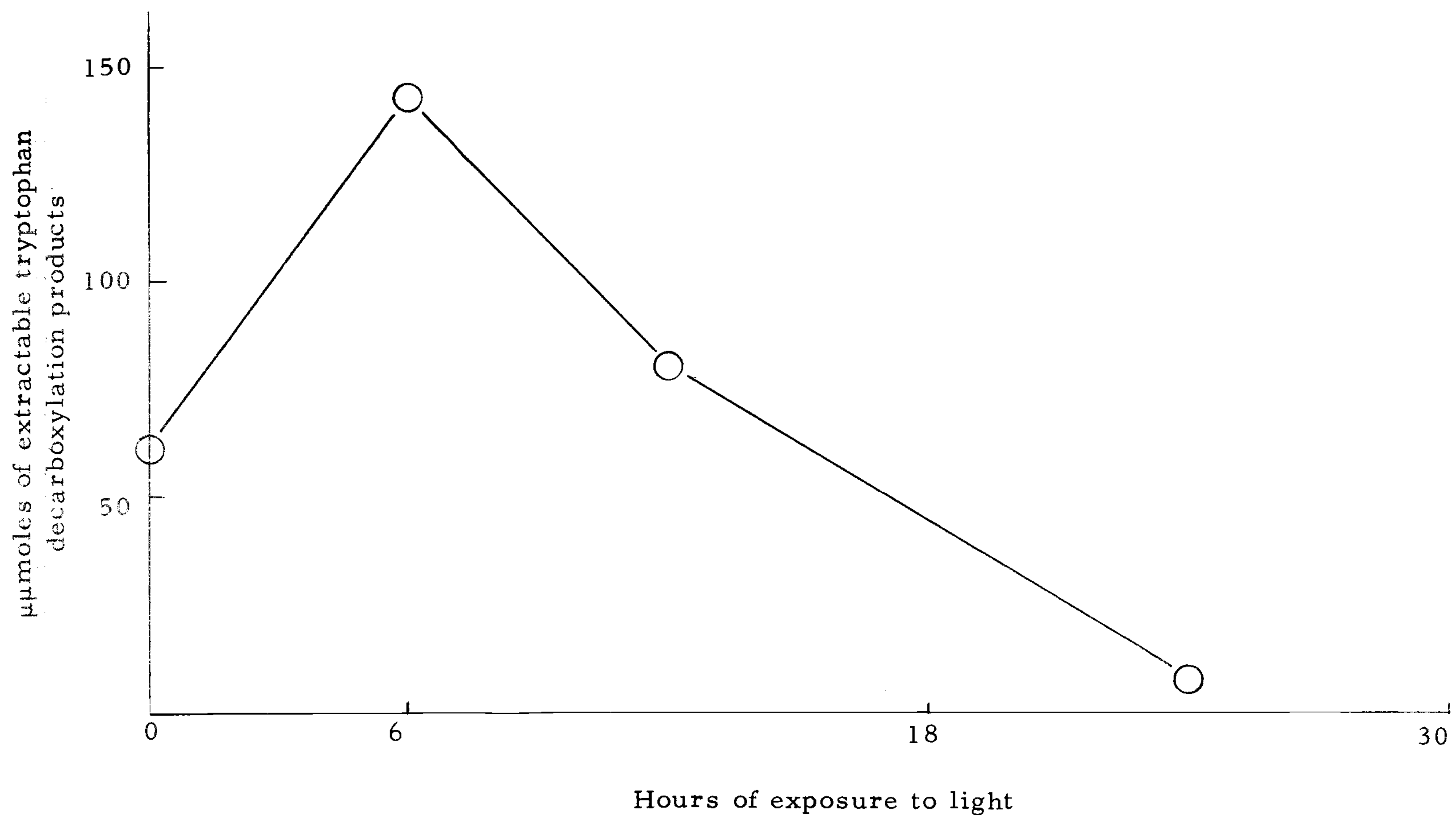

Figure 2. Time sequence of the light inhibition of the tryptophan decarboxylating enzyme in the leaf sections of Little Marvel pea seedlings.

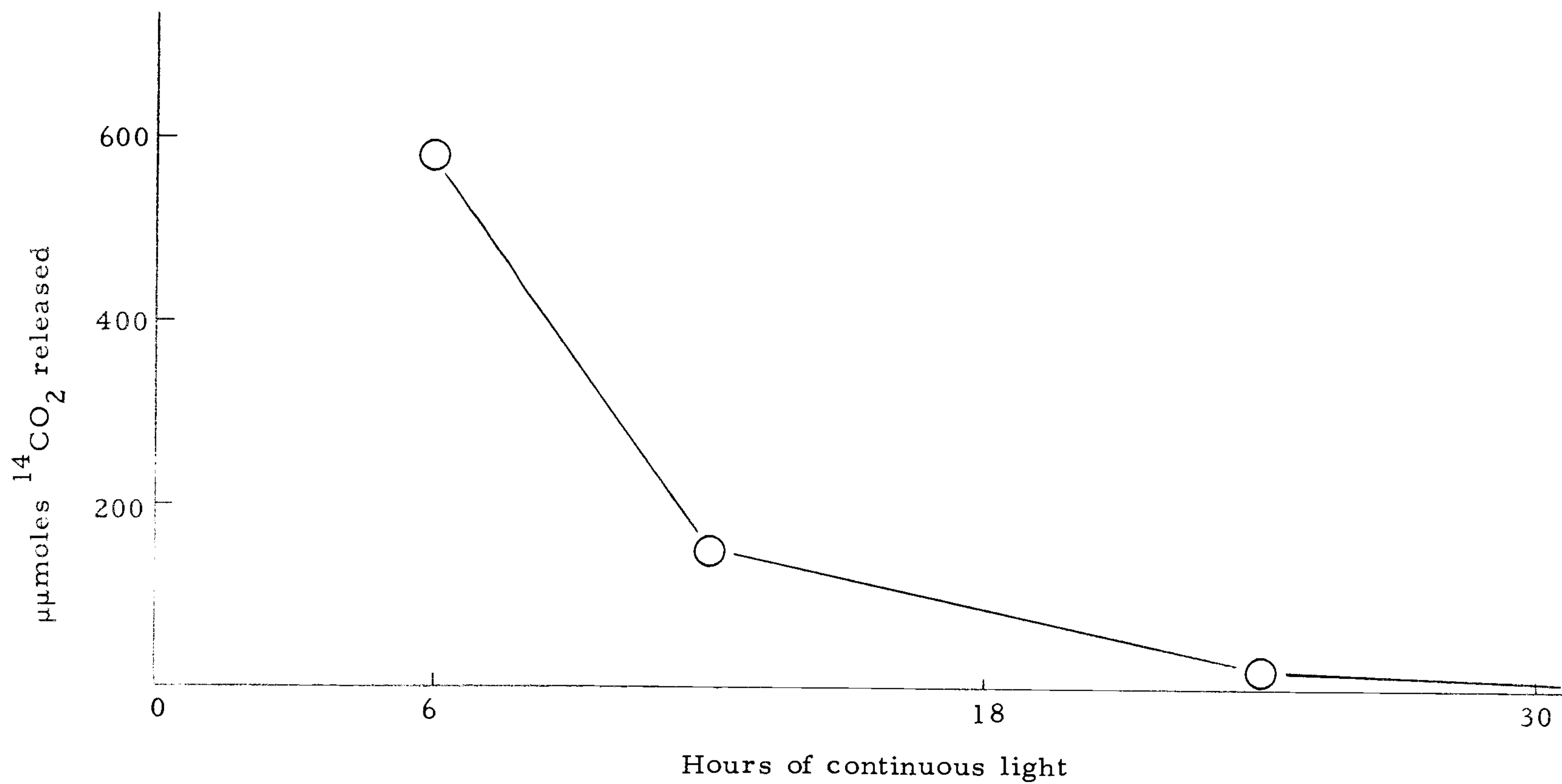

Figure 3. Time sequence of the light inhibition of the tryptophan decarboxylating enzyme in the upper stem section of Alaska pea seedlings (Reed, 1968).

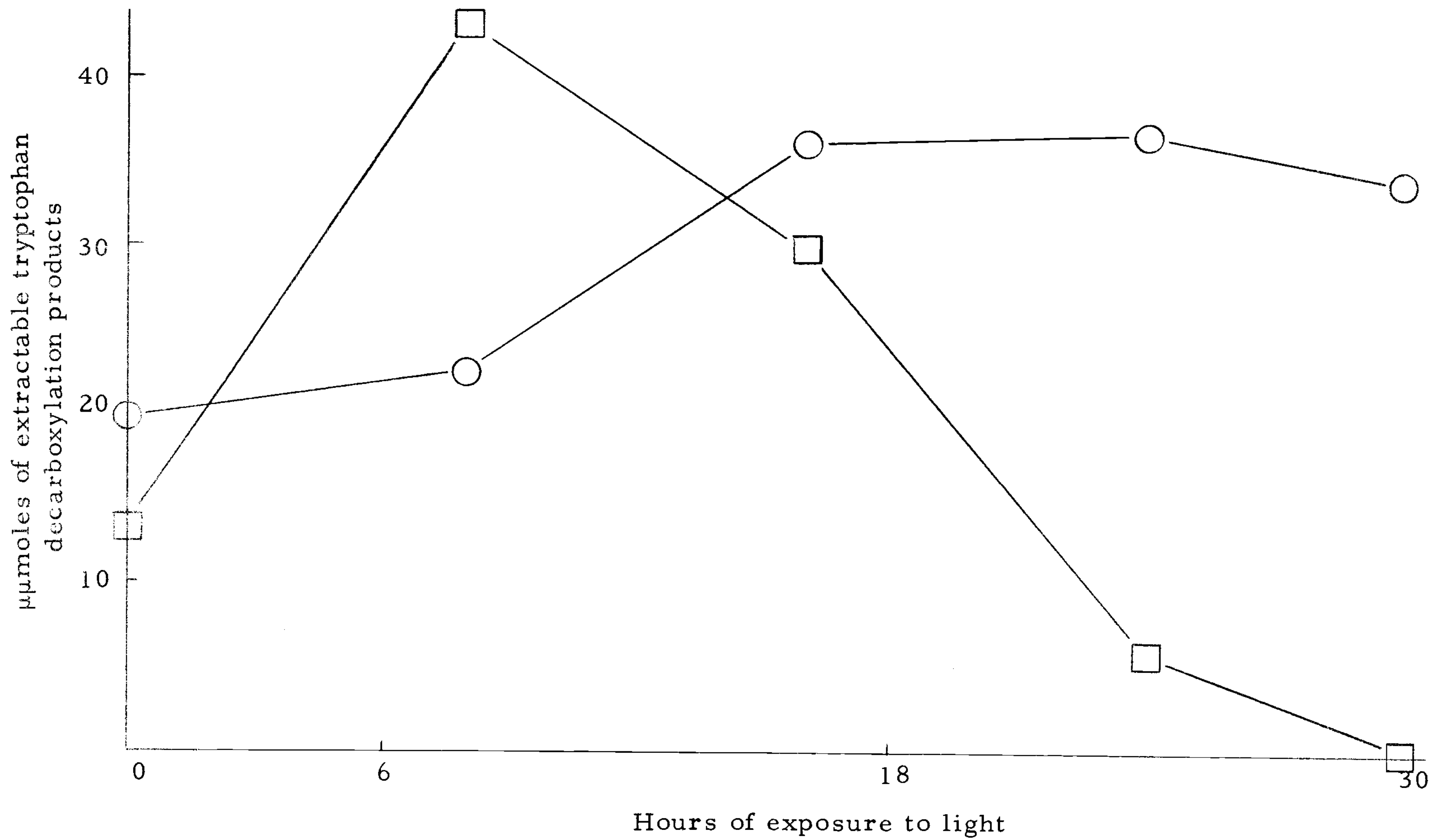

Figure 4. Time sequence of the light inhibition of the tryptophan decarboxylating enzyme in the upper stem sections of Little Marvel and Alaska pea seedlings.(○ Little Marvel seedlings, □ Alaska seedlings)

Table 2. Summary of data on the light inhibition of IAA oxidase in the seedlings.

Hours of light exposure	Optical density$_{562}$					
	Leaf		Upper stem		1. Leaf	
	LM*	Alaska	LM*	Alaska	LM*	Alaska
0	0.139	0.185	0.043	0.035	0.050	0.046
6	0.154	0.190	0.053	0.035	0.070	0.046
18	0.031		0.087		0.072	
19		0.068		0.068		0.015
29		0.037		< 0.001		< 0.001
30	0.037		0.096		< 0.001	

*Little Marvel

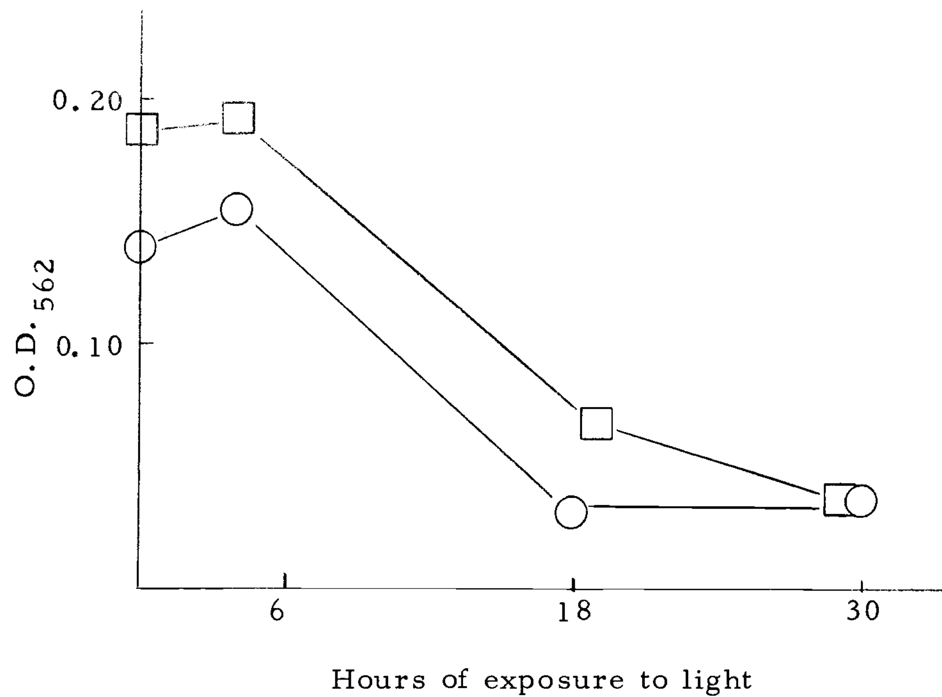

Figure 5. Time sequence of the light inhibition of IAA oxidase in the leaf sections of Little Marvel and Alaska pea seedlings. (○ Little Marvel seedlings, □ Alaska seedlings)

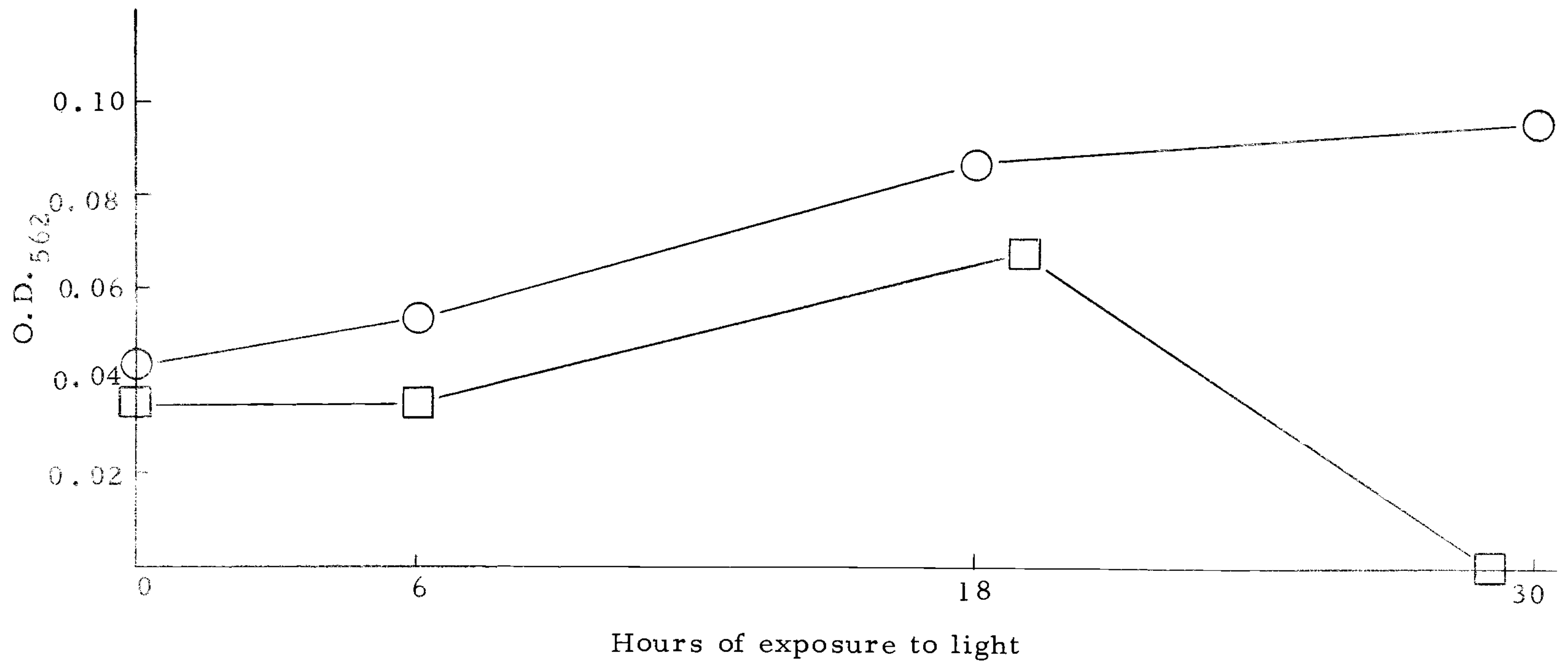

Figure 6. Time sequence of the light inhibition of IAA oxidase in the upper stem sections of Little Marvel and Alaska pea seedlings. (○ Little Marvel seedlings, □ Alaska seedlings)

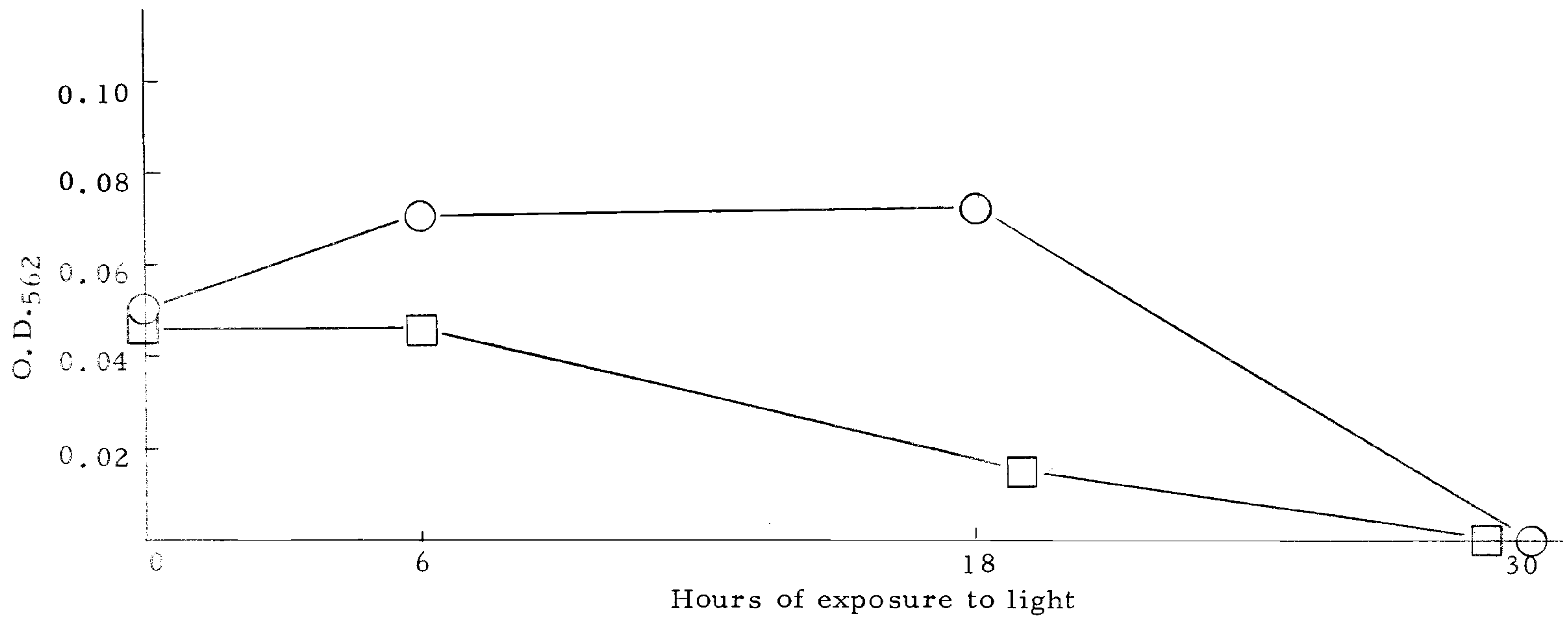

Figure 7. Time sequence of the light inhibition of IAA oxidase in the lower stem sections of Little Marvel and Alaska pea seedlings. (○ Little Marvel seedlings, □ Alaska seedlings)

Table 3. Peroxidase activity after constant light exposure of seedlings.

Hours of light exposure	Optical density$_{470}$					
	Leaf		Upper stem		Lower stem	
	LM*	Alaska	LM*	Alaska	LM*	Alaska
0	0.098	0.110	0.032	0.043	0.021	0.029
6	0.109	0.100	0.035	0.045	0.020	0.032
18	0.117		0.035		0.028	
19		0.134		0.054		0.042
30	0.125		0.040		0.028	
31		0.145		0.075		0.046

*Little Marvel

Table 4. Protein content of pea seedlings exposed to continuous light.

Hours of light exposure	μg protein/mg fresh tissue weight					
	Leaf		Upper stem		Lower stem	
	LM*	Alaska	LM*	Alaska	LM*	Alaska
0	9.9	8.4	3.7	2.3	1.6	3.3
6	7.8	8.3	2.8	2.9	1.8	0.9
18	10.0	9.6	4.1	4.1	2.1	2.8
30	10.5	6.1	3.9	2.7	0.8	1.1

*Little Marvel

Table 5. Enzyme activities of effluent fractions from Sephadex G-25 chromatography.

Fraction number	Tryptophan assay cpm	Peroxidase assay $O.D._{470}$	IAA oxidase assay $O.D._{562}$	Protein $O.D._{490}$
1	338	0.055	0.023	0.063
2	1440	1.1	0.442	0.173
3	1158	1.7	0.532	0.270
4	781	0.85	0.441	0.142
5	383	0.22	0.328	0.114
6	246	0.21	0.168	0.043
7	571	0.084	0.096	0.024
8	1701	0.056	0.038	0.033
9	1556	0.035	0.032	0.028
10	1532			
11	980			
12	733			

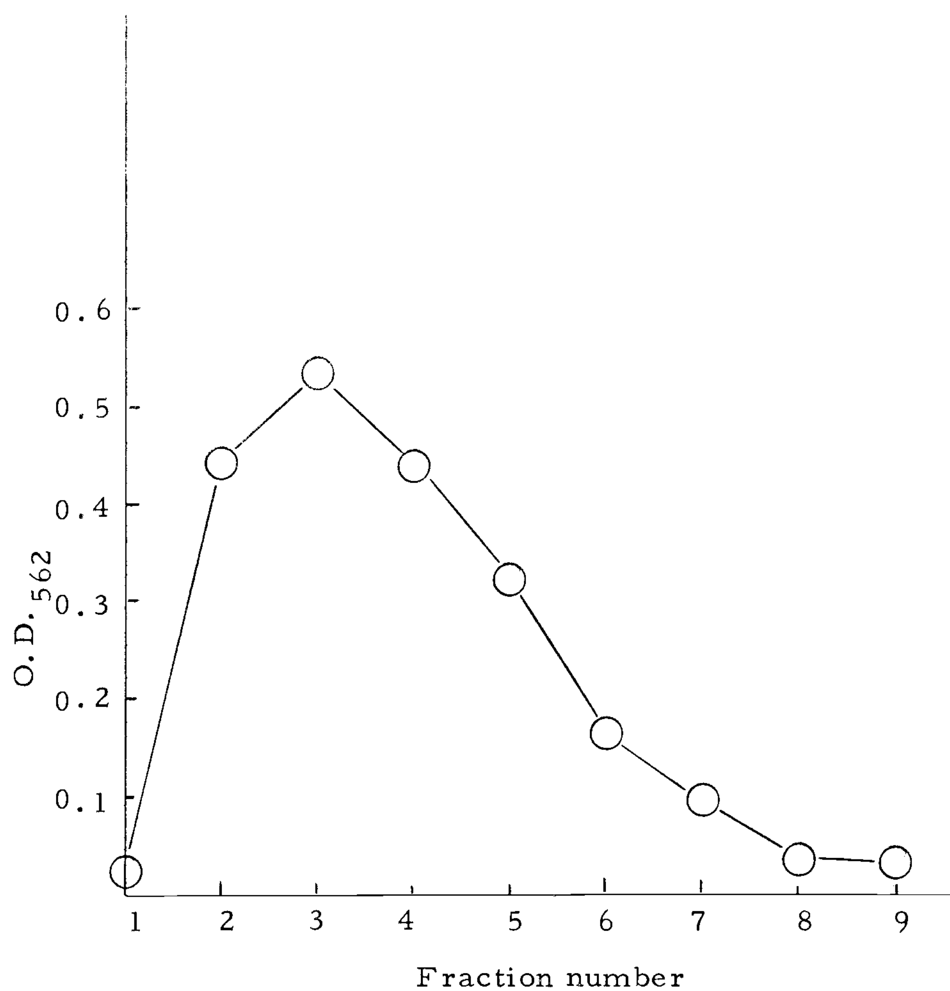

Figure 8. IAA oxidase assay of the eluate fractions from a Sephadex G-25 column. The precipitate from a 70%-90% ammonium sulfate fractionation of Alaska pea seedling epicotyl sap was resuspended in a minimum amount of buffer and applied to the column.

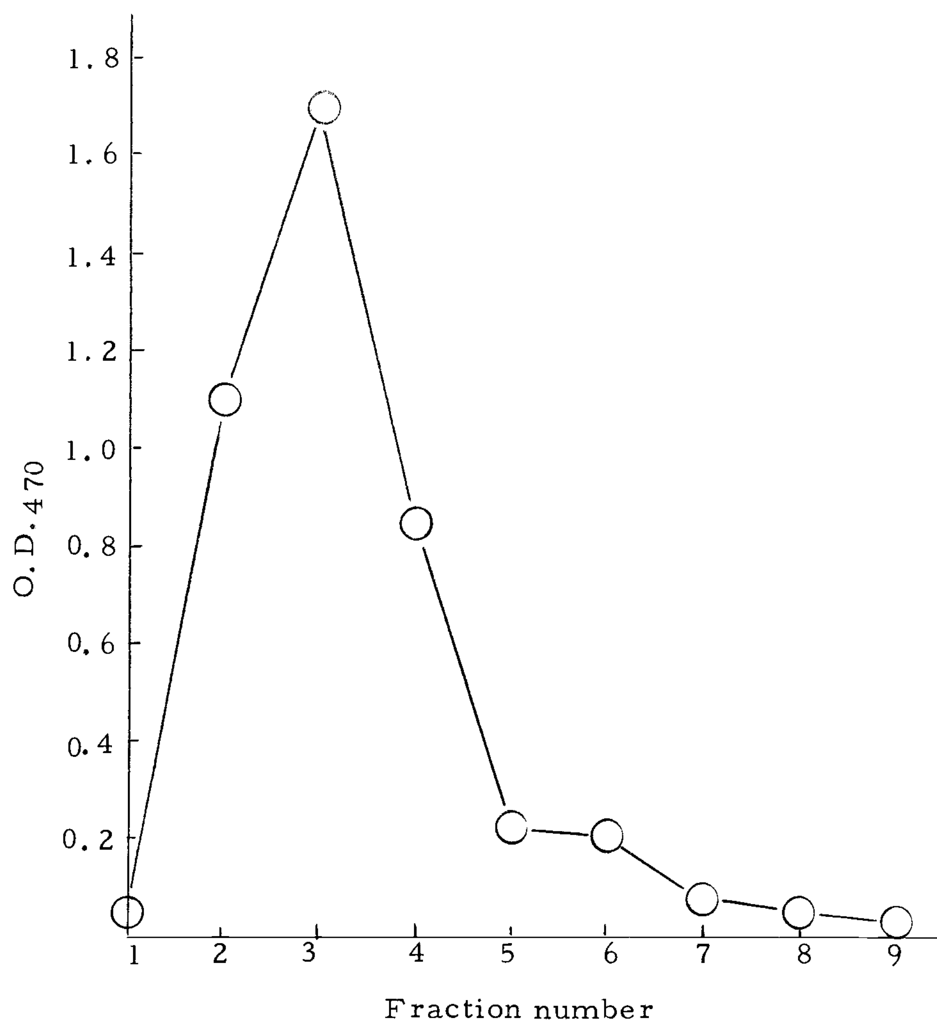

Figure 9. Peroxidase assay of the eluate fractions from a Sephadex G-25 column. The precipitate from a 70%-90% ammonium sulfate fractionation of Alaska pea seedling epicotyl sap was resuspended in a minimum amount of buffer and applied to the column.

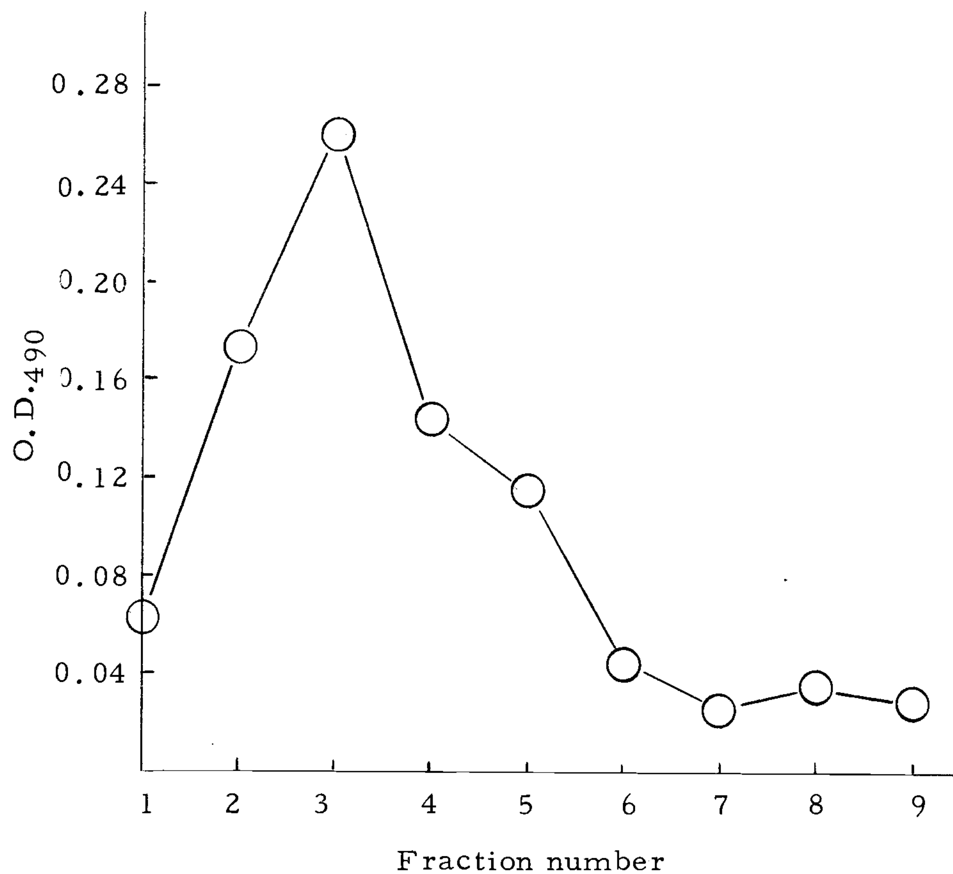

Figure 10. Protein determination of the eluate fractions from a Sephadex G-25 column. The precipitate from a 70%-90% ammonium sulfate fractionation of Alaska pea seedling epicotyl sap was resuspended in a minimum amount of buffer and applied to the column.

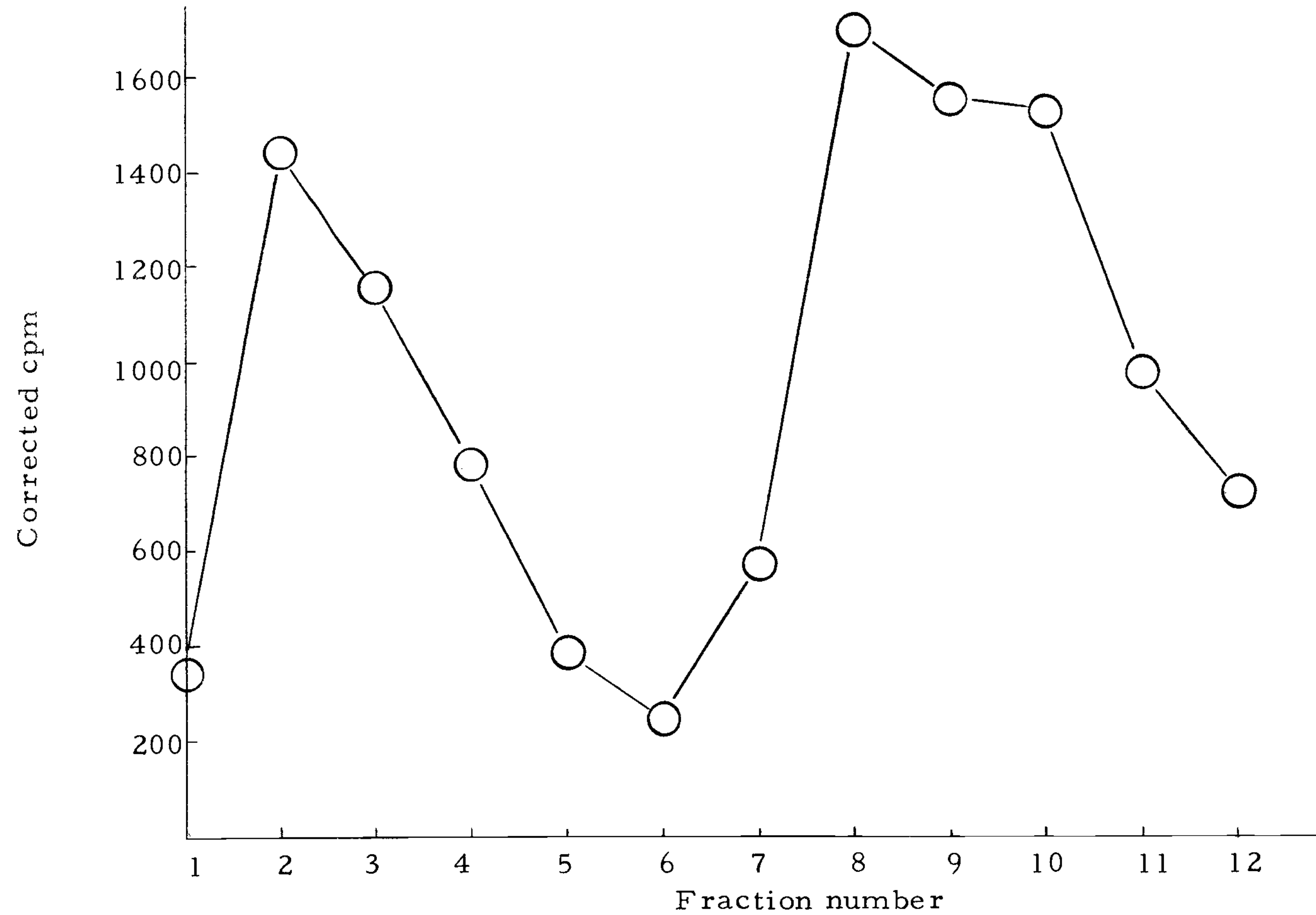

Figure 11. An assay of the tryptophan decarboxylating enzyme activity of the eluate fractions from a Sephadex G-25 column. The precipitate from a 70%-90% ammonium sulfate fractionation of Alaska pea seedling epicotyl sap was resuspended in a minimum amount of buffer and applied to the column.

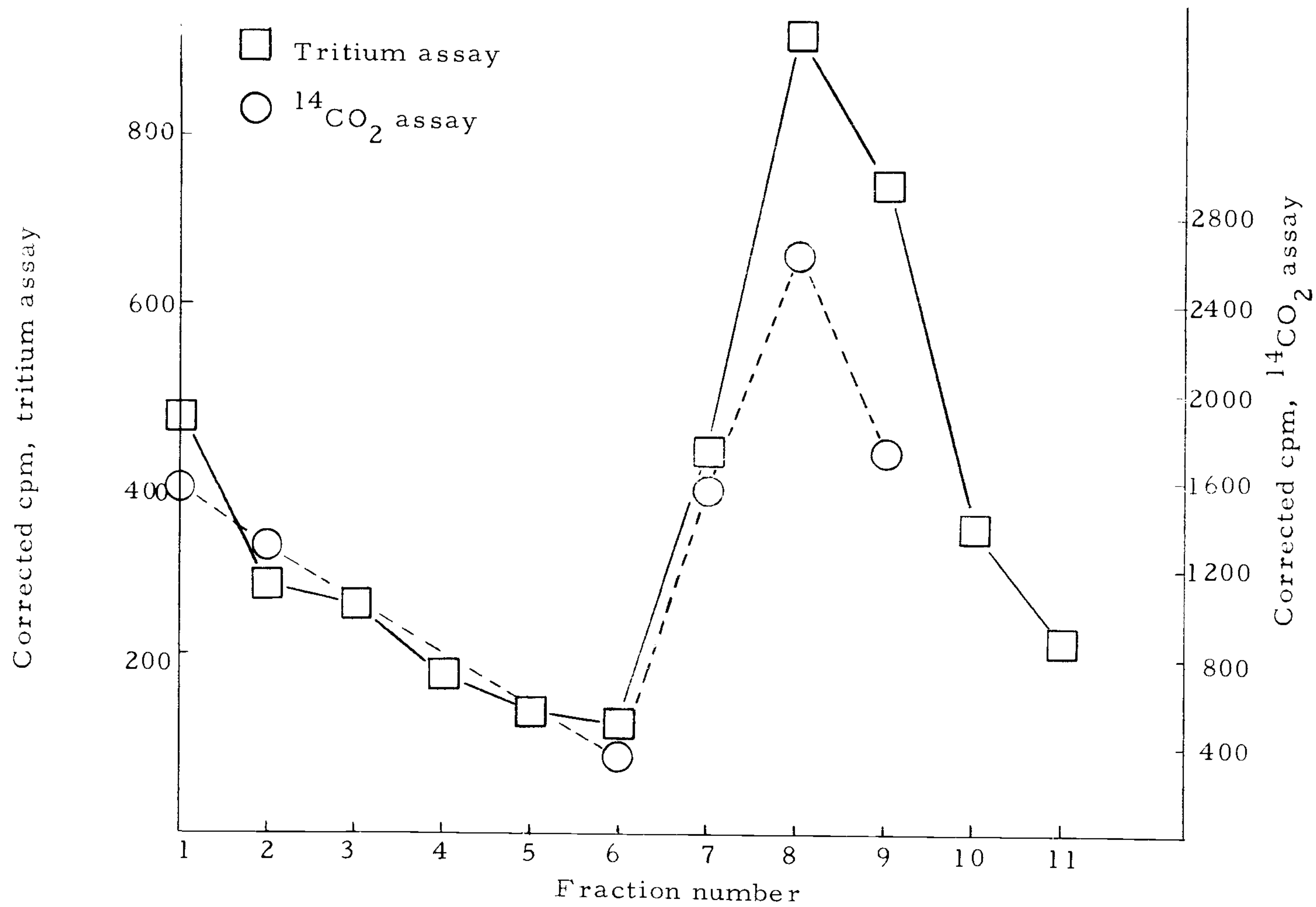

Figure 12. A comparison of the tryptophan-^{3}H-(G) and tryptophan-1-^{14}C assays for the same fractions of eluate from a Sephadex G-25 column. The precipitate from a 70%-90% ammonium sulfate fractionation of Alaska pea seedling epicotyl sap was resuspended in a minimum amount of buffer and applied to the column.

Table 6. Inhibition of Alaska pea seedling homogenates by catechol.

	I = 0	I = 10^{-6}M	I = 4 × 10^{-6}M	I = 10^{-5}M
μμmoles of extractable oxidation products (40 mμmoles tryptophan)	6.74 5.10	5.19	2.30	1.25 1.34
μμmoles of extractable oxidation products (100 mμmoles tryptophan)	11.20 15.32	13.03	6.22	4.80 3.19
μμmoles of extractable oxidation products (400 mμmoles tryptophan)	42.70 31.45	35.68	17.35	11.12 8.14
Intercept of best line through points (Lineweaver-Burk plot)	0.012	0.007	0.006	0.0016
Standard error	0.010	0.004	0.014	0.032
Slope of line	6.41	7.39	16.97	30.27

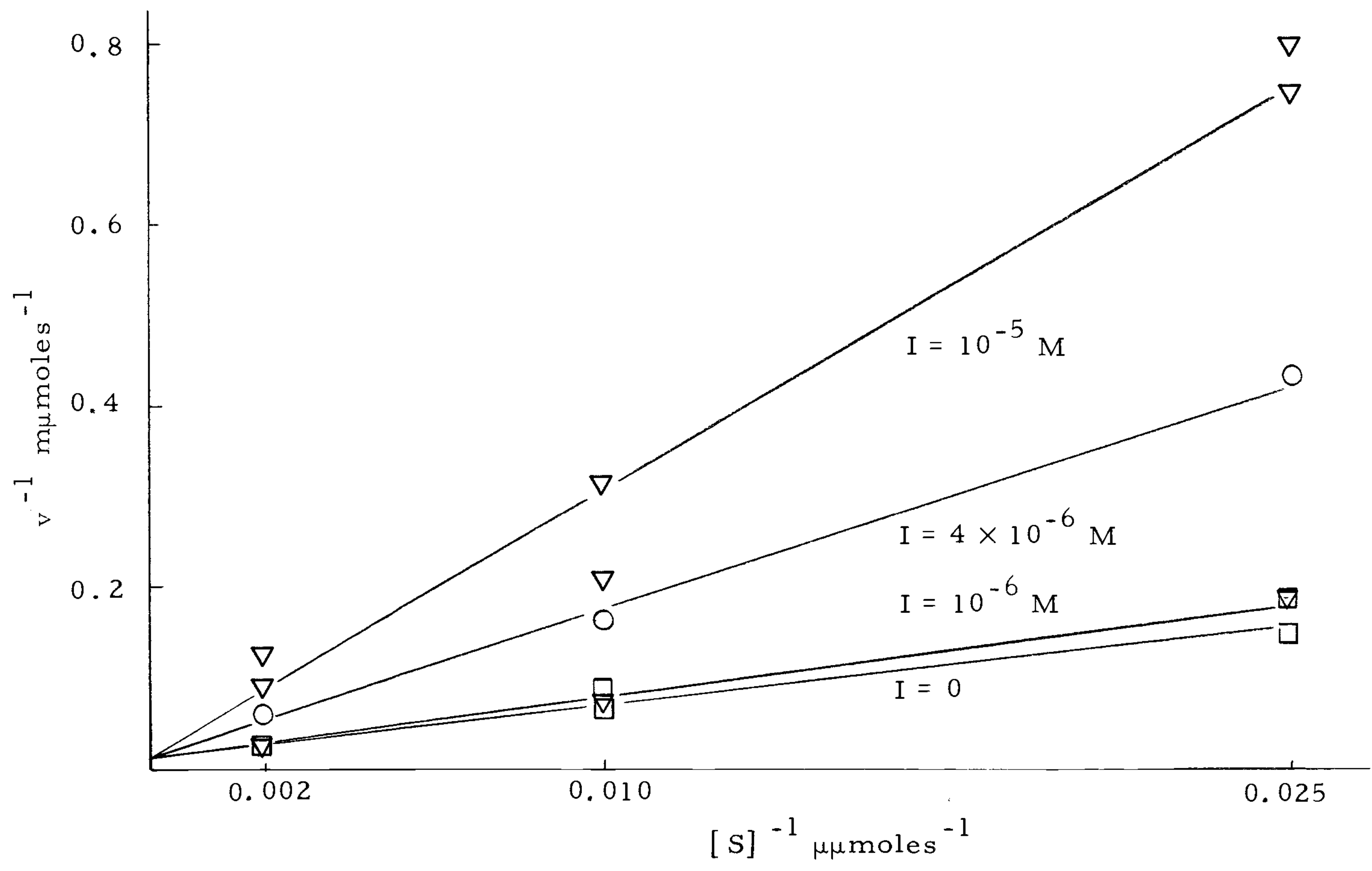

Figure 13. Lineweaver-Burk plot of the tryptophan decarboxylating enzyme from pea seedling homogenates. The inhibitor used was catechol.

Table 7. Inhibitor effects on tryptophan decarboxylation.

Compound	Concentration	Percent inhibition
	μM	
Kaempferol	4	36
	8	90
	40	100
Quercetin	4	22
	8	65
	40	100
Chlorogenic acid	1	63
	5	95
	10	100
Caffeic acid	5	92
	10	97
	100	98
D(-) Quinic acid	100	0
MK-485	0, 3	22
	1	40
	3	80
	10	93
Catechol	100	100
Resorcinol	100	100

RESULTS

Light Inhibition Studies

Reed (1968) determined that the tryptophan decarboxylating enzyme in etiolated pea epicotyls was inhibited within 24 hours by exposure of the seedling plants to continuous light. This prompted the study of the decarboxylating enzyme activity in different sections of the epicotyl. The different sections of pea epicotyl used are shown and labeled in Figure 1. These sections are used throughout the light inhibition assays. Alaska and Little Marvel pea seedlings are used to ascertain the change in inhibitor activity between different varieties of peas.

Tryptophan Decarboxylating Enzyme

Figure 2 shows the time sequence of the light inhibition of tryptophan decarboxylation activity of the leaf section of Little Marvel peas. The curve resembles that of Figure 3, in which Reed (1968) also showed that the inhibitor from preparations of light-exposed plants would inhibit enzymic activity in etiolated pea homogenates. In preliminary experiments, homogenates from light-inhibited Alaska leaf sections inhibited the activity of stem homogenates of etiolated peas. The difference in decarboxylase activity in the upper

stem portions of Alaska and Little Marvel pea seedlings is shown in Figure 4. The Alaska seedlings are completely inhibited within 30 hours while the Little Marvel seedlings appear to be slightly activated. The data used for the figures is shown in Table 1.

Indoleacetic Acid Oxidase

Figures 5, 6 and 7 show the resultant IAA oxidase activity of the leaf, upper stem, and lower stem segments, respectively, after exposure to given periods of constant light. The leaf sections of both varieties of peas show a 75% loss of activity within 30 hours. The activity drops to a residual value and is not completely inhibited. IAA oxidase in the upper stem sections of Little Marvel pea seedlings parallel the activity of the tryptophan decarboxylating enzyme in that the activity is not inhibited by exposure to light. Light inhibition of the lower stem sections was studied in order to make a comparison with the upper stem sections. Unlike IAA oxidase activity of the upper stem sections, the activity of the lower stem sections of both varieties of pea seedlings are completely inhibited within 30 hours. The data used for the figures is shown in Table 2.

Peroxidase

Peroxidase activity was assayed to determine the effect of constant exposure to light upon the guaiacol-active peroxidase

isozymes. A summary of the data on the leaf section, the upper stem section, and the lower stem section (Table 3), show that guaiacol-active peroxidase was not inhibited by constant exposure to light. Unlike the IAA oxidase and tryptophan decarboxylating enzyme, there was no stimulation of activity at six to ten hours.

Protein

Micro-Kjeldahl determinations of the protein content of the two varieties of pea seedlings were performed and expressed as μg protein per mg fresh weight. The assays show essentially no change in the amount of protein in the leaf sections, upper stem sections, and lower stem sections (Table 4) during the light exposure period.

Sephadex Chromatography

Sephadex G-25 column chromatography was conducted on the 90% saturated ammonium sulfate precipitable protein from the cell sap of etiolated pea epicotyls. The IAA oxidase activity in the effluent fractions is given in Figure 8. The activity peak of peroxidase is shown in Figure 9. Figure 10 shows the fractions in which the majority of the protein was eluted off the column. With the exception of the tryptophan decarboxylating enzyme, the activity peaks of the assayed enzymes coincide with the protein peak. The tryptophan decarboxylating enzyme assay (Figure 11) shows a small activity

peak coinciding with the protein peak and also at fraction numbers eight and nine a larger activity peak. The protein assay shows a slight, reproducible increase at fraction eight. The activity peak is not due to an unrelated reaction of the tritiated tryptophan causing radioactivity to be extracted since the same peak can be shown with tryptophan-1-^{14}C. Figure 12 gives the results of experiments with both substrates performed upon the same Sephadex eluate fractions. The data used for the figures is shown in Table 5.

Inhibitor Determination

A known inhibitor of the tryptophan decarboxylating enzyme, catechol (Reed, 1968), was used to determine the type of inhibition. The lines between the points and the intercept were determined on a CDC 3300 computer. A Lineweaver-Burk plot (Figure 13) was used and the conclusion drawn that the inhibition appears to be competitive. Table 6 shows the data necessary to draw the Lineweaver-Burk plot and the pertinent data from the computer printout.

Table 7 shows the percent inhibition of the tryptophan decarboxylating enzyme with various phenolic inhibitors. The MK-485 was the first compound studied and its inhibition of the tryptophan decarboxylating enzyme led to the survey of the phenolic inhibitors (Reed and Crecelius, 1967). As can be seen from the table, phenolic compounds are very potent inhibitors of the tryptophan decarboxylating enzyme activity.

DISCUSSION

Plant peroxidases have a very broad hydrogen-donor specificity. They have been implicated in many processes, including IAA oxidation. The specific tryptophan decarboxylating enzyme found in plants (Reed and Crecelius, 1967) has several of the characteristics of IAA oxidase and therefore may also be a function of an isozyme of peroxidase. They both require oxygen, and they catalyze the decarboxylation of their respective substrates. Like peroxidase and IAA oxidase, the activity of the tryptophan decarboxylating enzyme may be precipitated only by high ammonium sulfate concentrations. This probably indicates either a low molecular weight or possibly a high carbohydrate content associated with the protein. Macnicol (1966) showed differences among the various peroxidase isozymes isolated from peas in the relative rates of guaiacol peroxidation and IAA oxidation. Sequeira and Mineo (1966) felt that horseradish peroxidase oxidation of IAA was due to a more specific component of the peroxidase. In this thesis, studies showed that the guaiacol-active peroxidase in etiolated pea seedlings was not inhibited by exposure of the seedlings to continuous light and thus differed from both the IAA oxidase activity and the tryptophan decarboxylating activity in this respect. Wiltshire (1953) felt that tryptophan oxidation was catalyzed by pea peroxidase and that

hydrogen peroxide was required for the reaction. He followed the reaction by the absorption maxima at 270 and 370 mμ, and by oxygen uptake. Although the isolation procedures for preparing homogenates for enzyme assays were similar to those presented in Wiltshire's paper, it was found in this work that the inhibition of the tryptophan decarboxylating activity follows that of IAA oxidase and not peroxidase. Also, preliminary experiments showed no detectable decarboxylation of tryptophan-1-^{14}C with horseradish peroxidase.

In 1961, Hinman, Bauman and Lang found that IAA oxidation to 3-methyleneoxindole was catalyzed by horseradish peroxidase without added hydrogen peroxide. Later, as part of their survey of reactions of indole compounds with oxidizing agents, Hinman and Lang (1965) found no reaction between peroxidase and tryptophan after 94 hours, measured spectrophotometrically.

The IAA oxidase assay which was developed by Meudt and Gaines (1967) and utilizes the p-dimethylaminocinnamaldehyde reagent is specific for the IAA oxidation products described by Hinman et al. (1961). Therefore, unlike the Salkowski assay, which measures loss of IAA, the increase in oxidation products can be directly measured.

Early work on the light inhibition of IAA oxidase present in pea seedlings was done by Hillman and Galston (1957). In contrast to the experiments presented in this thesis, they exposed etiolated

Alaska pea seedlings to red light for short time periods, then left them in the dark for various lengths of time before harvesting. The inhibition caused in this way could be reversed by dilution or by dialysis of the homogenates. In contrast, the tryptophan decarboxylating enzyme did not recover appreciable activity by dialyzing (Reed and Crecelius, 1967).

Interestingly, a study with a constant exposure of seedlings to light had not been done before with either IAA oxidase or the tryptophan decarboxylating enzyme. When etiolated seedlings were exposed to constant white light throughout the inhibitor study, the inhibition of the tryptophan decarboxylating enzyme and IAA oxidase paralleled each other very closely, both for the varieties of pea seedlings and the parts of the seedlings used (Figures 2 and 3, Table 1).

IAA oxidase (Bottomley, Galston, and Stowe, 1966) and the tryptophan decarboxylating enzyme were both inhibited by flavonoid compounds which were quercetin and kaempferol derivatives. These compounds have been characterized in peas by Furuya, Galston and Stowe (1962) and increase with exposure of the pea plants to light. In green pea plants, the petiole and tendril of the youngest leaf, and the second youngest leaf and stipule have the highest quercetin glycoside concentration. The highest kaempferol glycoside concentration is found in the second youngest leaf and stipule and the youngest

leaf and stipule. Lower in content of both compounds are the internode tissues (Bottomley, Smith and Galston, 1966).

As the flavonoid compounds inhibit IAA oxidase, they could play a role in both the control of IAA content of plants and IAA's role as a plant growth regulator. Since IAA oxidase converts IAA to oxindole type of compounds, the presence of the enzyme would inhibit growth in plants by lowering the IAA concentration. If, however, oxindoles or intermediates in their formation play a role in plant growth regulation as suggested by several workers (Meudt, 1967; Still, Fukuyama and Moyed, 1965; Tuli and Moyed, 1966), then a balance in plant growth regulation may be exerted by IAA oxidase activity. This is discussed in greater detail later in the discussion. A high concentration of the flavonoid compounds in the areas of the plant where growth is required would permit IAA to stimulate growth. The rapid inhibition of IAA oxidase in the leaf tissue of etiolated pea seedlings, for example would permit growth of the tissues needed for photosynthesis and continuance of life in the plant. The lack of inhibition in the upper stem portion of Little Marvel pea seedlings could account for the dwarf nature of the pea plant, as the uninhibited IAA oxidase would destroy IAA, preventing it from stimulating growth in the stem. Galston (1967) has shown an inverse correlation between the age of the plant tissue and the IAA oxidase activity, which affects the tissue's ability to grow.

Another school of thought (Meudt, 1967) feels that it is not IAA but IAA oxidation products which promote growth. In this case the oxidative enzymic decarboxylation of tryptophan and its parallelism to the action of IAA oxidase on indoleacetic acid could account for the studies which have shown conversion of tryptophan to IAA by bioassay (Lantican and Muir, 1967). Since tryptophan is decarboxylated but tryptamine is not the product (Reed, 1968), it has been proposed that tryptophan, like IAA, goes through oxindole intermediates in its degradation. Possibly IAA and tryptophan go through the same or very similar intermediates, accounting for the bioassay stimulation of growth for both compounds.

Valdovinos and Ernest (1966) have reported the stimulation of tryptophan decarboxylation by gibberellic acid in preparations of light-grown Little Marvel pea apical tissue. The upper stem tissue of Little Marvel pea seedlings seems to be unique among the epicotyl tissues examined in that it is not inhibited by constant exposure to light within the 30 hour period of the experiments. It seems improbable that Valdovinos and coworkers could show this work in Alaska pea seedlings, because of the complete inhibition of activity in the upper stem portions after exposure to light. They have not, as yet, characterized the indole product of tryptophan decarboxylation.

The retardation of the tryptophan decarboxylating enzyme activity on the Sephadex G-25 column could be due to the enzyme

being a very small protein. It is unlikely that there is any ionic binding to the column, considering the ionic strength of the buffer used.

Due to the unavailability of the kaempferol and quercetin glycosides, and the low solubilities of kaempferol and quercetin, it was decided to use the simplest polyphenol, catechol, for the inhibitor studies. Rabin and Klein (1956) have examined chlorogenic acid as an inhibitor of IAA oxidase and have found it to be competitive. Chlorogenic acid is a component of the quercetin glycoside and contains a diphenolic ring similar to catechol.

Since possibly both IAA oxidase and the tryptophan decarboxylating enzyme are activated by low concentration of the kaempferol glycoside, the activation observed at six to ten hours during the light inhibition studies could be due to a rise in the concentration of the kaempferol derivative (Russell and Galston, 1966). The inhibition could then possibly be due to the hydroxylation of the kaempferol derivative to make the quercetin derivative, or the increased concentration of the kaempferol derivative.

This work points to the need for more detailed work on the biosynthesis and distribution of the flavonoid compounds in plants. The entire question of the metabolic growth regulation by indoleacetic acid, or by indoleacetic acid or tryptophan oxidation products may be related to the control exerted *in vivo* by these compounds.

BIBLIOGRAPHY

Bottomley, W., H. Smith and A. W. Galston. 1966. Flavonoid complexes in Pisum sativum. III. The effect of light on the synthesis of kaempferol and quercetin complexes. Phytochemistry 5:117-123.

Furuya, M., A. W. Galston and B. B. Stowe. 1962. Isolation from peas of co-factors and inhibitors of indolyl-3-acetic acid oxidase. Nature 193:456-457.

Galston, A. W. 1967. Regulatory systems in higher plants. American Scientist 55:144-160.

Hillman, W. S. and A. W. Galston. 1957. Inductive control of indoleacetic acid oxidase activity by red and near infrared light. Plant Physiology 32:129-135.

Hinman, R. O., C. Bauman and J. Lang. 1961. The conversion of indole-3-acetic acid to 3-methyleneoxindole in the presence of peroxidase. Biochemical and Biophysical Research Communications 5:250-254.

Hinman, R. L. and J. Lang. 1965. Peroxidase-catalyzed oxidation of indole-3-acetic acid. Biochemistry 4:144-158.

Lantican, B. P. and R. M. Muir. 1967. Isolation and properties of the enzyme system forming indoleacetic acid. Plant Physiology 42:1158-1160.

Lang, C. A. 1958. Simple microdetermination of Kjeldahl nitrogen in biological materials. Analytical Chemistry 30:1692-1694.

Libbert, E. S. et al. 1966. The influence of epiphytic bacteriae on auxin metabolism. Planta 68:327-334.

Lovenberg, W., H. Weissbach and S. Udenfriend. 1962. Aromatic L-amino acid decarboxylase. Journal of Biological Chemistry 237:89-93.

Macnicol, P. K. 1966. Peroxidases of the Alaska pea. Archives of Biochemistry and Biophysics 117:347-356.

Mason, H. S. 1957. Mechanisms of oxygen metabolism. Advances in Enzymology 19:79-213.

Meudt, W. J. 1967. Studies on the oxidation of indole-3-acetic acid by peroxidase enzymes. Annals of the New York Academy of Sciences 144:118-128.

Meudt, W. J. and T. P. Gaines. 1967. Studies on the oxidation of indole-3-acetic acid by peroxidase enzymes. I. Colorimetric determination of indole-3-acetic acid oxidation products. Plant Physiology 42:1395-1399.

Moore, T. C. and C. A. Shaner. 1967. Biosynthesis of indoleacetic acid from tryptophan-^{14}C in cell-free extracts of pea shoot tips. Plant Physiology 42:1787-1796.

Morgan, P. W. 1964. Distribution of indoleacetic acid oxidase and inhibitors in light-grown cotton. Plant Physiology 39:741-746.

Rabin, R. S. and R. M. Klein. 1956. Chlorogenic acid as a competitive inhibitor of indoleacetic acid oxidase. Archives of Biochemistry and Biophysics 70:11-15.

Ray, P. M. 1958. Destruction of auxin. Annual Reviews of Plant Physiology 9:81-118.

Reed, D. J. 1968. Oxidative decarboxylation of tryptophan in peas. In: The physiology and biochemistry of plant growth substances, ed. by G. Setterfield and F. Wightman. Ottawa, Runge Press. (In press)

Reed, D. J. and J. Crecelius. 1967. Light-induced inhibition of tryptophan decarboxylation in cell-free extracts of pea epicotyls. Plant Physiology 42:1303-1306.

Russell, D. W. and A. W. Galston. 1967. Flavonoid complexes in _Pisum sativum_. IV. The effect of red light on synthesis of kaempferol complexes and on growth in sub-apical internode tissues. Phytochemistry 6:791-797.

Sequeira, L. and L. Mineo. 1966. Partial purification and kinetics of indoleacetic acid oxidase from tobacco roots. Plant Physiology 41:1200-1208.

Shannon, L. M. Professor, University of California, Dept. of Plant Biochemistry. Personal communication. Los Angeles, California. September 6, 1968.

Siegel, B. Z. and A. W. Galston. 1967. Indoleacetic acid oxidase activity of apoperoxidase. Science 157:1557-1559.

Still, C. C., T. T. Fukuyama and H. S. Moyed. 1965. Inhibitory oxidation products of indole-3-acetic acid: Mechanism of action and route of detoxification. The Journal of Biological Chemistry 240:2612-2618.

Still, C. C., C. C. Oliver and H. S. Moyed. 1965. Inhibitory oxidation products of indole-3-acetic acid: Enzymic formation and detoxification by pea seedlings. Science 149:1249-1251.

Tomaszewski, M. and K. V. Thimann. 1966. Interactions of phenolic acids, metallic ions and chelating agents on auxin-induced growth. Plant Physiology 41:1443-1454.

Tuli, V. and H. S. Moyed. 1967. Inhibitory oxidation products of indole-3-acetic acid: 3-hydroxymethyloxindole and 3-methyleneoxindole as plant metabolites. Plant Physiology 42:425-430.

Valdovinos, J. G. and L. C. Ernest. 1966. Gibberellin-enhanced CO_2 release from tryptophan-1-^{14}C in plant apical tissue. Plant Physiology 41:1551-1552.

Wiltshire, G. H. 1953. The oxidation of tryptophan in pea-seedling tissues and extracts. Biochemical Journal 55:408-417.

Winter, A. 1966. A hypothetical route for the biogenesis of IAA. Planta 71:229-239.