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THE UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

PHYSICAL AND KINETIC STUDY OF TWO ISOPEROXIDASES FROM TOBACCO SUSPENSION CULTURE WR-132 GROWN UNDER DARKNESS

A DISSERTATION

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degree of

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PHYSICAL AND KINETIC STUDY OF TWO ISOPEROXIDASES FROM TOBACCO SUSPENSION CULTURE WR-132 GROWN UNDER DARKNESS

APPROVED BY

Eddie Carol Smith

Stanley C. Nutz

Arthur E. Johnson

John A. Veltcha

Simon H. Wender

DISSERTATION COMMITTEE

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Their is no adequate word expressions to thank my parents, who sacrificed many things so that I could reach this goal.

I owe a debt to my wife, Hee Sup, for preparation of this manuscript and for her other assistance during this research.

DEDICATION

To my parents

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ABSTRACT

Two major isoperoxidases produced from *Nicotiana tabacum* tissue culture WR-132 grown in total darkness have been isolated utilizing ammonium sulfate precipitation followed by CM-cellulose chromatography and DEAE-cellulose chromatography.

The cathodic isoperoxidase, designated C_n, was eluted by 50 mM phosphate buffer, pH 6.0, from a CM-cellulose column. The anodic isoperoxidase, called A_f, was isolated by eluting stepwise a DEAE-cellulose column with 40 mM phosphate buffer, pH 6.0. Further purification of both isoperoxidases to homogeneity, as ascertained by disc electrophoresis, was achieved by Sephadex G-150 filtration.

Molecular weights of the two isoperoxidases as determined by SDS electrophoresis were 53,000 for anodic isoperoxidase A_f and 30,000 for cathodic isoperoxidase C_n. Gel filtration chromatography on Sephadex G-150 yielded values of 54,000 for isoperoxidase A_f and 28,000 for isoperoxidase C_n.

Of the two isoperoxidases, only A_f contained carbohydrate. Approximately 5 to 6% of the molecule is carbohydrate, with the hexosamine content being about 0.7% of the dry weight. Chromatography of neutral sugars after hydrolysis revealed that xylose and mannose are major monosaccharide constituents of the carbohydrate portion of isoperoxidase A_f.

Using guaiacol as a substrate, Michaelis constants were found to be 4 mM and 13 mM for isoperoxidase A_f and C_n, respectively. The pH optimum for the guaiacol oxidation of both isoperoxidases is 6.5. Effector studies

with naturally occurring phenolic compounds revealed that the two isoenzymes differ considerably in catalytic ability. The guaiacol oxidation of isoperoxidase A_f was stimulated by p-coumaric acid and scopoletin. Such an activation was not observed for isoperoxidase C_n. However, ferulic acid and caffeic acid stimulated the guaiacol oxidation of both isoenzymes. Esculetin, esculin, chlorogenic acid and catechol were found to be inhibitors. Scopolin and IAA had no effect on the guaiacol oxidation of either isoenzyme.

Substrate studies with naturally occurring phenolic compounds also verified that the two isoperoxidases A_f and C_n differ significantly in substrate preference. Relative substrate oxidation ratios for both isoenzymes revealed that A_f utilized scopoletin much more readily than any other phenolic compound tested. On the other hand, ferulic acid was a preferential substrate for isoperoxidase C_n. Scopoletin is not significantly oxidized by isoperoxidase C_n. Kinetic properties of both isoperoxidases against four naturally occurring phenolic substrates have been determined.

The peptide map of trypsin digests of isoperoxidase C_n revealed definite differences as well as a marked similarity of this isoperoxidase to other isoperoxidases normally present in tobacco tissue culture WR-132. Comparison of peptide patterns of isoperoxidase A_f and C_n indicate that many peptides of both isoperoxidases overlap each other, even though the two isoenzymes have quite different electrophoretic mobilities. Although the tryptic peptide pattern of isoperoxidase A_f appears to be identical with that of isoperoxidase A_3 from tobacco callus culture W-38, none of the other isoperoxidases studied from tobacco suspension culture WR-132 and tobacco callus culture W-38 is identical with any other one with a different letter or number designation.

The electrophoretic mobilities of most of the new isoperoxidases produced from tobacco suspension culture WR-132 grown in total darkness are the same as those of the isoperoxidases normally present in tobacco callus culture W-38.

Since tobacco callus culture W-38 contains a whole array of isoperoxidases, it is proposed that a derepression of certain isoperoxidases occurs when tobacco suspension cultures WR-132 are transferred into total darkness. The derepression of isoperoxidase A_f in total darkness was indicated by comparing the physico-chemical properties and fingerprinting pattern of isoperoxidase A_f from WR-132 in the dark and isoperoxidase A_3 from tobacco callus culture W-38, and finding them to be identical.

CHAPTER I

INTRODUCTION

Peroxidase (EC. 1.11.1.7) is an enzyme which has been implicated in the regulation of cell growth and differentiation in a large number of different plants (1,2). Since the enzyme does not exhibit absolute specificity, numerous catalytic functions have been reported for peroxidase in higher plants. These include the oxidation of indole-3-acetic aicd (IAA) (3); the oxidation of reduced pyridine nucleotides (4); the conversion of methional to ethylene (5); the oxidation of chalcones (6); the oxidation of pyridoxal related compounds (7); the oxidation of some phenolic compounds (8,9,10,11); and lignification (12).

The existence of multiple forms of peroxidase has been reported for many plants (13). In order to determine the preferred role of individual isoperoxidases in plant metabolism and the physiological reasons for existence of multiple forms of peroxidase, individual isoperoxidases from many different plants have been isolated and characterized (14,15,16,17,18,19, 20). The study of single isoperoxidases has been extended to the determination of amino acid sequence (21). Such studies with individual isoperoxidases indicate that physical, kinetic properties and substrate preferences of isoperoxidases from a single source may vary significantly.

Changes in the peroxidase patterns, as visualized by electrophoresis

or an increase in activity, occur during aging (22), after application of growth regulators (23,24,25), and after wounding and infection (26,27) in plants and plant tissue cultures. Although there is no consistent effect yet apparent on the peroxidase pattern or on increase in activity due to changing situations, possible physiological roles have been suggested for some of the individual isoperoxidases based on such studies (23,27).

Induction and repression of peroxidase isoenzymes by IAA in tobacco pith tissues cultured *in vitro* have long been known (28). IAA at low concentrations in tobacco callus culture induced two fast-migrating IAA oxidase isoenzymes, but at high concentrations it increased the level of other IAA oxidases with low and moderate electrophoretic mobilities, according to Lee (23). The IAA oxidase isoenzyme fractions obtained in Lee's study all possessed peroxidase activity. Furthermore, the development of the two fast-migrating IAA oxidases was associated with a tumor type growth. Depending upon concentrations used, kinetin, gibberellic acid and other synthetic auxins also affected the development of fast-migrating IAA oxidases (23,24, 25). Lee (29) later found that the relative IAA oxidase activity per unit peroxidase activity varied with the subcellular localization, and high concentration of kinetin increased the relative IAA oxidase activity.

Variations in isoperoxidase patterns are not confined to the effect of plant growth regulators described above. Tobacco callus cultures grown on low calcium ion concentrations in the medium have been shown to contain increased amounts of two slowly migrating anodic isoperoxidases (called A_1 and A_2) and all cathodic isoperoxidases (30). However, the amount of a rapidly migrating anodic isoperoxidase (designated A_3) was unchanged at all levels of calcium ion concentration. Since calcium ion concentration is directly related to the amount of lignification, differential involvement

of these isoperoxidases in lignification was speculated. Further study by Schafer et al. (31) demonstrated a stimulatory effect of scopoletin on the guaiacol oxidizing activity of isoperoxidase A_3 and none on another isoperoxidase, A_1, from the same source. Continuing studies on isoperoxidase A_3 by Reigh et al. (10) have verified that scopoletin is actually a substrate for this isoperoxidase. On the other hand, ferulic acid appeared to be a preferential substrate for isoperoxidases A_1 and A_2, and for two other cathodic isoperoxidases (11,18).

From these studies, it seems that different peroxidase isoenzymes may have different physiological functions *in vivo* depending upon cellular location or substrate specificities against different physiological substrates.

Relatively little is known about the effect of light or darkness, and temperature on the isoperoxidase activity of plants and plant tissue cultures. Penel and Greppin (32) found that after transfer to continuous light (4000 lux) of short-day (8 hour during 4 to 8 weeks) grown spinach plants, the activity of acidic peroxidase was enhanced, whereas that of the basic peroxidase was lowered. There was also some alteration in the isoperoxidase composition. De Jong et al. (33) found that the major isoperoxidases released into the medium by tobacco suspension tissue culture WR-132 varied with the temperature maintained for growth. They further reported that the culture temperature also influenced the isoperoxidase pattern of the cells. McCown et al. (34), on the other hand, have reported that isoperoxidase patterns of *Dianthus* callus cultures were not influenced qualitatively by growth conditions involving four combinations of environments: dark and light, warm and cold. The study of Leu et al. (35), however, firmly demonstrated that the amount of illumination has a definite influence on the

appearance of certain isoperoxidases both in the media and in the cells. The effect was more drastic in cultures grown under complete darkness than in those grown under intense light.

Legrand and coworkers (36,37) have studied the effect of darkness and light on the peroxidase activity and phenolic compounds content in *Cichorium intybus* L. leaf tissues cultured *in vitro*. Although the isoperoxidase pattern is the same in both illuminated and non-illuminated tissues, the peroxidase level increased in the dark-grown *Cichorium* leaf as compared with that in the light-grown leaf. However, the opposite was the case for the content of phenolics. The increase in peroxidase activity and the decrease in phenolic compounds have also been observed when tissues cultured previously under continous illumination are placed in darkness. Furthermore, a considerable reduction in growth was observed when *Cichorium* cultures were grown in darkness as compared to the cultures grown under continuous illumination (1600 lux). Since peroxidized caffeic acid or chlorogenic acid, which are naturally present in *Cichorium*, inhibited guaiacol oxidation by horseradish peroxidase, they proposed the light-dark control of peroxidase activity in *Cichorium* through the inverse variation of endogenous phenolics.

Thus, there appears more than a casual relationship among the degree of illumination, production or activity of isoperoxidases, phenolic compounds and plant growth. In the present study, two major isoperoxidases produced from *Nicotiana tabacum* tissue culture WR-132 grown in total darkness have been isolated. The kinetic and physical properties of both isoenzymes were compared to the other isoperoxidases normally present in tobacco tissue culture to ascertain what effect darkness has upon the production or function of the new isoperoxidases.

CHAPTER II

MATERIALS AND METHODS

WR-132 Tobacco Suspension Culture

The source material for the isoenzymes of peroxidase used in this work was a cell line of tobacco suspension culture WR-132 (*Nicotiana tabacum* L., var. Xanthi). The cell line was obtained from Dr. A. C. Olson, U. S. Department of Agriculture, Albany, California. The culture medium (Table I) was that of Murashige and Skoog (38) except that 2,4-dichlorophenoxyacetic acid was used instead of indole-3-acetic acid, and kinetin was deleted. Two g of wet cells of stock culture were aseptically transferred, in a laminar flow hood (Agnew-Higgins), into a 125 ml Erlenmeyer flask containing 50 ml of culture medium. Growth was accomplished in constant agitation on a reciprocal shaker (70 rpm). After growing the cells at room temperature under weak continous illumination (about 1 foot candle), they were transferred and grown in complete darkness at 21°C in a growth chamber.

Electrophoresis

A) Starch Gel Electrophoresis

Starch gel electrophoresis was performed using a modified Smithies apparatus (39). The gel was 11% starch (Electrostarch Co.) in 5 mM histidine (pH 7.0). Electrophoresis was accomplished at a constant voltage of 400 volts for approximately 4 hours in a cold room, using 0.41 M

TABLE I

REVISED WR-132 CULTURE MEDIUM

Compound	mg/l	Compound	mg/l
Sucrose	3000	KH_2PO_4	170
myo-Inositol	100	Na_2EDTA	37.3
Glycine	20	$FeSO_4 \cdot 7H_2O$	27.8
Thiamine·HCl	0.1	H_3BO_3	6.2
Niacin	0.5	$MnSO_4 \cdot 4H_2O$	22.3
Pyridoxine·HCl	0.5	$ZnSO_4 \cdot 4H_2O$	8.6
2,4-Dichlorophenoxy-acetic acid	0.5	KI	0.83
NH_4NO_3	1650	$Na_2MoO_4 \cdot 2H_2O$	0.25
KNO_3	1900	$CuSO_4 \cdot 5H_2O$	0.025
$CaCl_2 \cdot 2H_2O$	440	$CoCl_2 \cdot 6H_2O$	0.025
$MgSO_4 \cdot 7H_2O$	370		

sodium citrate (pH 7.0) as a bridge buffer. Peroxidase bands were visualized by placing the gels in a solution of 100 mg 3-amino-9-ethyl carbazole in 10 ml of dimethylformamide, 185 ml of 50 mM acetate buffer (pH 5.0), 10 ml of 100 mM $CaCl_2$ and 0.2 ml of 30% H_2O_2 (40).

B) Anodic Disc Gel Electrophoresis

Anodic disc gel electrophoresis was performed using a Buchler Polyanalyst apparatus according to the method of Ornstein and Davis (41). Gels were 7.5% acrylamide and 0.2% N,N'-methylene bisacrylamide. The running pH was 9.3 with bromphenol blue as the tracking dye. After completion of electrophoresis, peroxidase bands were visualized by placing

gels in a mixture of 2 parts 1% guaiacol, 2 parts 50 mM sodium phosphate buffer (pH 6.0) and 1 part 0.2% H_2O_2. Protein staining was done using 0.25% wt/vol Coomassie Brilliant Blue in 45% (v/v) methanol in 9% (v/v) acetic acid for 4-5 hours, according to the procedure of Weber and coworkers (42).

C) Cathodic Disc Gel Electrophoresis

Cathodic disc gel electrophoresis was performed in the same manner as anodic electrophoresis except that the electrical current was reversed. Potassium persulfate was used as the gel polymerization catalyst. The running pH was 4.3 with methyl green used as the tracking dye.

Peroxidase Assays

Guaiacol

Peroxidase assays with respect to guaiacol as substrate were accomplished using a procedure based upon that of Lance (43) which was modified by Schafer (30). Assays contained guaiacol, 5 mM H_2O_2 and 40 mM phosphate buffer (pH 6.5). The reaction was initiated by the addition of 0.1 ml of enzyme, and velocity of the reaction was monitored by measuring the increase in absorbance at 470 nm. Guaiacol concentration was adjusted to Km values of each isoperoxidase in effector studies.

Scopoletin

A typical assay solution contained 1.2 mM scopoletin, 5 mM H_2O_2 and 40 mM sodium citrate buffer (pH 5.0). Assays were initiated by adding 0.1 ml of enzyme preparation and the increase in absorbance of oxidation product(s) at 450 nm was recorded (44).

Ferulic Acid

The peroxidase catalyzed oxidation of ferulic acid was monitored by measuring the disappearance of ferulic acid absorbance at 320 nm. A typical assay contained 0.2 mM ferulic acid, 5 mM H_2O_2, and 40 mM sodium citrate

buffer (pH 5.0). The assay was initiated by addition of enzyme (45,46).

Esculetin

A typical assay contained 1.2 mM esculetin, 5 mM H_2O_2 and 40 mM phosphate buffer (pH 7.5). Assays were initiated by adding enzyme preparation, and increase in absorbance at 469 nm was recorded (44).

Chlorogenic Acid

Chlorogenic acid assays were done by monitoring the absorbance of product(s) at 400 nm. An assay solution contained 1.2 mM chlorogenic acid, 5 mM H_2O_2 and 40 mM citrate buffer (pH 5.0). The reaction was initiated by the addition of 0.1 ml of enzyme preparation (45).

All enzyme assays and absorption spectra utilized a Varian Techtron UV-Visible Model 635 spectrophotometer equipped with a two pen recorder and automatic cell programmer.

Protein Determination

Protein concentrations were determined by the method of Lowry et al. (47).

Chemicals

Chemical	Source
Guaiacol	Eastman Chemical Co.
Scopoletin	Sigma Chemical Co.
Ferulic acid	Aldrich Chemical Co.
Esculetin	Fluka AG
Chlorogenic acid	Fluka AG
Catechol	Fluka AG
para-Coumaric acid	Calbiochem
Caffeic acid	Calbiochem

(cont'd)

Chemical	Source
IAA	Sigma Chemical Co.
Esculin	Nutritional Biochemical Co.
Starch	Electrostarch Co.
DEAE-cellulose	Reeve Angel Corp.
CM-cellulose	Reeve Angel Corp.
Sephadex G-150	Pharmacia Fine Chemicals Incorp.
Polyclar AT	GAF Corp., Chemical Division

Trans-cinammic acid and scopolin had previously been synthesized in this laboratory. Standard enzymes which were obtained from Worthington Biochemical Co. are: creatine kinase and β-galactosidase. Enzymes which were obtained from Sigma Chemical Co. are: lipoxidase, egg albumin, α-chymotrypsinogen, serum albumin and trypsin. All chemicals used in carbohydrate determination were obtained from the Sigma Chemical Company.

CHAPTER III

ISOPEROXIDASE PATTERN OF WR-132 TOBACCO CULTURE GROWN IN TOTAL DARKNESS

Starch gel electrophoresis of the crude extracts of WR-132 tobacco cells grown under dim light (10 lux) indicated that no anodic isoperoxidases were present in the cell extracts. However, one anodic band appeared in the growth medium. Four major cathodic isoperoxidase bands from tissue extracts and three cathodic bands from the medium were observed, while another isoperoxidase, designated C_a, also appeared frequently in the cell extracts of normally grown cultures. These cathodic peroxidases were similar in mobilities to those reported by Stafford and Galston (2). The mobilities of these isoperoxidases are shown in Fig. 1 and 2.

Preparation of Media and Cell Extracts

WR-132 culture cells were harvested every 10 days using a preweighed glass tube containing a sintered glass filter. The media were used directly for starch gel electrophoresis. Approximately 50 grams of cells were washed thoroughly with 50 mM phosphate buffer (pH 6.0) and homogenized in a Sorvall Omnimixer at 6,000 rpm for 10 min. with 100 ml of 50 mM phosphate buffer (pH 6.0), 50 g of glass beads, and 30 g of washed, hydrated polyclar AT. The homogenates were centrifuged for 20 min at 27,000 X g (Sorvall RC 2-B centrifuge), and the resulting supernatant was

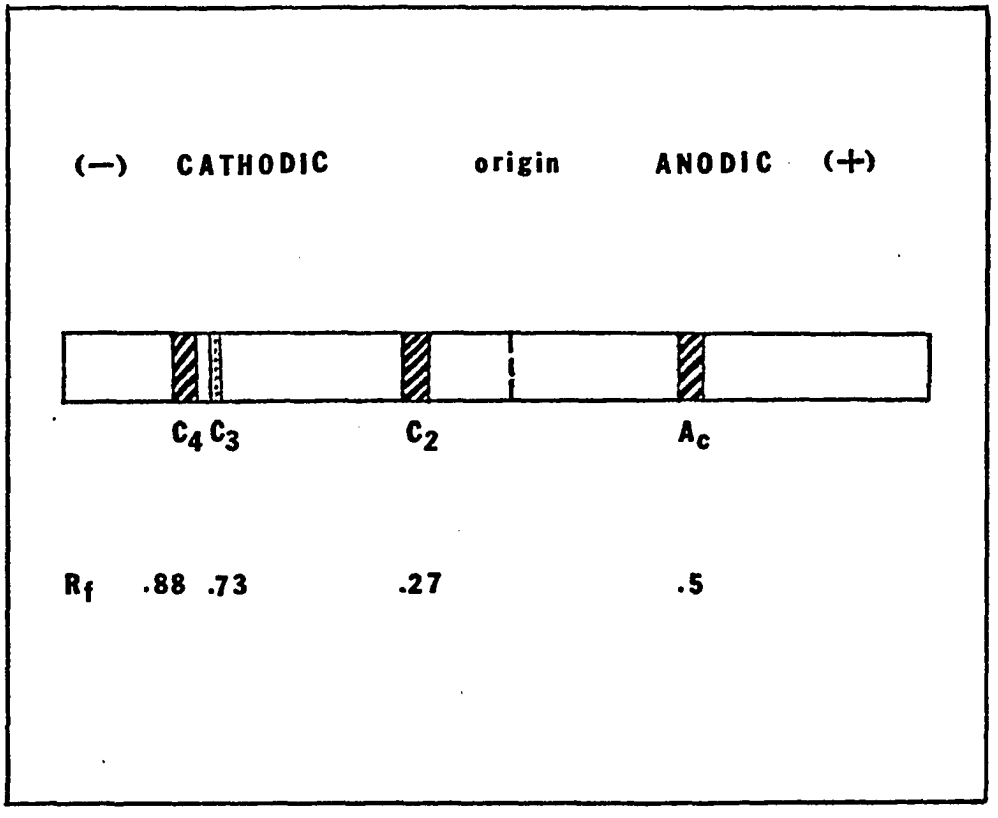


Figure 1. STARCH GEL ELECTROPHORETIC PATTERN AT pH 7.0 OF ISOPEROXIDASES FROM MEDIA OF TOBACCO SUSPENSION CULTURE WR-132.

strong weak

Intensity of isoperoxidase bands as visualized by 3-amino-9-ethyl carbazole.
One hundred fifty µl of media was placed on the origin.

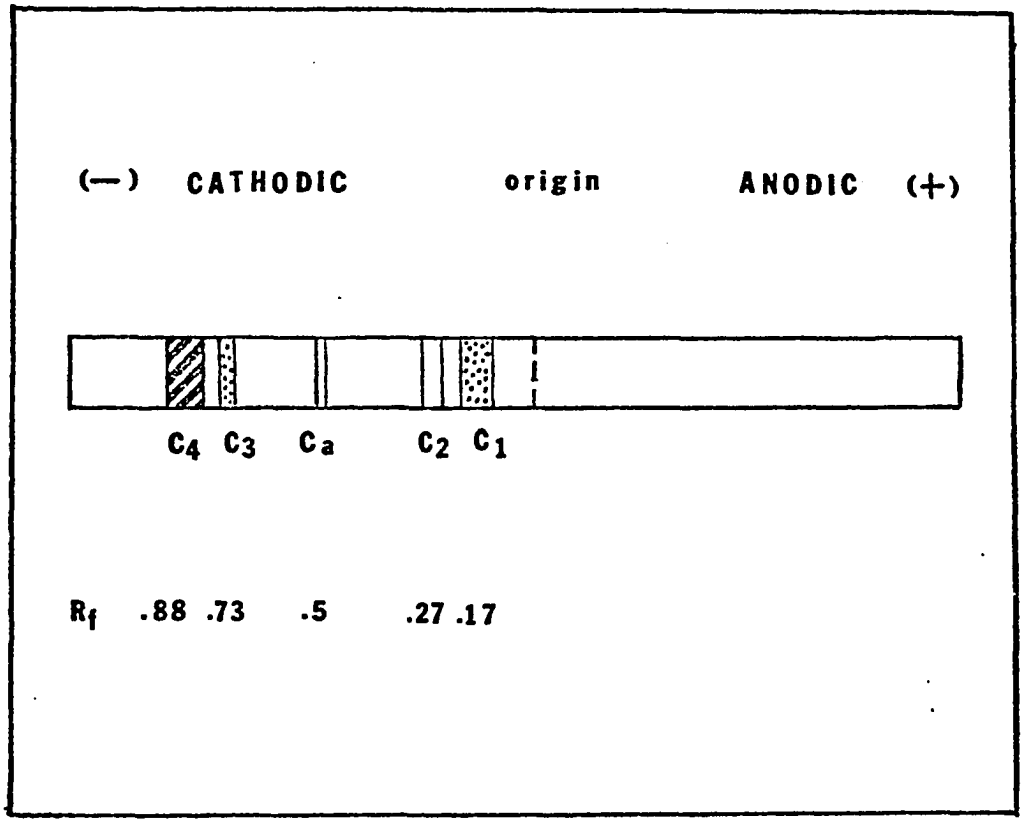


Figure 2. STARCH GEL ELECTROPHORETIC PATTERN AT pH 7.0 OF ISOPEROXIDASES FROM CELL EXTRACTS OF TOBACCO SUSPENSION CULTURE WR-132.

strong weak very weak

Intensity of isoperoxidase bands as visualized by 3-amino-9-ethyl carbazole.
One hundred fifty µl of cell extracts, as prepared according to page 10, was placed on the origin.

retained for further analysis.

Isoperoxidase Patterns of Media of WR-132 Cultures Grown In Total Darkness

In order to understand more about the effect of darkness on the production of the isoperoxidases in WR-132 suspension cells, 10 passages (10 days growth per passage) of these cells were grown in complete darkness. The resulting isoperoxidase patterns of the cell extracts and media were analyzed and compared to the isoperoxidase patterns of those grown under dim light conditions. Since crude extracts of tobacco callus culture W-38 when subjected to starch gel electrophoresis exhibit a wide range of anodic and cathodic isoperoxidases (2), that pattern was used as the standard designation for the isoperoxidase bands (Fig. 3). Similar to those cells under usual growth conditions in 10 lux, the cells grown in darkness were harvested at 10 day interval, and the peroxidases in the media were analyzed by starch gel electrophoresis. In the medium of the dark grown cells, there were qualitative and quantitative changes in the isoperoxidase patterns. As soon as the cells were transferred from dim light to complete darkness, a new anodic isoperoxidase, designated A_f, appeared in the media. The mobility of this isoperoxidase is the same as that of isoperoxidase A_3 from tobacco callus culture W-38 (Fig. 3). As will be shown in later pages, A_f seems to be identical with the A_3 from W-38, and so A_3 will be used also for A_f from WR-132 grown in darkness. The intensity of the isoperoxidase $A_f(A_3)$ increased to a maximum about third passage, and then decreased after that. In addition to the isoperoxidase $A_f(A_3)$, other anodic isoperoxidases A_a, A_b, and A_d appeared during the 3rd, 4th, and 5th passages in darkness (Table 2). The mobility of A_a and A_b were comparable to the slowest migrating undesignated isoperoxidase and

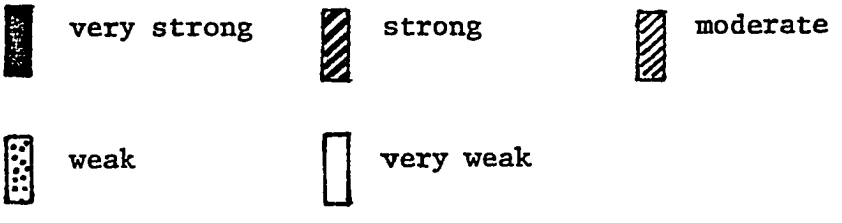
Figure 3. COMPARISON OF ISOPEROXIDASE PATTERNS BETWEEN TOBACCO CALLUS CULTURE W-38 AND TOBACCO SUSPENSION CULTURE WR-132.

(a). Cell extracts of W-38 callus culture.

(b). Fifth passage medium of WR-132 suspension culture grown in total darkness.

(c). Fifth passage cell extracts of WR-132 suspension culture grown in total darkness.

Intensity of isoperoxidase bands as visualized by 3-amino-9-ethyl carbazole.



One hundred fifty µl of media or cell extracts, as prepared according to page 10, were placed on the origin.

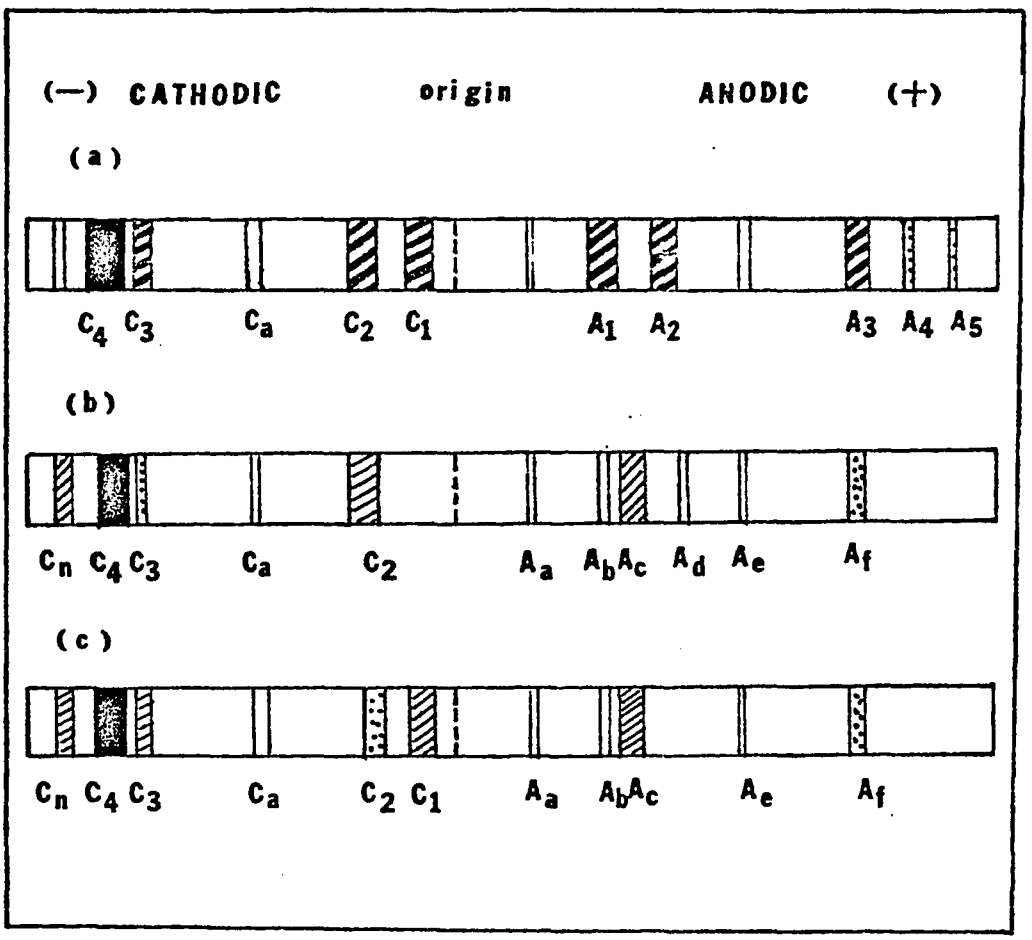

(—) CATHODIC
origin
ANODIC (+)
(a)
C_4 C_3 C_a C_2 C_1 A_1 A_2 A_3 A_4 A_5
(b)
C_n C_4 C_3 C_a C_2 A_a A_b A_c A_d A_e A_f
(c)
C_n C_4 C_3 C_a C_2 C_1 A_a A_b A_c A_e A_f

TABLE 2

ISOPEROXIDASES IN THE MEDIA OF DARK CELLS

	Relative intensity of staining*										
	Isoperoxidase										
	C_n	C_4	C_3	C_a	C_2	A_a	A_b	A_c	A_d	A_e	A_f
	Relative mobility**										
Age (10 days) passage no.	1.0	0.88	0.73	0.5	0.27	0.25	0.4	0.5	0.61	0.81	1.2
Normal	-	H	W	-	H	-	-	H	-	-	-
Dark I	-	H	W	-	H	-	-	H	-	-	W
Dark II	-	H	W	-	H	-	-	VH	-	-	W
Dark III	-	H	W	-	H	-	-	H	VW	-	M
Dark IV	W	VH	W	-	M	-	-	H	VW	-	W
Dark V	M	VH	W	VW	M	VW	VW	M	VW	VW	W
Dark VI	W	H	W	VW	M	-	-	M	-	-	W
Dark VII	VW	H	W	-	M	-	-	M	-	-	-
Dark VIII	-	M	W	-	M	-	-	W	-	-	-
Dark IX	-	M	W	-	M	-	-	W	-	-	-
Dark X	-	M	W	-	M	-	-	W	-	-	-

Cells were grown for 10 days in weak light (10 lux), and then grown for 10 passages of 10 days each in the dark. Cells were harvested at 10 days interval, and the peroxidases in the media were analyzed by starch gel electrophoresis.

* Relative intensities, using 3-amino-9-ethyl carbazole as substrate, are given as; VH = very heavy, H = heavy, M = moderate, W = weak, VW = very weak, and - = not detected.

** Relative mobility of each peroxidase was calculated by dividing the distance from the origin traveled by isoperoxidase C_n into the distance traveled from the origin by the isoperoxidase named.

isoperoxidase A_1 respectively of callus culture W-38. The mobility of A_d, however, was not comparable to any isoperoxidase present in callus culture W-38, although the mobility was similar to isoperoxidase A_2 of tobacco callus culture W-38 (Fig. 3). A weakly staining isoperoxidase A_e was visible at the 5th passage. The mobility of A_e appears the same as an undesignated isoperoxidase present in W-38 crude extracts (Fig. 3). A new cathodic isoperoxidase, designated C_n, which migrated more rapidly toward the cathode than any other cathodic isoperoxidases, appeared as growth progressed to the 4th passage. This level became maximal at the 10th day of the 4th and 5th passages. Isoperoxidase C_n could also be found in W-38 crude extracts, although the intensity of C_n was extremely weak when compared to other isoperoxidases present in that culture. After the 6th passage, the staining intensity of C_n decreased, and it eventually disappeared after the 7th passage (Table 2). As far as the other cathodic isoperoxidase bands are concerned, the amount of C_4 increased at the 4th and 5th passages, and then decreased after the 6th passage. Isoperoxidase C_2 remained constant at an elevated level during the first four passages, but decreased after that. Isoperoxidase C_3 maintained a relatively constant level during all the passages tested. The anodic isoperoxidase A_c level remained constant during the first three passages in the dark and then decreased, beginning with the 5th passage. The mobility of A_c appears to be different from isoperoxidases A_1 and A_2 of tobacco callus culture W-38. These results are summarized in Table 2.

Leu (48) reported that isoperoxidase C_n appeared in the medium within 2 days after the cells were placed in the dark. She also reported that weakly staining anodic isoperoxidase A_f appeared in the media of the third and fourth passages of dark cells, but did not appear in the non-growing

fifth passages. In the present study, the isoperoxidase C_n appeared only at the 4th and 5th passages in the medium and cells grown in the darkness. An anodic isoperoxidase A_f, however, appeared, beginning with the first passage and continuing up to the sixth passage. The decrease in intensity of C_2 as growth progressed during the 4th passage in total darkness was not comparable to the results of Leu who observed the decrease of C_2 beginning with the 2nd passage in the total darkness. However, the appearance of the anodic isoperoxidases A_a, A_b, A_d and A_e during the 5th passage agrees well with the results of Leu.

Isoperoxidase Patterns of Cell Extracts of WR-132 Culture Grown In Total Darkness

The peroxidase isozyme patterns of the cell extracts of WR-132 tissue grown in total darkness are shown in Table 3. No differences in staining of cathodic isoperoxidase C_2, C_a, C_3, and C_4 could be seen in the first three passages of dark grown cells and the normal grown cells. However, the new cathodic isoperoxidase C_n appeared at the 4th and 5th passage of the dark grown cells. This cathodic isoperoxidase has the same mobility as the one found in the media of dark grown cells. Another new cathodic C_b, which has a electrophoretic mobility between C_a and C_3, also appeared at the 4th passage, and remained until the 6th passage. This isoperoxidase is usually absent in extracts of cell grown under normal conditions. The other cathodic isoperoxidases C_1, C_3, and C_4 from the dark grown cells increased during the 4th and 5th passages. The intensity of these isoperoxidases decreased as the growth continued above the 6th passage and remained at the same level as in the normally grown cell. Isoperoxidase C_2 was not detected in the first and second passages of dark grown cell extracts, but reappeared at the 3rd passage at levels comparable to normally

TABLE 3

ISOPEROXIDASES IN EXTRACTS OF DARK CELLS

	Relative intensity of staining*										
	Isoperoxidase										
	C_n	C_4	C_3	C_b	C_a	C_2	C_1	A_a	A_b	A_c	A_f
	Relative mobility**										
Age (10 days) passage no.	1.0	0.88	0.73	0.62	0.5	0.27	0.17	0.25	0.4	0.5	1.2
Normal	-	H	W	-	VW	VW	W	-	-	-	-
Dark I	-	H	W	-	VW	-	W	-	-	-	-
Dark II	-	H	W	-	VW	-	W	-	-	-	VW
Dark III	-	H	W	-	W	VW	W	-	-	-	VW
Dark IV	VW	VH	W	VW	M	VW	W	VW	VW	W	VW
Dark V	M	VH	M	VW	M	W	M	VW	W	M	-
Dark VI	-	H	M	VW	W	VW	M	-	VW	W	-
Dark VII	-	H	M	-	W	-	W	-	-	VW	-
Dark VIII	-	H	M	-	W	-	W	-	-	VW	-
Dark IX	-	H	M	-	W	-	W	-	-	-	-
Dark X	-	H	M	-	W	-	W	-	-	-	-

Cells were grown for 10 days in weak light (10 lux) and then grown for 10 passages of 10 days each in the dark. Cells were harvested at 10 days interval, and the peroxidases in the cell extracts were separated by starch gel electrophoresis.

* Relative intensities, using 3-amino-9-ethyl carbazole as substrate, are given as; VH = very heavy, H = heavy, M = moderate, W = weak, VW = very weak, and - = not detected.

** Relative mobility of each peroxidase was calculated by dividing the distance from the origin traveled by isoperoxidase C_n into the distance traveled from the origin by the isoperoxidase named.

grown cells and then remained at that level until the 6th passage. This C_2 level decreased again after the 6th passage and was absent in later stages.

No anodic isoperoxidases appeared in the first three passages of cell extracts grown in the dark except isoperoxidase A_f which appeared beginning with the 2nd passage. However, three anodics A_a, A_b and A_c appeared in the cell extracts of dark cells around the 4th passage. The staining intensity of these anodic isoperoxidase increased at the 4th and 5th passages and then gradually decreased after the 5th passage. A weakly staining intensity A_e was also occasionally visible at the 5th passage. Changes in the amounts of the isoperoxidases with passage, as estimated from staining intensities, are indicated in Table 3. The isoperoxidase pattern of dark cells in this study was also not completely comparable to the results of Leu (48). Although the appearance of isoperoxidase C_n, A_a, A_b and A_e in the dark grown cell extracts of the 4th and 5th passages agrees well with the results of Leu, two additional isoperoxidases C_b and A_c appeared in the cell extracts beginning with the 4th passage in the present study. The appearance of isoperoxidase A_f was also not observed by Leu.

Cells Grown In Darkness

Cells grown under 10 lux intensity of light (normal condition) were bright yellow and the medium was greenish-yellow in color. For cells grown in total darkness these same colors were observed for the first three passages. However, the color of the cells changed to grayish white, and the medium turned to light greenish-yellow beginning with the 4th passage in the darkness. These observations are in contrast to those of Leu(48) who reported that the color of the dark cells changed to brown, and the

medium turned to light brown after day 8 of the fourth passage. In this study, cells and medium remained the same during the 5th passage as they had been in the 4th passage. After the 6th passage, the color of the cells and medium gradually returned to almost normal colors.

The fresh weights of the cells grown in the dark is shown in Fig. 4. The fresh weight (g/flask) of the 10 day grown cells of normal condition was usually about 13 grams. When grown in the dark, the fresh weights of the cells decreased and became minimal (around 7 to 8 grams/flask) at the 5th passage. From the 6th passage on, the fresh weights of the dark grown cells began to increase. However, the fresh weights of cells in all 10 passages in darkness were less than those of cells grown in 10 lux.

DISCUSSION

The results of the present study demonstrate that the amount of illumination has a definite influence on the physiological events relating to the production of isoperoxidases, although the cessation of growth during the fifth passage of dark cells observed by Leu (48) could not be confirmed. The cessation of growth found by Leu during the fifth passage of dark-grown cells indicated the necessity of light for the continued growth of those cells. In the present study, considerable reduction of growth under darkness was demonstrated as indicated in Fig. 4. The fresh weight (7-8 g/flask) of the 5th passage cells grown in darkness was approximately half of that of normal cells (13-14 g/flask). It was also noted that the fresh weight of the 5th passage dark cells in the present study corresponds to that of the 4th passage dark cells reported by Leu (48). It is possible that the discrepancy between the two studies could be due to the difference in age of two cultures used in each study since the tobacco suspension culture WR-132

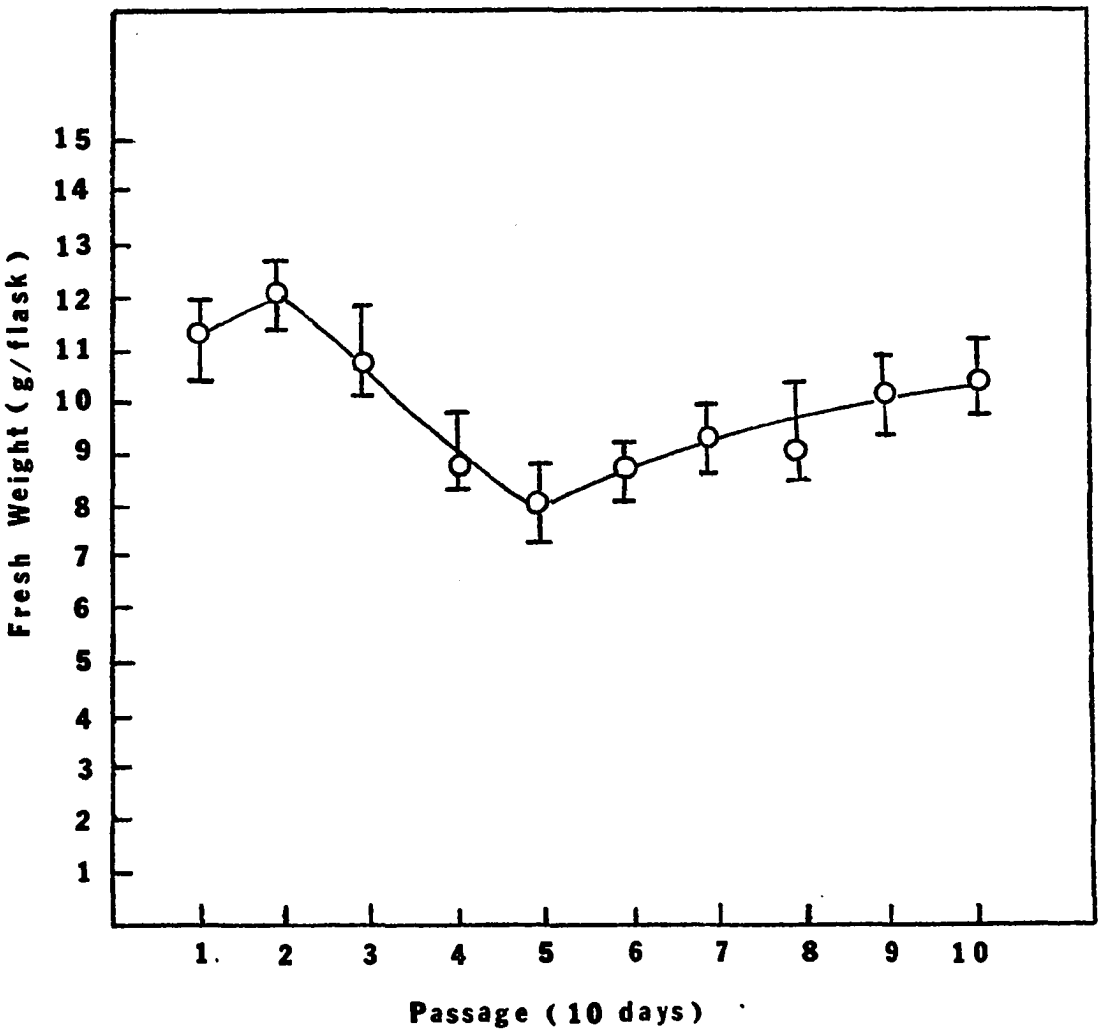


Figure 4. FRESH WEIGHTS OF TOBACCO CELLS GROWN UNDER DARKNESS.

Deviations from mean value are shown.

Cells grown under normal (dim light) condition weigh about 13-14 g/flask.

employed in the present study was obtained more recently from Dr. Olson and was young and vigorous, whereas the cells used by Leu were much older and were obtained several years earlier from Dr. Olson.

De Jong et al. (33) found that the major isoperoxidases released into the medium by WR-132 tobacco suspension cells varied with culture temperature. They also found little correlation between the excreted isoperoxidases and the intracellular ones at 13°C. On the other hand, Legrand (37) reported that the isoperoxidase pattern was the same in both illuminated and non-illuminated tissue of *Cichorium* culture. The results of Legrand, however, indicated an increase in peroxidase activity and an increase in isoperoxidase band A_1 in the dark-grown *Cichorium* culture when compared to the one grown under continuous illumination (1600 lux). This is in contrast to the results of the present study, since both quantitative and qualitative changes in the isoperoxidase pattern were observed in the medium and cells of dark-grown tobacco cultures.

The fact that most new isoperoxidases released into the medium of the dark cells were also present in the crude extracts of dark cells at approximately the same passage may possibly indicate an alteration of the protein synthesizing mechanism rather than a protein releasing mechanism when the cells are transferred to the dark.

Perhaps the most striking observation made in this study is that when WR-132 tobacco cultures reach the fourth and fifth passage in total darkness, the isoperoxidase pattern of the medium and cells becomes similar to that of W-38 callus tobacco culture. The isoperoxidase pattern of the medium of dark cells when compared to that of an extract of W-38 cells, as obtained by starch gel electrophoresis, showed identical mobilities of isoperoxidase A_b and A_f to those of isoperoxidase A_1 and A_3 respectively (Fig. 3).

Furthermore, isoperoxidases which have the same mobilities as isoperoxidase C_n, A_a and A_e of the medium of dark-grown cells were also present in the cell extracts of W-38 callus culture. The only exception was the appearance of A_d in the medium at the fifth passage of dark-grown cells. This isoperoxidase was absent in the cell extracts of W-38 tobacco tissue culture. These observations indicate that derepression of isoperoxidases occur when WR-132 tobacco suspension cultures are transferred to complete darkness. The transitory appearance of the new isoperoxidases at the 4th and 5th passages of dark-grown cells to normal color after the 6th passage probably indicate that there are two cell types present at the 4th and 5th passages: those that turn color, and those that don't. The ones which turn color also produce new isoperoxidases and then die, leaving the other (normal-colored) cells to continue after the 6th passage. This view is also consistent with Fig. 4 and the results of Leu (48), which were described earlier in discussion section.

Legrand et al. (36) have reported that the increase of peroxidase activity in the dark-grown *Cichorium* leaf as compared to the light-grown is probably due to the light-dark control of peroxidase activity through inverse variation of endogenous phenolic compounds. This proposal was based on the fact that phenolic extracts of the tissues inhibited the guaiacol oxidation of peroxidases. Since the isoperoxidase pattern, observed by Legrand (37), was the same in both illuminated and non-illuminated *Cichorium* leaf, the derepression of tobacco isoperoxidases in the present study, however, may not be explained by a simple decrease in the phenolic compounds present in the dark-grown cells.

Several other workers reported variations in the peroxidase pattern and/or activity when plants or plant tissue cultures were subjected to various stress condition such as aging (22), wounding or infection (26,27), gamma-irradiation (49), or mineral deficiency (50). However, none of the changes

so far has been linked conclusively with any metabolic processes in which peroxidase has been implicated, such as oxidation of IAA, oxidation of phenolic compounds and lignification.

The involvement of peroxidase in plant resistance to pathogens has not been settled. Vance et al. (27) have reported that attempted penetration of *Helminthosporium avenae* into leaf discs of a non-compatible host, reed canarygrass, was accompanied by an increase in peroxidase activity, in particular in the walls at the penetration sites, and in wall lignification. The authors have suggested that the resistance mechanism involves an induction of three cathodic isoperoxidases in the challenged tissue. Birecka et al. (26), however, suggested that infection-induced peroxidase-enhancement may result from a non-specific response to injury.

The increased peroxidase activity in slowly growing tissues (51) and dwarf (52) plants suggests that peroxidase may be a growth-inhibiting factor in the plant. Since some peroxidases have been known to degrade IAA (3), IAA content may be reduced to a subnormal level by a high IAA oxidase activity.

Palmieri et al. (53) found that two mutants of tomato, *Lycopersicon esculentum* Mill, contained abnormally high amounts of peroxidase-IAA oxidase activity. The reduction in length and weight of mutant organs was associated with high peroxidase-IAA oxidase activities and with the derepression of the root-specific band C_2. They suggest, therefore, that the two tomato mutants, *olivacea* and *monstrosa*, are examples of auxin-related mutations controlling the synthesis of a group of proteins related in function.

Whatever the mechanism of induction, repression and derepression of peroxidases are, it likely will be essential to isolate and study the physicochemical, catalytic properties of individual isoperoxidase since the effect of environmental stress conditions on peroxidase pattern and activity is obviously quite complex.

CHAPTER IV

ISOLATION AND PHYSICAL PROPERTIES OF ISOPEROXIDASE A_f AND C_n

Although the physiological significance of multiple forms of peroxidase remains largely unknown, the findings of numerous workers indicate the probable importance of peroxidases in plant tissue metabolism. In order to determine the metabolic role of peroxidases and physiological reasons for the existence of multiple forms of peroxidase, the individual isoperoxidases have been isolated and their kinetic and physical properties have been studied (Chapter I). Kinetic and physical properties of different isoperoxidases isolated from a single source showed both marked differences or similarities, depending upon the isoperoxidases studied.

The new major isoperoxidases, A_f and C_n, which appear and increase during certain passages of WR-132 tobacco cells in darkness suggest the need for studying a possible relationship of these isoperoxidase to the molecular metabolic processes occurring in darkness. So far, no work has been reported on the isolation and characterization of the specific isoperoxidases which increased or were primarily produced in the dark.

Preprartion of Enzymes

Since isoperoxidases A_f and C_n were found in much larger amounts in the medium than in the cell extracts of the dark-grown culture, the medium was the laboratory source used for isolation of A_f and C_n. However, A_f

and C_n could be isolated from the cell extracts after breaking the cells by the method described in Chapter III. When isoperoxidase A_f was the object of investigation, the medium from the third passage of dark grown cells was used. Alternately, when isoperoxidase C_n was being isolated, the media from the 4th and 5th passages were collected, since a higher amount of C_n was present at those passages.

Thirty flasks of WR-132 tobacco suspension culture were filtered through a Buchner funnel. The filtrates (approximately 1 liter) were subjected to ammonium sulfate precipitation. The protein which precipitated between 20 and 90% saturation with respect to ammonium sulfate was collected by centrifugation (27,000 X g for 20 min) and redissolved in a minimum volume of 5 mM phosphate buffer (pH 6.0). This solution was then dialyzed against 100 volumes of distilled water for 24 hours. The water was changed three times during the period of dialysis. Fifteen ml of the dialyzed sample containing approximately 15-20 mg protein was applied to a carboxymethyl cellulose (CM-cellulose) column preequilibrated with 5 mM phosphate buffer (pH 6.0) or distilled water. Initial elution was begun with distilled water and the flow rate was adjusted to approximately 20 ml per hour. After collecting 8 ml fractions with the Gilson Escargot Fractionator, Model SC-15, the eluate was spot-checked for peroxidase activity using a solution of 2 parts 1% guaiacol, 1 part 0.5% H_2O_2 and 2 parts 40 mM phosphate buffer (pH 6.0).

The water eluate from the CM-cellulose column contained largely isoperoxidases A_c and A_f. This water eluate containing A_c and A_f was concentrated by lyophilization and was redissolved in a minimal amount (5 ml) of distilled water. The concentrate was placed onto a diethyl-aminoethyl cellulose (DEAE-cellulose) column preequilibrated with distilled water or 5 mM phosphate buffer (pH 6.0). Isoperoxidase A_c was eluted with 10 mM

phosphate buffer (pH 6.0), and isoperoxidase A_f was eluted with 40 mM phosphate buffer (pH 6.0) following a stepwise elution (10 → 20 → 40 → 80) with the pH 6.0 phosphate buffer. The most active fractions of each isoperoxidase, as judged by the spot assay against guaiacol, were combined and subjected to starch gel electrophoresis and anodic disc gel electrophoresis for determination of the purity of each isoenzyme. Those fractions found to be free from other contaminating proteins were lyophilized. The dry samples were redissolved and adjusted to the proper dilution for enzyme assays. The isolation scheme is summarized in Table 4.

Isoperoxidase C_n was isolated by stepwise elution of the CM-cellulose column which was previously washed thoroughly with distilled water to remove all anodic proteins. The elution was performed with phosphate buffer (pH 6.0) from 10 mM to 100 mM (10 → 20 → 40 → 50 → 100) (Table 4). The isoperoxidase C_n could be isolated most successfully with a elution of 50 mM phosphate buffer (pH 6.0). The most active C_n fractions from the 50 mM elutions were collected and the fractions were further analyzed by starch gel electrophoresis and cathodic disc gel electrophoresis. Occasionally, isoperoxidase C_n co-eluted with a small amount of isoperoxidase C_3, which could be separated from C_n using Sephadex G-150 filtration.

Molecular Weight Determination

The molecular weights of isoperoxidases A_f and C_n were estimated by two different methods. The first method was gel filtration chromatography using Sephadex G-150 according to the method of Andrews (54). A 90 X 2.5 cm Ace glass column was packed with Sephadex G-150 which had previously been equilibrated in a 5 mM phosphate buffer (pH 6.0). Two mg of each protein standard and lyophilized isoperoxidase A_f and C_n were dissolved in 2 ml of the 5 mM phosphate buffer, pH 6.0. After adding a few crystals

TABLE 4

ISOLATION PROCEDURE FOR ISOPEROXIDASES A_f and C_n

FROM MEDIA OF WR-132 CULTURE

Step I

Filter WR-132 culture using a Buchner funnel and wash thoroughly with distilled water. Save filtrate.

Step II

Bring filtrate to 20% saturation with solid $(NH_4)_2SO_4$. Centrifuge for 20 min. at 27,000 X g. Discard pellet.

Step III

Bring supernatant to 90% saturation with solid $(NH_4)_2SO_4$. Centrifuge for 20 min. at 27,000 X g. Redissolve pellet in small volume of 5 mM phosphate buffer (pH 6.0).

Step IV

Dialyze against 100 volumes of distilled H_2O for 24 hours with three changes.

Step V

CM-cellulose chromatography

Elution with distilled H_2O gives A_c and A_f. Lyophilize and redissolve in distilled H_2O

Step VI

DEAE-cellulose chromatography

Elution with 10 mM phosphate buffer (pH 6.0) → A_c.

Elution with 40 mM phosphate buffer (pH 6.0) → A_f.

Step V'

CM-cellulose chromatography

Stepwise elution with phosphate buffer (pH 6.0)

10 → 20 → 40 → 50 → 100 mM

Elution with 50 mM phosphate buffer (pH 6.0) → C_n

of sucrose to increase the density, the entire 2 ml sample was layered on the top of the column. The flow rate was approximately 5 ml per hour, and 2 ml fractions were collected using a Gilson Escargot Fractionator, Model SC-15. The absorbance at 280 nm was followed to detect the presence of protein. The peroxidase activity of appropriate fractions was located using a spot assay and starch gel electrophoresis. The molecular weights of A_f and C_n obtained from semi-logarithmic plots of molecular weight versus elution volume as shown in Fig. 5 were 54,000 and 28,000 respectively.

Molecular weights of isoperoxidases A_f and C_n were also determined utilizing the sodium dodecyl sulfate (SDS) gel electrophoresis procedure of Weber, Pringle and Osborn (42). Gels were prepared 7 cm in length with an acrylamide concentration of 7.5%. Two mg of each standard protein and samples of A_f and C_n were dissolved in 10 mM sodium phosphate (pH 7.0), and one part of this solution was mixed with nine parts of 10 mM sodium phosphate (pH 7.0) containing 1% SDS and 1% β-mercaptoethanol. This solution was incubated in a boiling water bath for 2 minutes and cooled to room temperature. One ml of this solution was mixed with 0.04 ml of 0.05% bromphenol blue and 0.04 ml of β-mercaptoethanol. A few crystals of sucrose were added to facilitate layering. One hundred µl of this final protein solution (approximately 0.1 mg of protein) was applied to the top of each electrophoresis gel.

After electrophoresis (8 mA/gel) for 4½ hours, gels were removed and placed in a staining solution of 0.25% wt/vol Coomassie Brilliant Blue in 45% (v/v) methanol and 9% (v/v) acetic acid for 4-6 hours. The gels were destained with 5% (v/v) methanol in 7.5% (v/v) acetic acid until dark protein bands were visible. Mobilities were calculated using the equation:

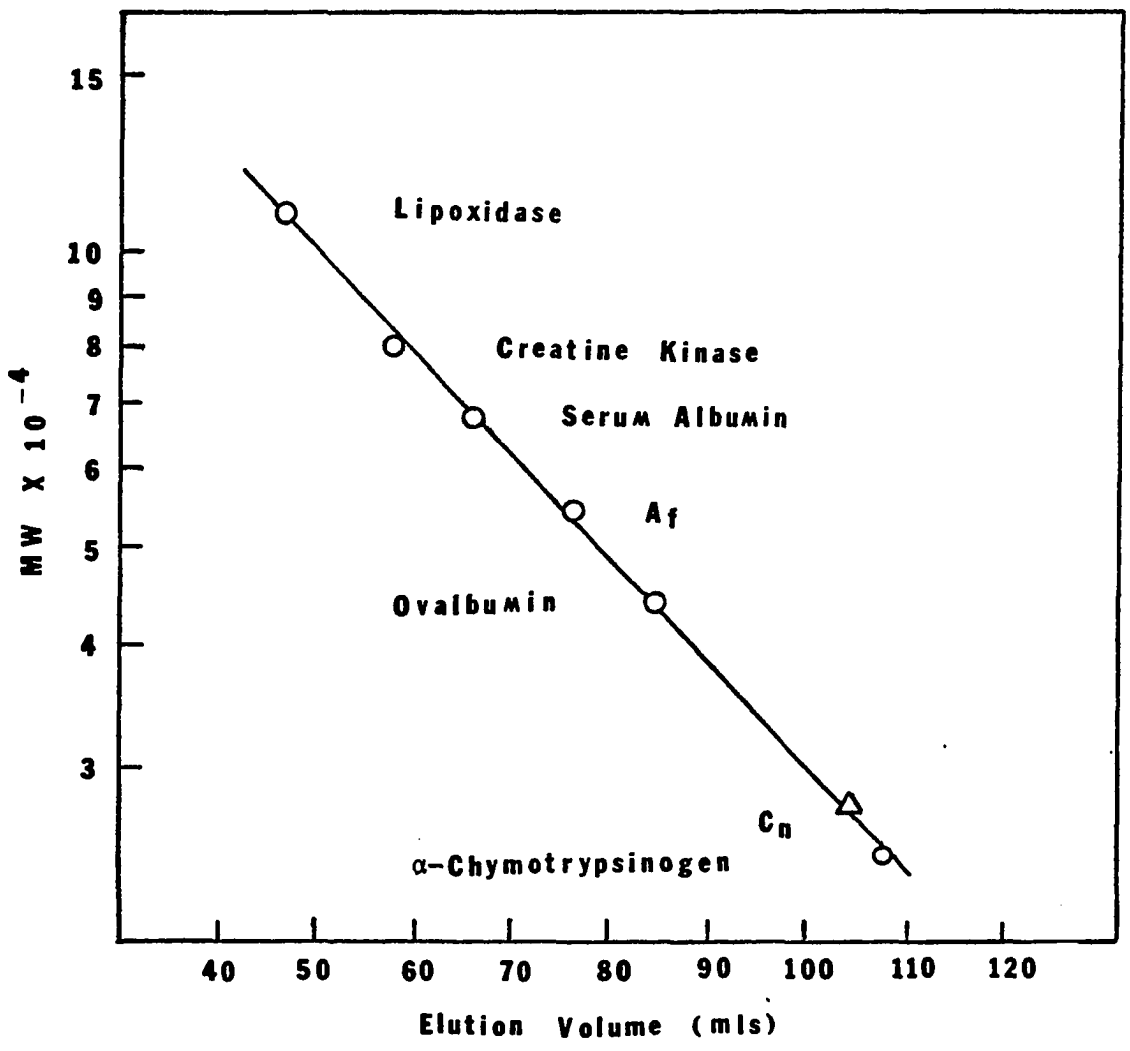


Figure 5. DETERMINATION OF MOLECULAR WEIGHTS OF ISOPEROXIDASES A_f AND C_n BY GEL FILTRATION ON SEPHADEX G-150

$$M = \frac{\text{distance of protein migration}}{\text{length of gel after destaining}} \times \frac{\text{length of gel before staining}}{\text{distance of dye migration}}$$

Molecular weights were calculated from a plot of log molecular weight versus mobilities for protein standards as indicated in Fig. 6. Molecular weights obtained by this method were 53,000 for isoperoxidase A_f and 30,000 for isoperoxidase C_n.

Glycoprotein Determination

The procedure described by Glossmann and Neville (55) was used to determine if isoperoxidases A_f and C_n contain carbohydrate. Following SDS electrophoresis, the gels were washed for 48 hours with 40% (v/v) methanol in 7% (v/v) acetic acid and were oxidized for 1 hour at 4°C with 1% periodic acid in 7% acetic acid in the dark. The gels were then incubated for 15 hours in Schiff's reagent at 4°C in the dark. Schiff's reagent was prepared according to the method of McGuckin and McKnezie (56). After incubation in Schiff's reagent, the gels were washed three to four times with 1% sodium bisulfite in 0.1 N HCl and stored in Schiff's reagent. Under these conditions, isoperoxidase A_f showed an intensely stained reddish pink band, while C_n did not. Isoperoxidase A_c was also a glycoprotein, as determined by this same procedure.

Total neutral sugars of three isoperoxidases C_4, A_c and A_f were determined on the intact protein with the anthrone reagent of Roe (57), using a mixture of galactose and mannose in a ratio of 1:1 as a standard. Isoperoxidase C_4 had been previously been shown, by Pickering (45), to contain carbohydrate. To determine carbohydrate composition of each isoperoxidase, neutral and amino sugars were released from the enzyme by hydrolysis at 100°C for 20 hours in 1.0 N H_2SO_4 in a vacuum. The hydrolysate was passed through a column of Dowex 50-X4 (H^+) (200 to 400 mesh) coupled to a column of Dowex 1-X8 (HCO_3^-) (200 to 400 mesh) according to the method of Boas (58).

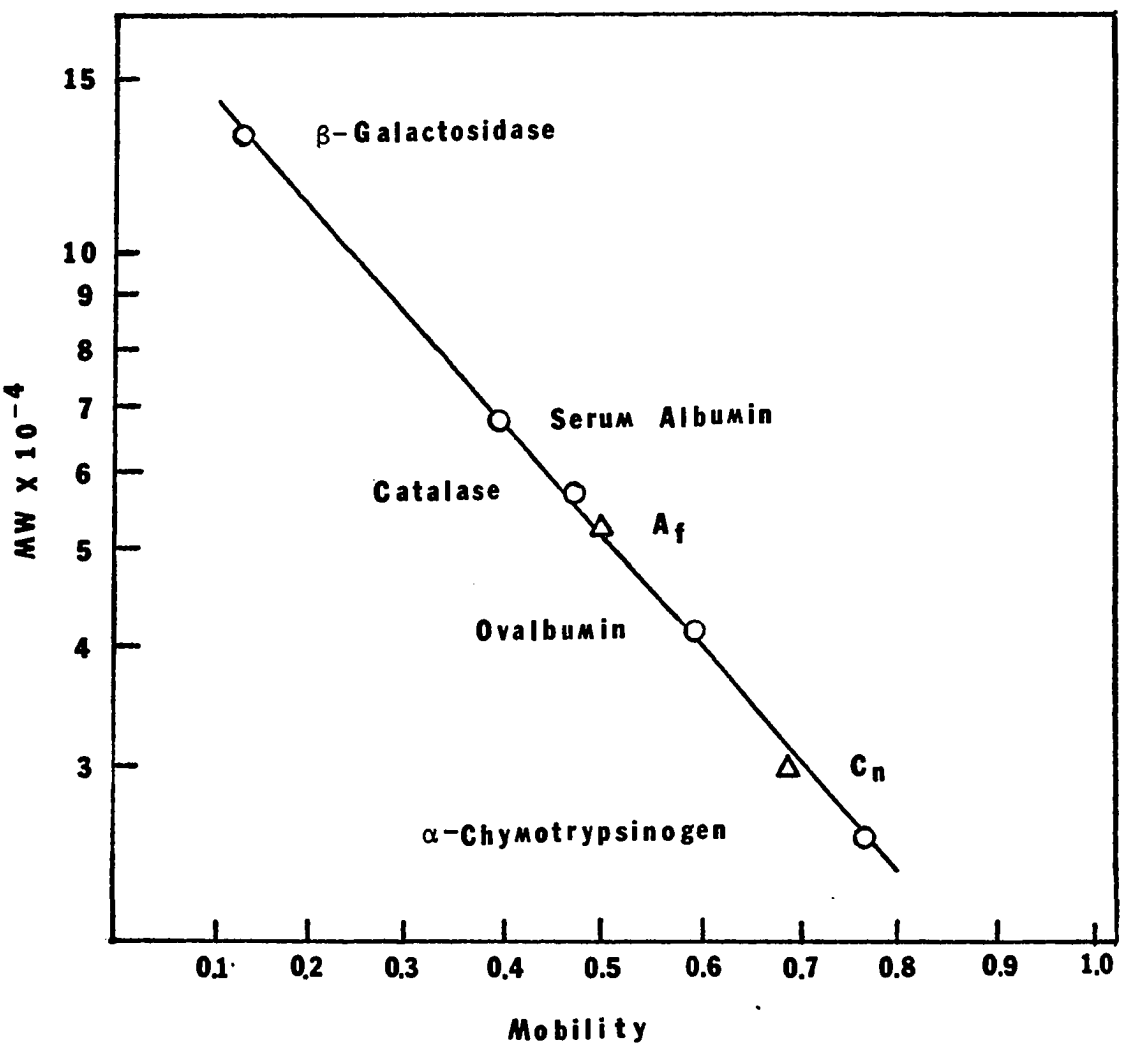


Figure 6. DETERMINATION OF MOLECULAR WEIGHTS OF ISOPEROXIDASES A_f AND C_n BY DODECYL SULFATE - POLYACRYLAMIDE GEL ELECTROPHORESIS.

The effluent and water wash from these columns were concentrated in a rotatory evaporator under reduced pressure at 40 - 45°C. The absorbed amino sugars were eluted from the Dowex 50-X4 (H^+) column with 2 N HCl and determined by the Elson-Morgan reaction (58). All of the hydrolyses were performed at a protein concentration of 2 to 3 mg except the determination of amino sugars. In this case, 10 mg of each protein was used.

For the identification of the neutral sugars, descending chromatography with butanol : ethanol : water (4:1:1) was employed, and individual sugars were detected by spraying with aniline hydrogen phthalate (59).

The results of carbohydrate analysis are summarized in Table 5. The neutral and amino sugars accounted for approximately 5 to 6% of the isoperoxidases. Qualitatively, the neutral sugar composition of isoperoxidases A_f and A_c was identical. Xylose and mannose were detected in both isoperoxidases. In contrast to isoperoxidases A_c and A_f, isoperoxidase C_4 contained arabinose and galactose.

In addition to these neutral sugars, very faint yellow spots could be found upon staining the chromatogram with aniline hydrogen phthalate. None of these faint spots, however, corresponded to any of the reference standard neutral sugars used: glucose, galactose, mannose, arabinose, xylose and fucose.

DISCUSSION

The value of 28,000 - 30,000 for the molecular weight of isoperoxidase C_n and 53,000 - 54,000 for isoperoxidase A_f indicate the significant difference of the two isoperoxidases in terms of physical properties, although both of them are the major peroxidases produced in the dark. The molecular weights of several isoperoxidases from tobacco tissue cultures are listed

TABLE 5

CARBOHYDRATE COMPOSITION OF ISOPEROXIDASES FROM TOBACCO SUSPENSION CULTURE WR-132

Carbohydrate Composition	Isoperoxidases		
	A_f	A_c	C_4
	g/100 g dry protein		
Free Sugar	5.1	4.2	4.8
Mannose	+	+	-
Xylose	+	+	-
Galactose	-	-	+
Arabinose	-	-	+
Amino Sugars	0.7	0.5	0.6

in Table 6. The value of 28,000 - 30,000 for isoperoxidase C_n is the smallest one among the tobacco tissue culture isoperoxidases studied in this laboratory.

A wide range of molecular weights has been reported in the literature for peroxidase isoenzymes from various sources. These include values of 38,700, 50,000, 51,000 and 51,600 for four turnip peroxidase isoenzymes (61); 33,400, 45,000, 57,000 for three ribosome-associated peroxidase isoenzymes from lentil roots (64); 40,000 and 50,000 for red alga peroxidase isoenzymes (65); 40,000 for five horseradish peroxidase isoenzymes (14); 60,000 for four peanut peroxidase isoenzymes (66); and 30,000, 40,500, 44,000 and 54,500 for Japanese-radish isoenzymes (67,68). As just shown, the molecular weights of peroxidase isoenzymes from various sources generally ranged between 30,000 to 60,000. Thus, the value of 28,000 - 30,000 for the molecular weight of isoperoxidase C_n is at the bottom of the range found by various research workers, and 53,000 - 54,000 for isoperoxidase A_f is well in the range of the reported plant peroxidases.

The conclusion of Rücker and Radola (69) that all isoperoxidases from tobacco tissue cultures have similar molecular weights is inconsistent with the above results for isoperoxidase A_f and C_n, although isoperoxidases from horseradish (14), and from peanut (66) and three cathodic isoperoxidases from tobacco suspension culture Hicks 2 (70) displayed the same molecular weight in a given system. The different molecular weights for isoperoxidases A_1, A_2, A_3, C_3 and C_4 from tobacco tissue culture WR-132 and W-38 (Table 6) also support the concept that all isoperoxidases from the same plant do not necessarily have the same molecular weights.

The identical molecular weights of isoperoxidase A_f from tobacco suspension culture WR-132 and isoperoxidase A_3 from tobacco callus culture

TABLE 6

MOLECULAR WEIGHTS OF ISOPEROXIDASES FROM TOBACCO TISSUE CULTURE WR-132 AND W-38

Source	Isozyme	Molecular Weight	
		Gel filtration	SDS electrophoresis
WR-132	*C_3	67,000	68,000
	*C_4	46,000	44,000
	C_n	28,000	30,000
	A_c	80,000	80,000
	A_f	54,000	53,000
W-38	*A_1	103,000	49,000
	*A_2	90,000	89,000
	**A_3	54,000	54,000

* Data taken from Powell et al. (18).

** Data taken from Reigh (44).

W-38 indicated the possibility that these two isoenzymes may be identical.

Since many isoperoxidases from different plant systems have been shown to contain carbohydrate components (14,60,61), SDS gels of A_f and C_n were stained for glycoprotein, utilizing the procedure of Glossmann and Neville (55). By using this procedure, isoperoxidases A_f and A_c were shown to contain carbohydrate. Isoperoxidase C_n, however, did not give a positive glycoprotein test. In addition to isoperoxidases A_f and A_c, isoperoxidase C_4 was also shown to contain carbohydrate while C_3 did not contain carbohydrate by the procedure described above (45).

Horseradish peroxidase, Japanese-radish peroxidase, and turnip peroxidase have all been reported to be glycoproteins. Acidic horseradish peroxidase A_1, A_2 and A_3, according to Shannon and coworkers (14), contain arabinose, galactose and mannosamine, whereas xylose, fucose, mannose, mannosamine and galactosamine were identified in their basic isoperoxidases B and C. Morita and Kameda (60) have reported that Japanese-radish peroxidase a contains mannose and xylose as principal monomeric components, in addition to arabinose and hexosamine. In contrast to horseradish peroxidases and Japanese-radish peroxidase, turnip peroxidases have been found by Mazza and coworkers (61) to contain glucose, mannose, fucose, and two amino sugars, glucosamine and galactosamine. Thus, carbohydrate composition of isoperoxidases from tobacco suspension culture WR-132 is similar to that of horseradish peroxidases and to Japanese-radish peroxidases.

The carbohydrate content of these particular tobacco isoperoxidases was rather low compared to that of some carbohydrate-containing peroxidases reported. Five horseradish peroxidases have been reported to contain approximately 18% (14); some turnip peroxidases have been shown to contain 12 to 18% carbohydrate (61), and Japanese-radish peroxidase a was reported to

contain approximately 20% carbohydrate (60). The values of 5 to 6% carbohydrate content of the isoperoxidase A_c, A_f and C_4 from tobacco suspension culture WR-132, however, are rather close to the value of Japanese-radish peroxidase c which contains approximately 5% carbohydrate (62).

CHAPTER V

KINETICS OF ISOPEROXIDASE A_f AND C_n WITH THE SYNTHETIC SUBSTRATE GUAIACOL

Introduction

Substrate studies with preparations of isoperoxidases A_f and C_n free from other contaminating proteins were begun with the synthetic substrate guaiacol. While other workers have reported that isoperoxidases from Alaska pea (16), egg plant leaves (17) and Japanese-radish (68) showed marked differences in ratios of rate constants with guaiacol as a substrate and in relative activity toward O-dianisidine/guaiacol, another group has reported that four cathodic isoperoxidases of horseradish have idential pH optima and specific activities with O-dianisidine (71).

So far, three isoperoxidases, A_1, A_2, and A_3, from tobacco tissue culture W-38 (44,46) and two isoperoxidases, C_3 and C_4 from tobacco tissue culture WR-132 (45) have been characterized, in our laboratory, using guaiacol as a substrate. All of these isoperoxidases showed a notable difference in Michaelis-Menten constants, although the pH optima of four isoperoxidases (A_1, A_2, C_3, and C_4) are the same (Table 7). Shinshi and Noguchi (70) have reported that seven isoperoxidase fractions from tobacco suspension culture, *Nicotiana tabacum* cultivar. Hicks 2, also showed the same pH optima against guaiacol oxidation. However, Mäder and Bopp (72) have shown that

three groups of isoperoxidases from tobacco callus culture, *Nicotiana tabacum* var. Burley, exhibited slightly different pH optima and Michaelis constants against guaiacol oxidation (72). The present study was performed to determine the kinetic properties of isoperoxidases A_f and C_n and the effect of a number of naturally occurring phenolic compounds on the guaiacol activity of these isoenzymes.

Results and Discussion

Isoperoxidases A_f and C_n appear to have a different catalytic activity with guaiacol as substrate. The pH optimum for the oxidation of guaiacol by isoperoxidase A_f and C_n is around 6.5 when the oxidation of guaiacol was followed spectrophotometrically at 470 nm by the method of Lance (43). The effect of pH on the guaiacol oxidizing activity of the two isoperoxidases is shown in Fig. 7 and 8. Fig. 9 and 10 illustrate the effect of substrate concentration for isoperoxidases A_f and C_n respectively, utilizing guaiacol as a substrate. Lineweaver-Burk plots of these data are linear and yield $S_{0.5}$ values of 4 mM and 13.3 mM for isoperoxidases A_f and C_n, respectively (Fig. 11 and 12).

A comparison of pH optima and Michaelis constants for guaiacol oxidation of isoperoxidases A_f and C_n to the other isoperoxidases normally present in tobacco tissue culture WR-132 and W-38 indicated that isoperoxidase A_f and isoperoxidase A_3 from tobacco tissue culture W-38 could be the same enzyme (Table 7). Both isoperoxidases have the same pH optimum and $S_{0.5}$ for the oxidation of guaiacol. On the other hand, isoperoxidase C_n might be quite different from other isoperoxidases so far isolated from tobacco tissue culture WR-132 and W-38 due to its unique high $S_{0.5}$ value (Table 7). It appears that isoperoxidase C_n has approximately a one-third to one-half lower affinity towards guaiacol, than other isoperoxidases from

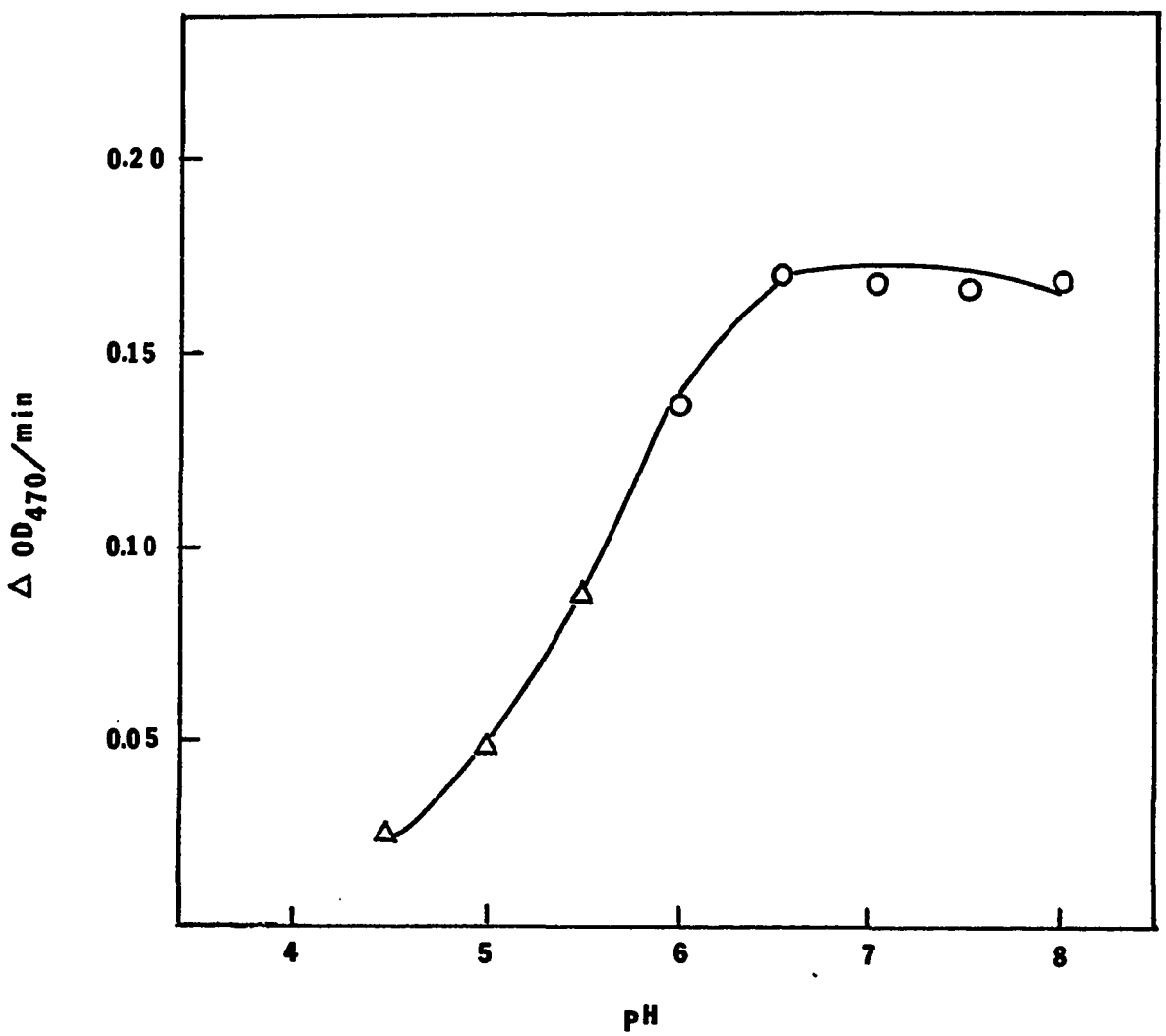


Figure 7. EFFECT OF pH ON THE OXIDATION OF GUAIACOL BY ISOPEROXIDASE A_f. Assays were run at 15 mM guaiacol and 5 mM H_2O_2 in 40 mM buffer. Δ - citrate, o - phosphate.

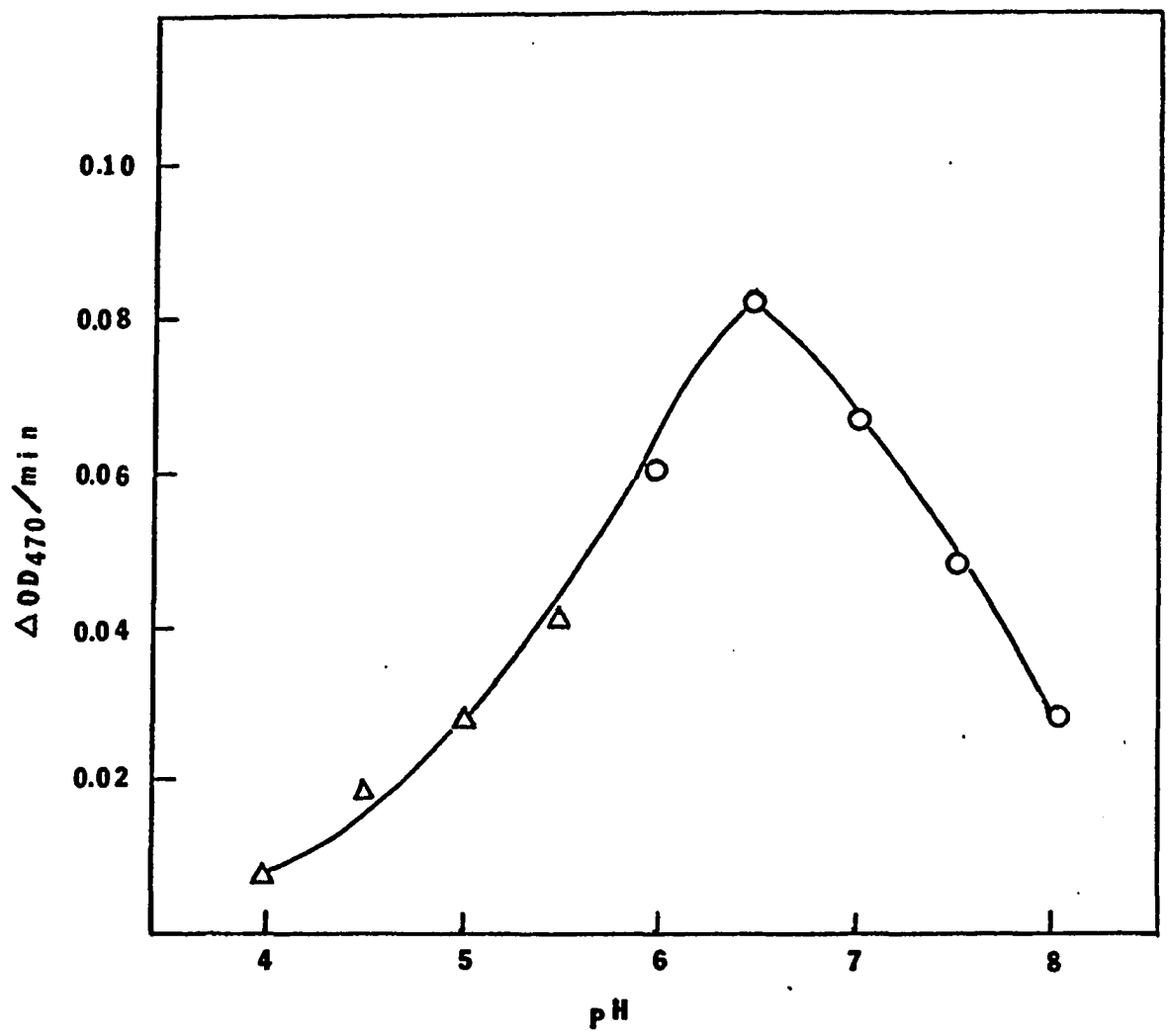


Figure 8. EFFECT OF pH ON THE OXIDATION OF GUAIACOL BY ISOPEROXIDASE C_n. Assays were run at 15 mM guaiacol and 5 mM H_2O_2 in 40 mM buffer. Δ - citrate, o - phosphate.

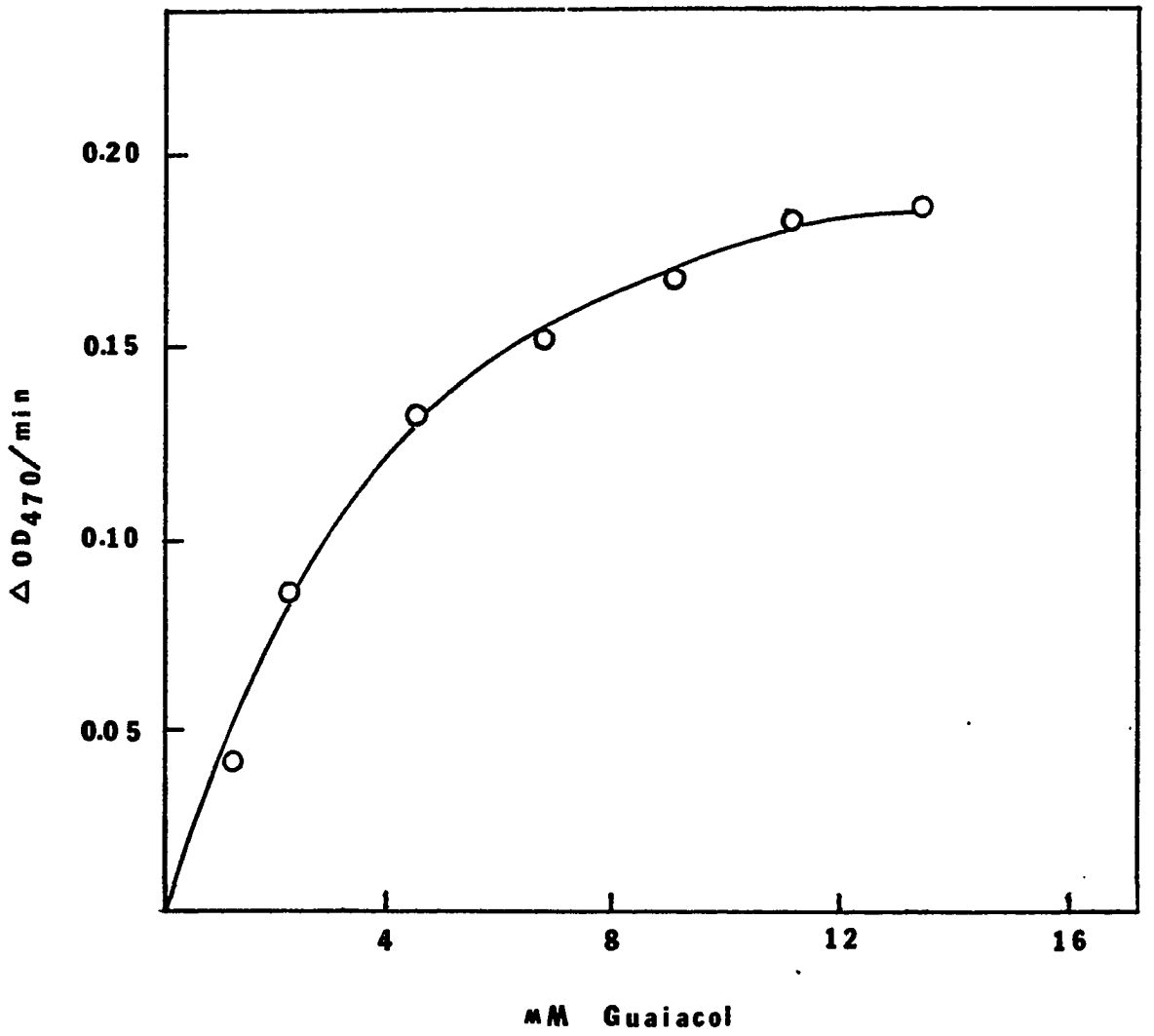


Figure 9. GUAIACOL SATURATION CURVE FOR ISOPEROXIDASE A_f. Assays were run at 5 mM H_2O_2 in 40 mM phosphate buffer pH 6.5.

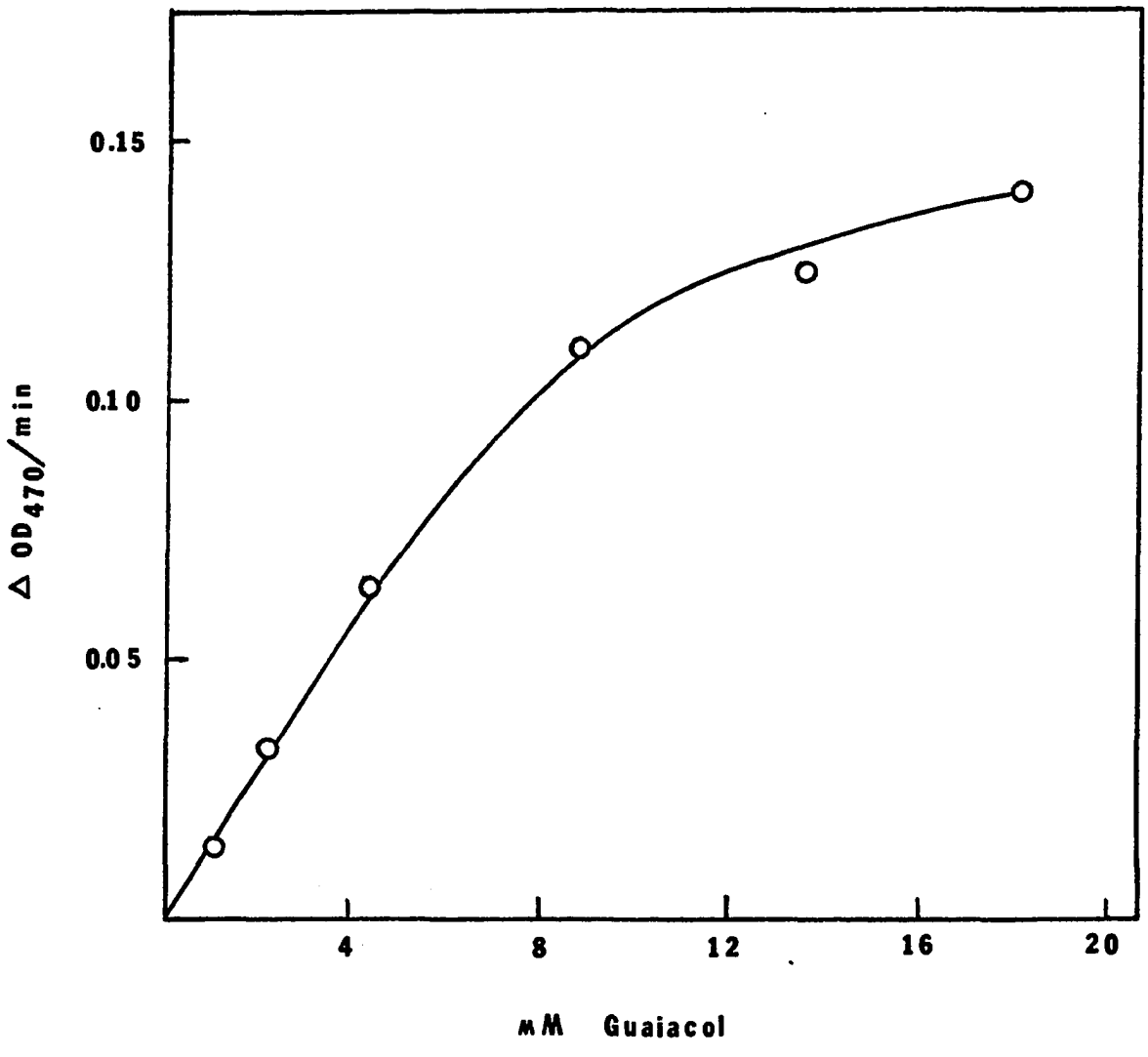


Figure 10. GUAIACOL SATURATION CURVE FOR ISOPEROXIDASE C_n. Assays were run at 5 mM H_2O_2 in 40 mM phosphate buffer, pH 6.5.

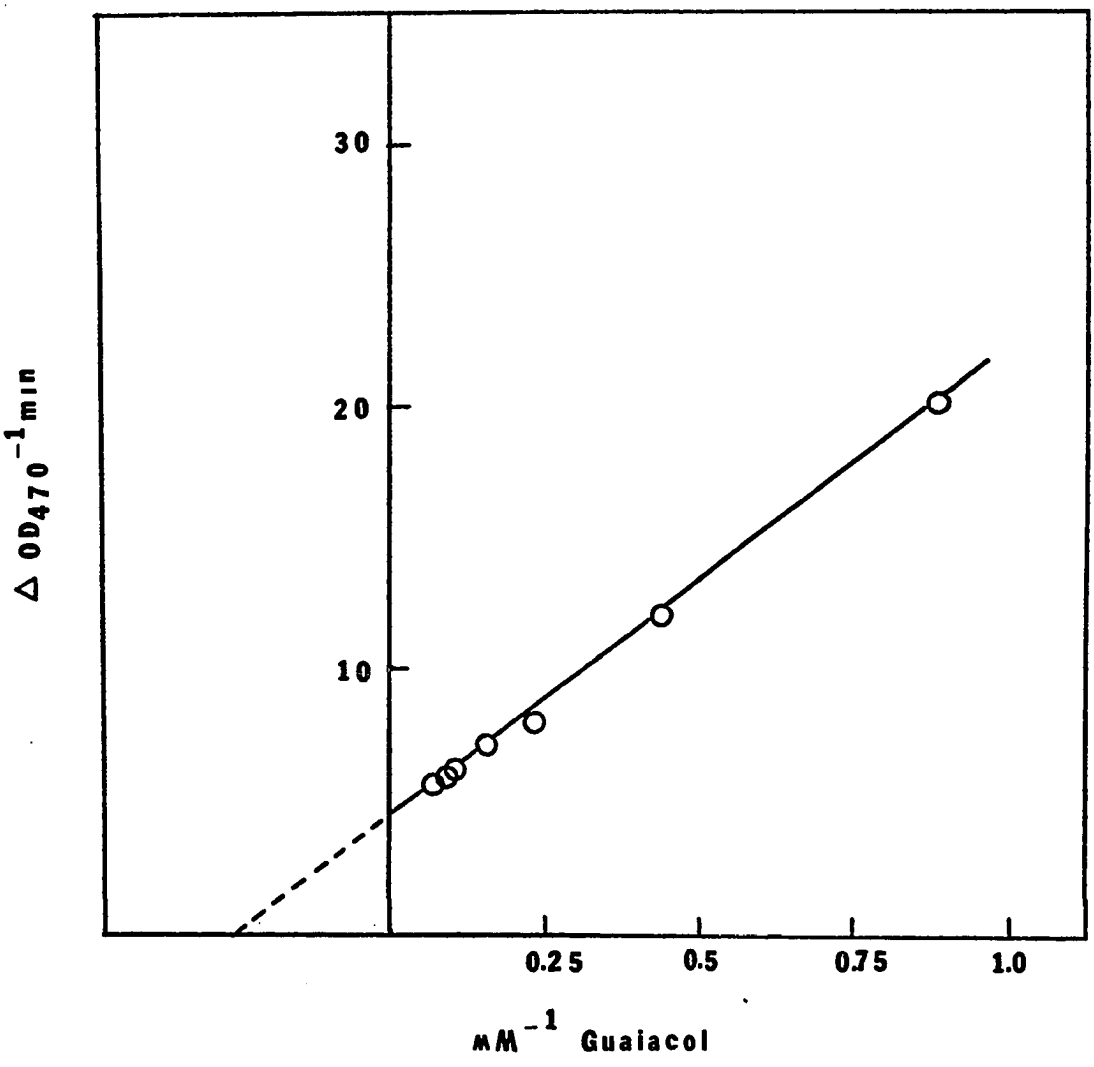


Figure 11. DOUBLE RECIPROCAL PLOT OF ACTIVITY VS. GUAIACOL CONCENTRATION FOR ISOPEROXIDASE A_f. Assays were run at 5 mM H_2O_2 in 40 mM phosphate buffer, pH 6.5.

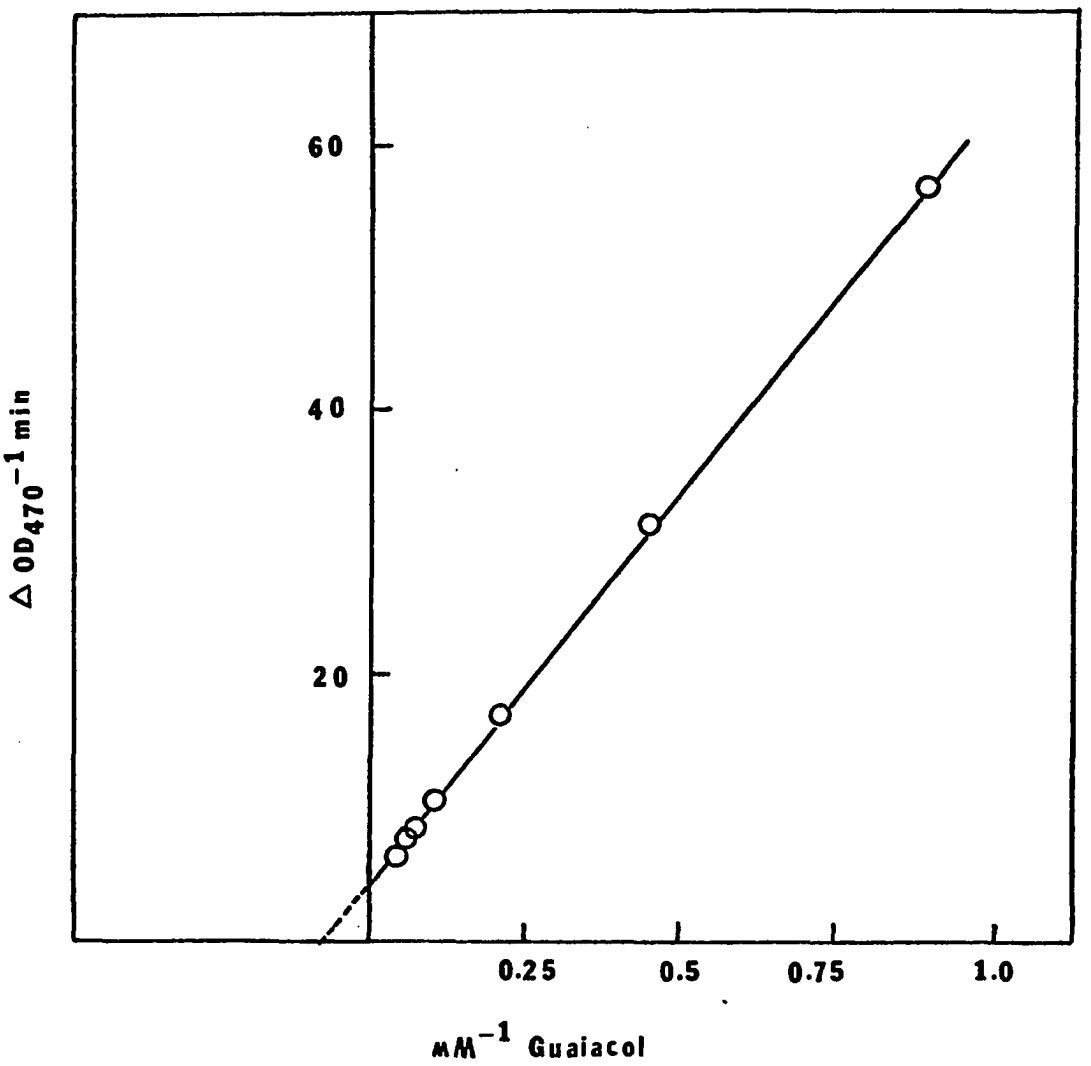


Figure 12. DOUBLE RECIPROCAL PLOT OF ACTIVITY VS. GUAIACOL CONCENTRATION FOR ISOPEROXIDASE C_n. Assays were run at 5 mM H_2O_2 in 40 mM phosphate buffer, pH 6.5.

TABLE 7

COMPARISON OF pH OPTIMA AND MICHAELIS CONSTANTS FOR GUAIACOL FOR ISOPEROXIDASES FROM TOBACCO TISSUE CULTURE

Isoperoxidases	Tobacco Culture (Strain)	pH optima	Km (mM)	Reference
A_1	W-38	6.0	5.0	(46)
A_2	W-38	6.0	5.8	
A_3	W-38	6.5	4.0	(44)
A_f	WR-132	6.5	4.0	-
C_3	WR-132	6.0	7.5	(45)
C_4	WR-132	6.0	4.0	
C_n	WR-132	6.5	13.3	-
Fraction				
G_I		5.5	3.28	
G_{II}	Burley	5.8	5.26	(72)
G_{III}		6.0	3.85	
Fraction				
A-2, A-1, N, C-1, C-2, C-3, C-4	Hicks 2	5.5	_	(70)

tobacco tissue culture WR-132 and W-38 when compared in this regard.

It is difficult to compare the data of isoperoxidases A_f and C_n to those of peroxidase fractions reported for *Nicotiana tabacum* var. Burley (72) and Hicks 2 strain (70) due to different reaction mixtures and non-homogeneity of the peroxidase fractions. Based on pH optima, $S_{0.5}$ data and electrophoretic mobilities, fast migrating anodic G_I, slow migrating anodic G_{II} and slow migrating cathodic G_{III} fo the Burley strain could possibly correspond to isoperoxidases A_3, A_1 (and/or A_2), and C_1 (and/or C_2), respectively (Fig. 3, Chapter 3 and Table 7). In contrast to the results obtained from tobacco tissue culture isoperoxidases, four isoperoxidases isolated from peanut suspension culture showed ten to thirty times higher $S_{0.5}$ values than isoperoxidases from the tobacco tissue culture system (19). It is apparent that these peanut isoperoxidases are different from the tobacco isoperoxidases of our laboratory.

Since the report of Goldacre and coworkers (73) that IAA oxidase activity of crude peroxidase from peas was stimulated by monophenols and inhibited by O- and p-diphenols, many phenolic compounds including ferulic acid, p-coumaric acids, and scopoletin have been reported to affect the activity of peroxidase preparations (9,74). The effect of several naturally occurring phenolics and related compounds on the guaiacol oxidizing activity of isoperoxidases A_f and C_n was examined to determine the possible involvement of these compounds in the physiological role of the two isoperoxidases. The assays were performed on the guaiacol concentration which corresponds to the $S_{0.5}$ value of both isoperoxidases, while H_2O_2, buffer and effector concentrations were fixed at 5 mM, 40 mM phosphate (pH 6.5) and 0.4 mM, respectively. As shown in Table 8, there is a significant difference between isoperoxidases A_f and C_n in the pattern of stimulation by the

phenolics. The guaiacol oxidation of isoperoxidase A_f was stimulated by p-coumaric acid and scopoletin. These stimulations were not observed for isoperoxidase C_n. However, ferulic acid and caffeic acid activated both isoenzymes.

Chlorogenic acid, esculetin, esculin and catechol were inhibitors of isoperoxidases A_f and C_n. Esculin(6-O-glucosylated esculetin) and catechol also inhibited both enzymes to almost the same degree.

Most of the compounds listed in Table 8 were also tested by Reigh (44) as possible effectors of the guaiacol oxidation of isoperoxidase A_3 from tobacco callus culture W-38. Although the degree of stimulation and inhibition by each of the effectors tested is different in isoperoxidase A_3 as compared to A_f, the pattern of stimulation and inhibition of both isoperoxidases by the phenolics is the same, except for stimulation of isoperoxidase A_f by p-coumaric acid (Table 8). The differences between the two studies are probably due to the difference in pH of the buffer used, since Reigh (44) used pH 5.5 citrate buffer instead of pH 6.5 phosphate buffer employed in the present study. Powell (46) and Pickering (45) studied possible effectors of the guaiacol oxidizing ability of four peroxidases (A_1, A_2, C_3 and C_4) from tobacco callus culture W-38 and tobacco suspension culture WR-132. As indicated in Table 8, all four isoperoxidases, as well as isoperoxidase C_n showed strikingly similar responses to the effectors utilized in this study. The only significant difference in the pattern of stimulation or inhibition by the phenolics in this case is the stimulation of isoperoxidases A_2 and C_4 by p-coumaric acid.

TABLE 8

EFFECT OF VARIOUS COMPOUNDS ON THE GUAIACOL ACTIVITY OF ISOPEROXIDASES FROM TOBACCO TISSUE CULTURE

Effector 0.4 mM	% of control activity						
	A_f	C_n	A_3^+	A_1^*	A_2^*	C_3^{**}	C_4^{**}
p-Coumaric acid	198	100	94	126	129	126	124
Caffeic acid	470	300	190	201	191	365	175
Ferulic acid	580	475	180	324	310	535	395
Scopoletin	450	95	165	100	100	105	102
Scopolin	100	103	-	100	100	100	100
Esculetin	18	0	10	14	15	16	15
Esculin	5	12	-	98	98	60	51
IAA (Indole-3-acetic acid)	98	97	-	93	86	96	100
Chlorogenic acid	15	5	-	5	4	8	8
Catechol	15	15	14	-	-	-	-
trans-Cinnamic acid	97	97	-	-	-	-	-

+ : Data supplied by Reigh (44).
* : Data supplied by Powell (46).
** : Data supplied by Pickering (45).

CHAPTER VI

KINETIC STUDIES OF ISOPEROXIDASES A_f AND C_n: POSSIBLE PHYSIOLOGICAL SUBSTRATES

Introduction

Although most physiological studies involving peroxidases and phenolic compounds have been centered on the effect of naturally occurring phenolic compounds on the IAA oxidase activity of these enzymes (8,9,74), it has not been established that all peroxidases oxidize IAA. Neither is every peroxidase that uses IAA limited to IAA as substrate. For example, Andreae (8) first demonstrated that a crude preparation of IAA oxidase from potatoes was capable of oxidizing scopoletin.

In this laboratory, Schafer et al. (31) demonstrated the stimulatory effect of scopoletin on the guaiacol oxidizing ability of isoperoxidase A_3 from tobacco tissue W-38, but scopoletin had practically no effect on another isoperoxidase A_1 from the same source. Reigh et al. (10) later demonstrated that scopoletin is actually a substrate for isoperoxidase A_3. Pickering et al. (11) showed that ferulic acid may act as a substrate for tobacco suspension culture isoperoxidases A_2 and C_4. Furthermore, none of these isoperoxidases studied in our laboratory has been shown to have IAA oxidase activity (44,45,46).

The apparent diversity of isoperoxidase activities suggests that investigations of individual isoperoxidases should be extended not only to IAA oxidation but also to other physiologically important phenolic compounds.

In the present study some naturally occurring phenolic compounds were tested as possible physiological substrates for isoperoxidases A_f and C_n. These include scopoletin, ferulic acid, esculetin and chlorogenic acid. All four compounds had considerable effect on the guaiacol activity of isoperoxidases A_f and C_n (Chapter V). In the following sections, the results of these studies on A_f and C_n will be compared to those obtained by other workers on other isoperoxidases.

Results and Discussion

Scopoletin (6-methoxy, 7-hydroxycoumarin)

When isoperoxidase A_f was added to a mixture of scopoletin, 5 mM H_2O_2 and 40 mM sodium citrate (pH 5.0), according to the method developed by Reigh (44) for scopoletin oxidation catalyzed by isoperoxidase A_3, a rapid formation of a blue intermediate was observed, followed by the appearance of yellow product(s). The absorption spectrum of the scopoletin reaction mixture before and after addition of isoperoxidase A_f had the same spectrum as reported by Reigh (44) for isoperoxidase A_3 reaction with scopoletin. The observation that the product formed from scopoletin absorbed at 450 nm while scopoletin absorbs negligibly at this wavelength served as the basis for an assay of this reaction. A typical assay contained 1.2 mM scopoletin, 5 mM H_2O_2 and 40 mM sodium citrate (pH 5.0). A_f was added to initiate the reaction, and the increase in absorbance at 450 nm was recorded. Usually lag times of from one to three minutes occurred, depending on enzyme concentration and pH, followed by linear rates for several minutes.

Fig. 13 shows the pH optimum for scopoletin oxidation of isoperoxidase A_f. The pH optimum was determined to be around 5.0. Fig. 14 shows the substrate saturation curve of isoperoxidase A_f against scopoletin. A Lineweaver-Burk plot (75) of these data is concave upward, although the saturation curve does not indicate clearly the sigmoidal nature (Fig. 15). A modified

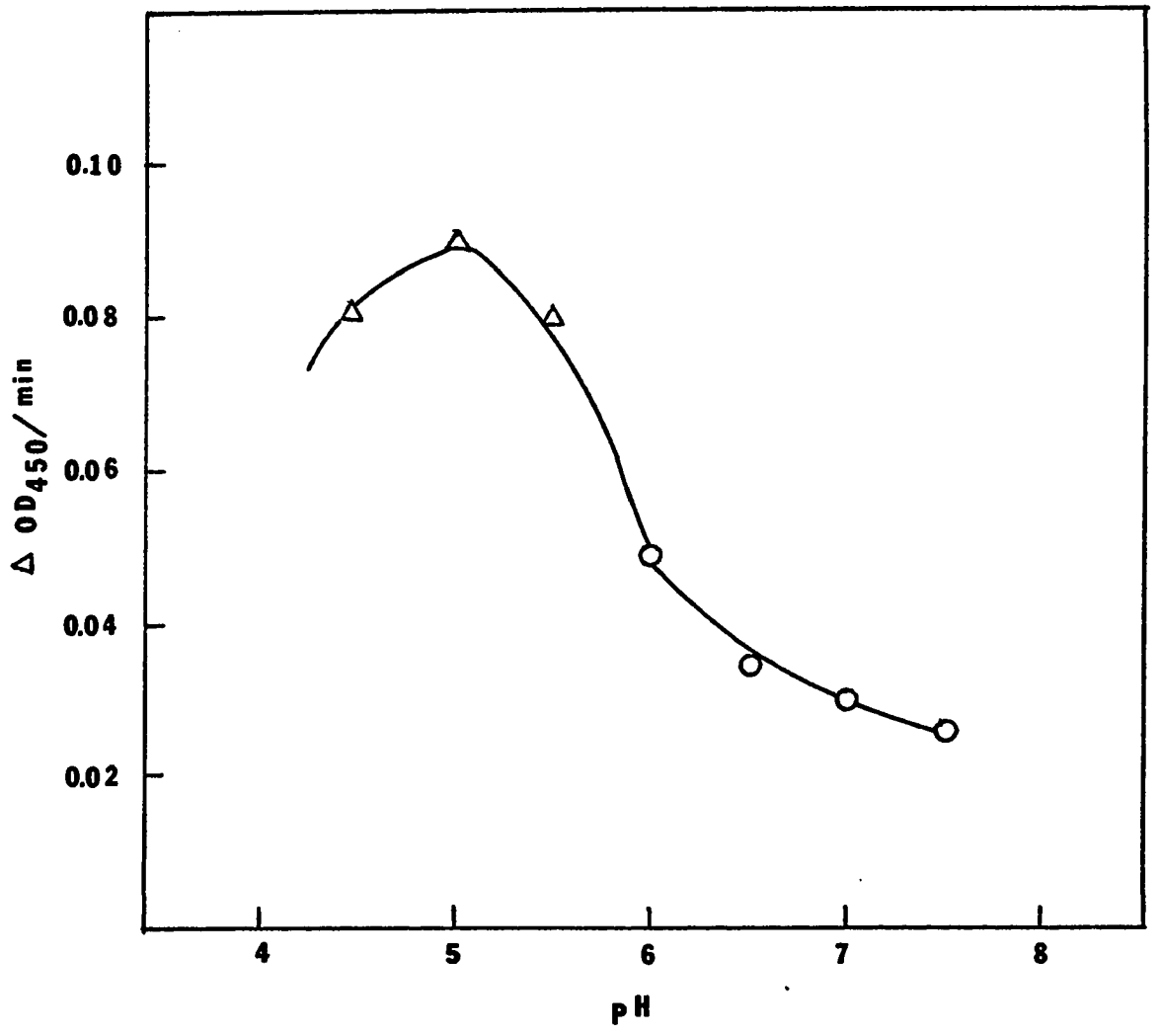


Figure 13. EFFECT OF pH ON THE SCOPOLETIN OXIDIZING ABILITY OF ISOPEROXIDASE A_f. Assays were run at 1.2 mM scopoletin and 5 mM H_2O_2 in 40 mM buffer. Δ - citrate, o - phosphate.

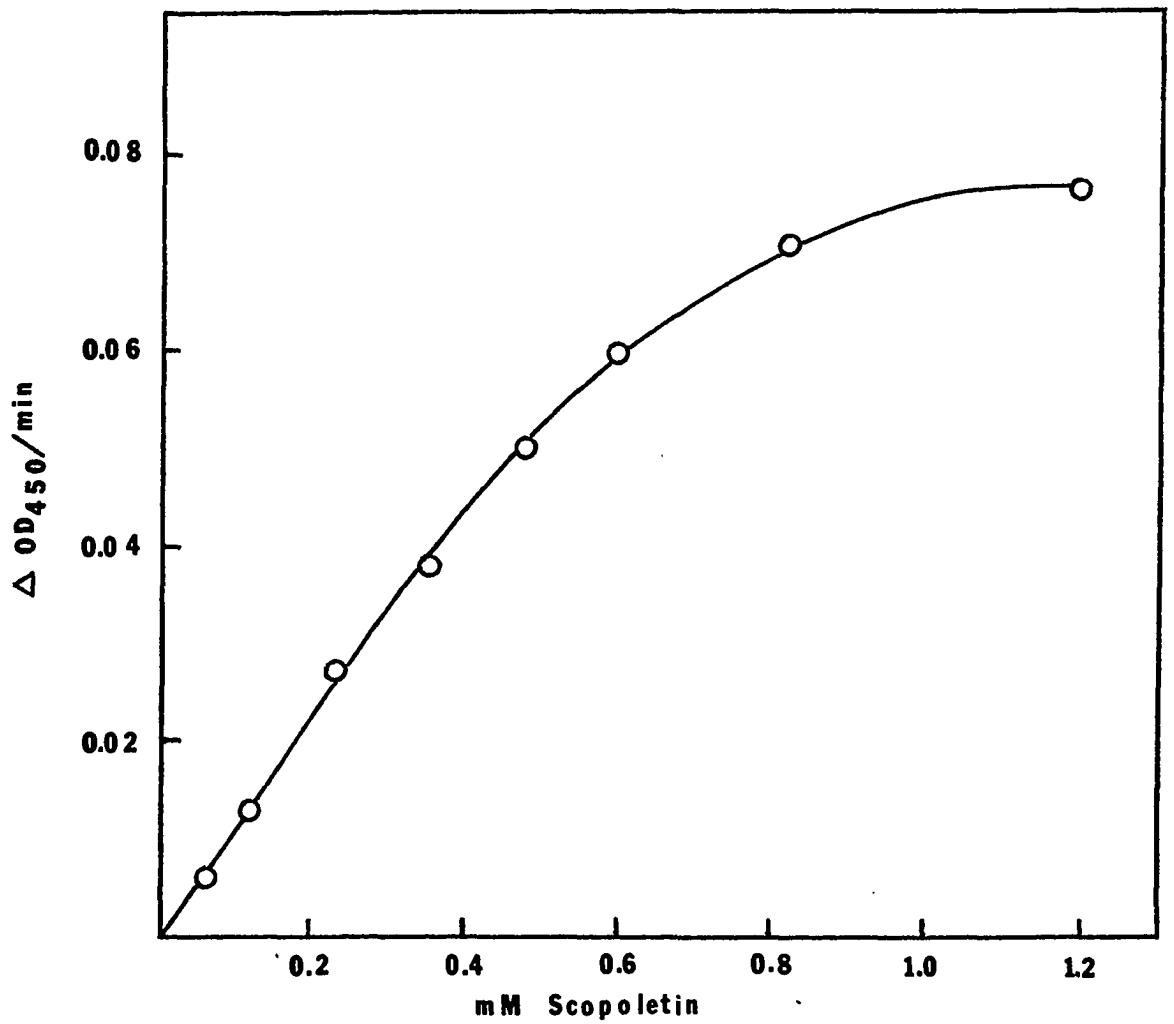


Figure 14. SCOPOLETIN SATURATION CURVE FOR ISOPEROXIDASE A_f.

Assays were run at 5 mM H_2O_2 in 40 mM sodium citrate (pH 5.0).

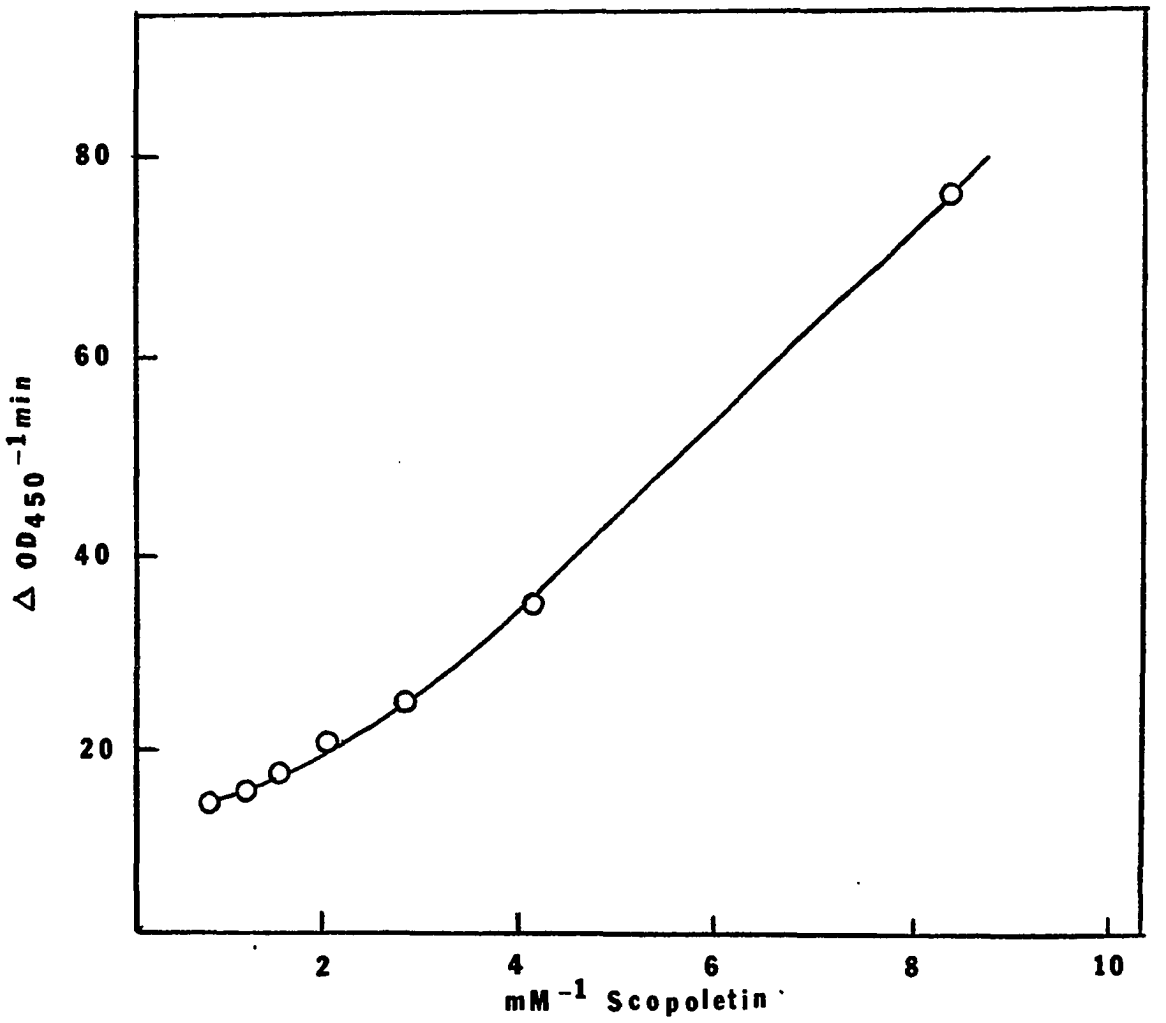


Figure 15. DOUBLE RECIPROCAL PLOT OF ACTIVITY VS. SCOPOLETIN CONCENTRATION FOR ISOPEROXIDASE A_f. Assays were run at 5 mM H_2O_2 in 40 mM sodium citrate (pH 5.0).

Lineweaver-Burk plot of 1/v versus $1/s^2$ yields a straight line and was used to determine $S_{0.5}$ (Fig. 16). The $S_{0.5}$ for scopoletin is about 0.22 mM (Table 9). A Hill plot (76) of these data is shown on Fig. 17. Although the nonlinearity of the Lineweaver-Burk plot and n>1 for the Hill plot are conditions necessary for substrate cooperative effects of enzymes having more than one binding site (77,78), an alternate multistep reaction mechanism may be responsible for the kinetics observed for scopoletin oxidation (44). The oxidation of scopoletin in the presence of a single subunit isoperoxidase A_3 from tobacco tissue culture W-38, appears to occur in more than a single step. The final product observed is yellow; however, at a pH less than six or with a high enzyme concentration, a blue intermediate was also noted (44). The isoperoxidase A_f oxidation of scopoletin revealed the same kind of phenomenon as isoperoxidase A_3. A blue intermediate was first observed at acidic pH (below pH 6.0) which changes slowly to a yellow final product; however, only the yellow final product could be observed at pH 7.0.

Based on kinetic data such as the pH optimum curve, the saturation curve and the Hill slope of scopoletin oxidation of isoperoxidase A_f described above, it is probable that isoperoxidase A_f and isoperoxidase A_3, which was isolated by Reigh (44) from tobacco callus culture W-38, may well be the same enzyme. Both isoenzymes exhibited a similar pH optimum (5.0 - 5.5), a sigmoidal saturation curve and a Hill slope (n=1.5) against scopoletin oxidation. The difference observed in $S_{0.5}$ value (Table 9) between isoperoxidases A_f and A_3 could possibly due to an alteration of either isoenzyme during purification or due to a contamination of isoperoxidase A_f or A_3 with other isoperoxidases which use scopoletin as a substrate.

Isoperoxidase C_n was also tested for scopoletin oxidation activity. At enzyme concentration in which ΔO.D 470/min for guaiacol was 0.15, the velocity (ΔOD/min) of the scopoletin reaction by isoperoxidase C_n was

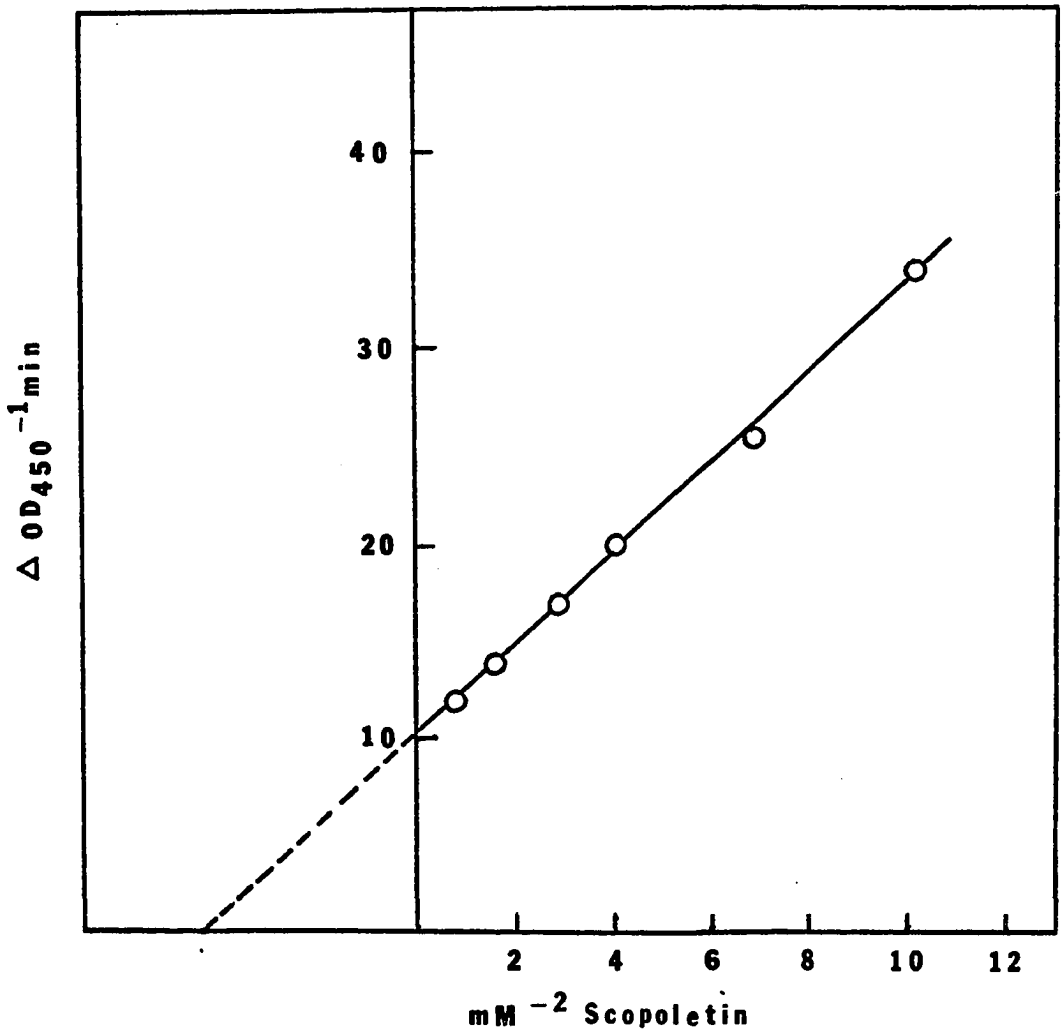


Figure 16. MODIFIED DOUBLE RECIPROCAL PLOT OF ACTIVITY VS. SCOPOLETIN CONCENTRATION FOR ISOPEROXIDASE A_f Assays were run at 5 mM H_2O_2 in 40 mM sodium citrate (pH 5.0).

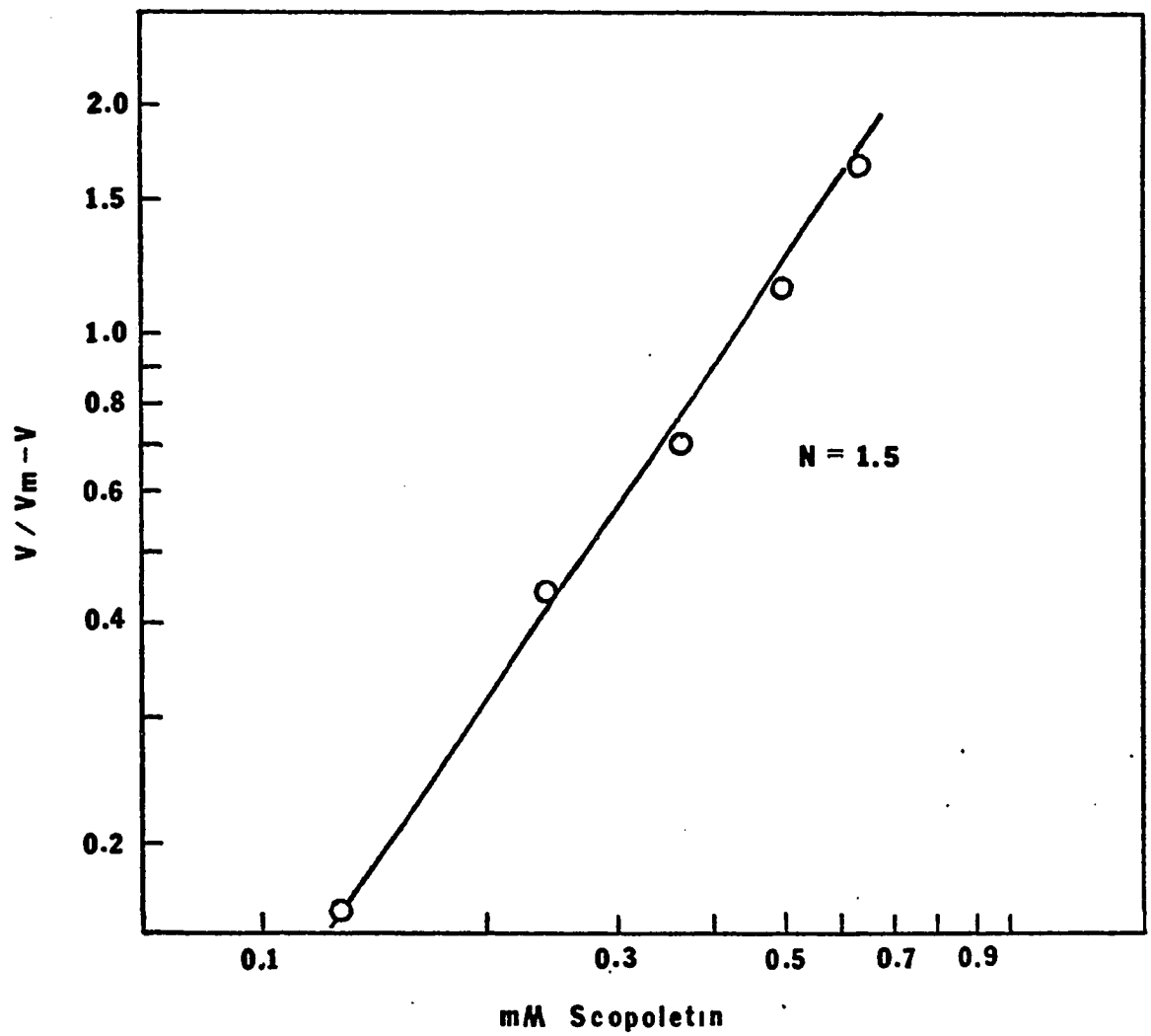


Figure 17. HILL PLOT OF ACTIVITY VS. SCOPOLETIN CONCENTRATION FOR ISOPEROXIDASE A_f. Assays were run at 5 mM H_2O_2 in 40 mM sodium citrate (pH 5.0).

TABLE 9

SUMMARY OF MICHAELIS CONSTANT AND pH OPTIMA FOR SOME PHENOLIC SUBSTRATES OF ISOPEROXIDASES

Substrate	Km (mM) Values						pH optima					
	A_f	C_n	A_3+	A_1*	A_2*	C_4**	A_f	C_n	A_3+	A_1*	A_2*	C_4**
Scopoletin	0.22	-	0.6	-	-	0.53	5.0	-	5.5	-	-	4.5
Esculetin	0.31	1.25	0.27	-	-	0.45	7.5	7.5	7.5	-	-	7.5
Ferulic Acid	0.29	0.19	-	0.4	0.4	0.3	5.5	5.0	-	4.5	5.0	5.5
Chlorogenic Acid	0.26	1.10	-	-	-	-	5.0	5.0	-	-	-	5.5

	G_I#	G_{II}	G_{III}	G_I	G_{II}	G_{III}
Scopoletin	0.235	0.95	-	-	-	-
Ferulic Acid	0.125	0.11	0.057	-	-	-
Chlorogenic Acid	0.625	0.8	0.15	5.8	5.5	5.5
Caffeic Acid	0.51	0.43	0.31	-	-	-

* W-38 strain : Data were supplied by Powell (46).
** WR-132 strain : Data were supplied by Pickering (45).
\+ W-38 strain : Data were supplied by Reigh (44).
\# *Nicotiana tabaccum* var. Burley strain : Data were taken from Mäder et al. (72)

negligible. However, if the reaction mixture was incubated several hours at room temperature, scopoletin was oxidized as judged by an increase in absorbance at 450 nm. Pickering (45), in his study on isoperoxidase C_4 from tobacco suspension culture, also observed the slow oxidation of scopoletin by isoperoxidase C_4. In this case, high enzyme concentration was required to measure scopoletin oxidation activity of isoperoxidase C_4. Isoperoxidase C_n, therefore, appears to be rather similar, in this respect, to isoperoxidase C_4.

Ferulic Acid (4-hydroxy, 3-methoxycinnamic acid)

When isoperoxidase A_f or C_n was added to a solution containing 0.6 mM ferulic acid and 5 mM H_2O_2, a faint pink color rapidly formed which was gradually replaced by a yellow color. The spectrum of the ferulic acid reaction mixture both before and after the addition of either isoperoxidase A_f or C_n showed a significant decrease in absorbance in the 320 nm region. This spectrum was very similar to the one obtained by Pickering (45) and Powell (46) for ferulic acid oxidation of isoperoxidases A_1, A_2, C_3 and C_4 except for the presence of a small peak around the 350 nm region. The decrease in absorbancy at 320 nm was used for an assay procedure for oxidation of ferulic acid by isoperoxidases A_f and C_n. A typical assay contained 0.2 mM ferulic acid, 5 mM H_2O_2 and 40 mM sodium citrate buffer (pH 5.0 or 5.5). Either isoperoxidase A_f or isoperoxidase C_n was added to initiate the reaction.

Figures 18 and 19 show the pH optima for ferulic acid oxidation to be 5.5 for isoperoxidase A_f and 5.0 for isoperoxidase C_n, respectively. The effect of ferulic acid concentration on the activity of isoperoxidase A_f and C_n is shown in Fig. 20 and 21. Both isoperoxidases appeared to follow simple Michaelis-Menten kinetics up to a ferulic acid concentration of about

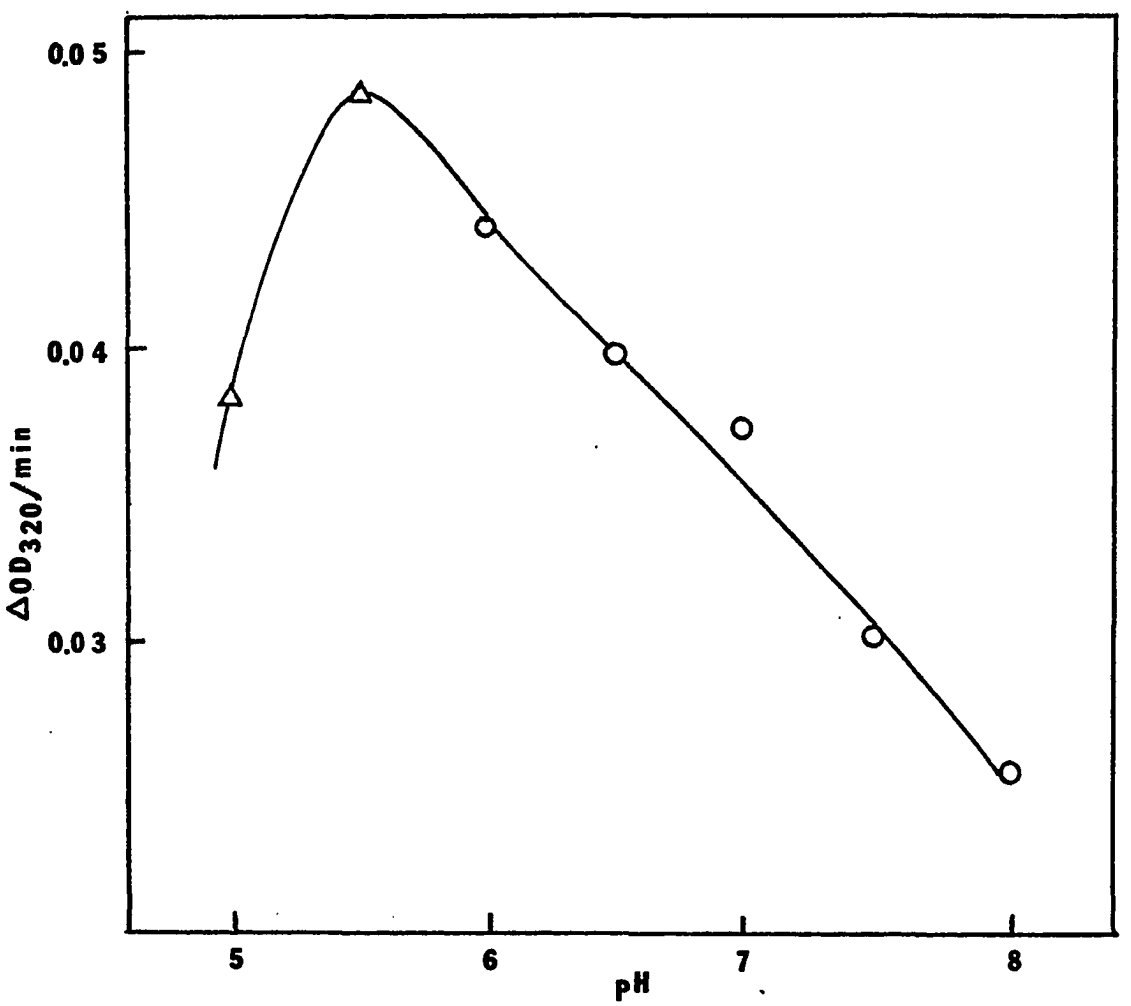


Figure 18. EFFECT OF pH ON THE FERULIC ACID OXIDATION BY ISOPEROXIDASE A_f. Assay solutions contained 0.2 mM ferulic acid and 5 mM H_2O_2 in 40 mM buffer. Δ - citrate, o - phosphate.

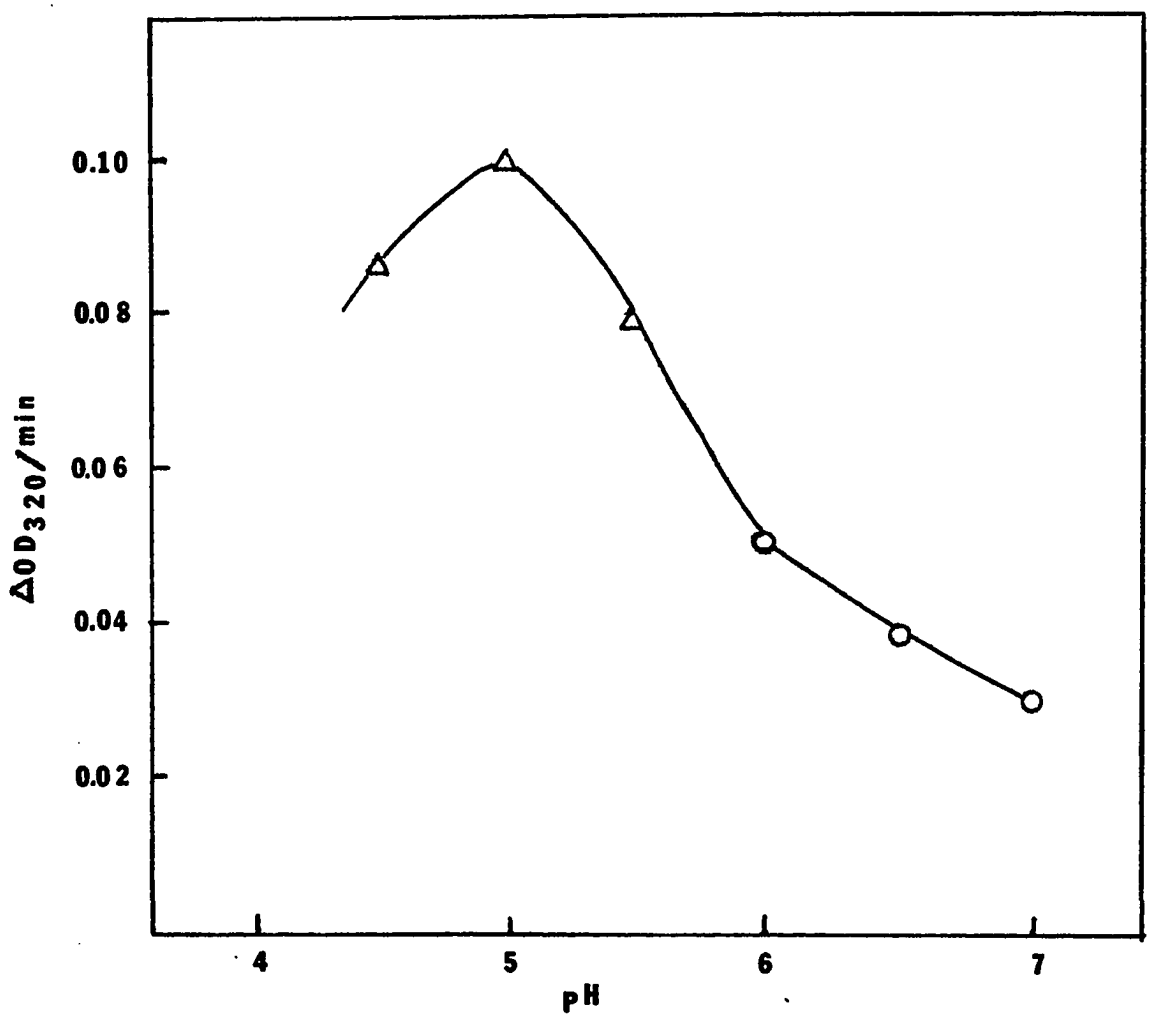


Figure 19. EFFECT OF pH ON THE FERULIC ACID OXIDATION BY ISOPEROXIDASE C_n. Assay solutions contained 0.2 mM ferulic acid and 5 mM H_2O_2 in 40 mM buffer. Δ - citrate, o - phosphate.

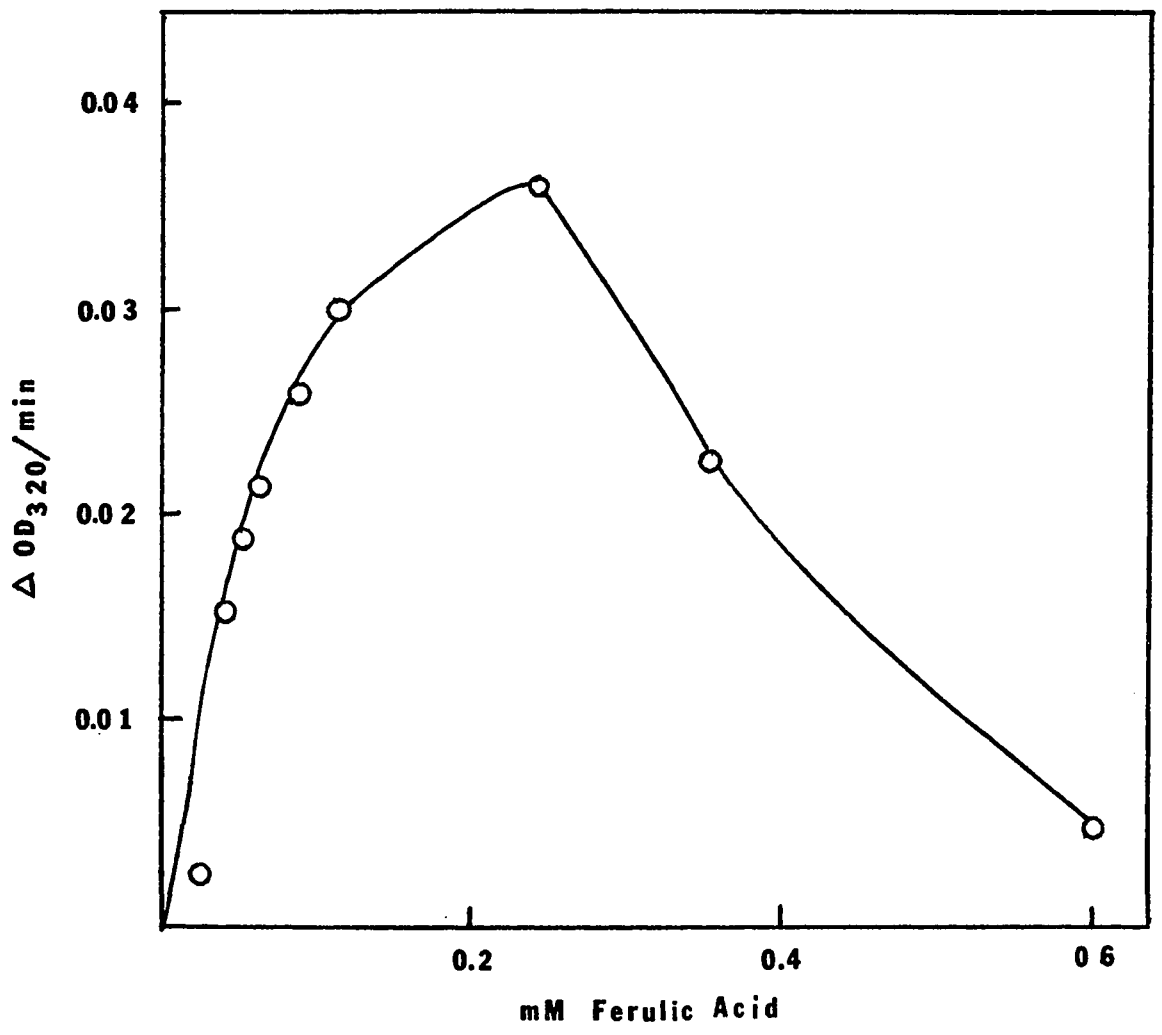


Figure 20. FERULIC ACID SATURATION CURVE FOR ISOPEROXIDASE A_f. Assays were run at 5 mM H_2O_2 in 40 mM sodium citrate buffer (pH 5.5).

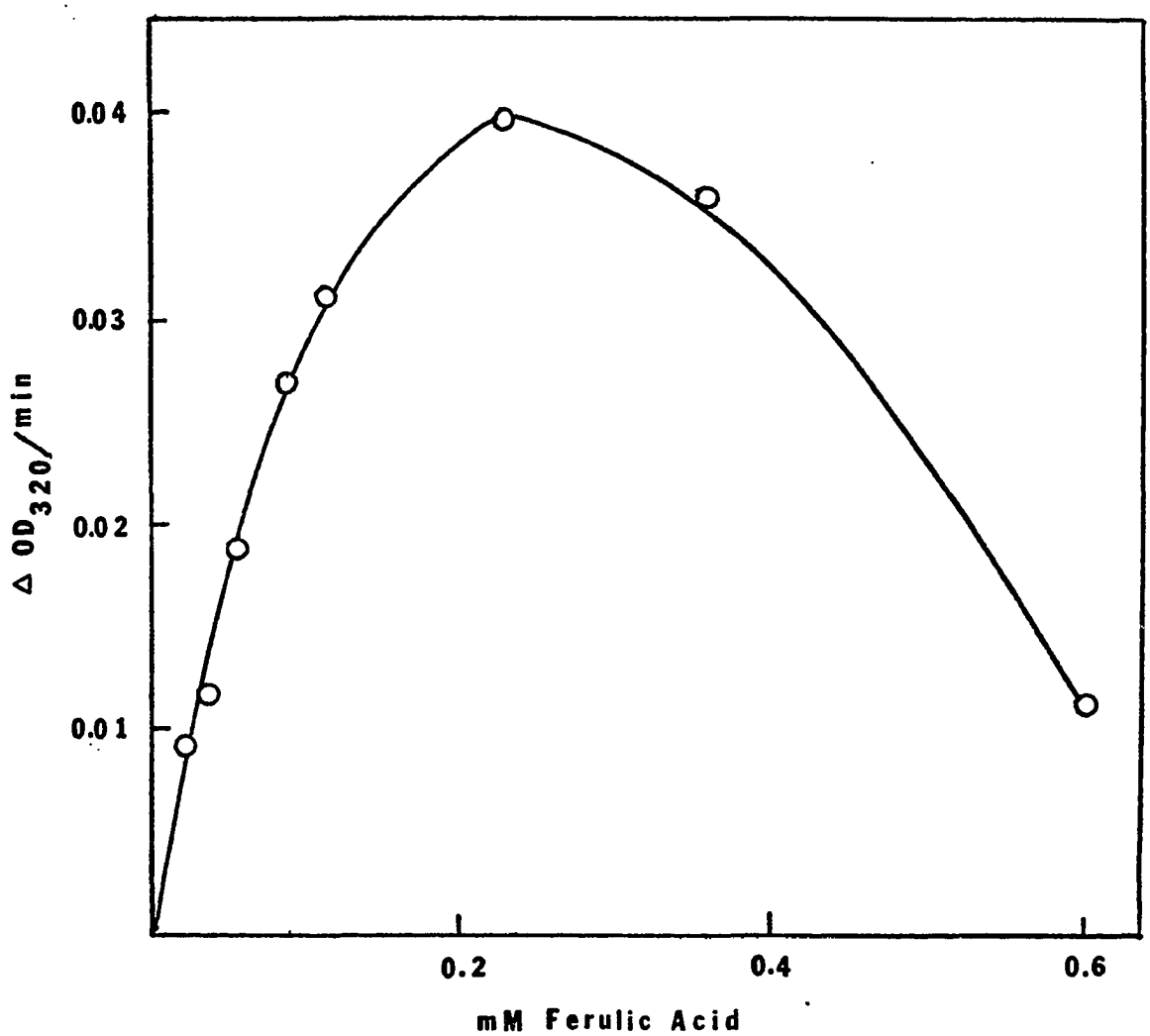


Figure 21. FERULIC ACID SATURATION CURVE FOR ISOPEROXIDASE C_n. Assays were run at 5 mM H_2O_2 in 40 mM sodium citrate buffer (pH 5.0).

0.25 mM. At this point the velocity decreased with an increase in ferulic acid concentration. Pickering (45) reported that if the isoperoxidase C_4 oxidation of ferulic acid was monitored at 360 nm, where products have higher absorbance than ferulic acid, the reaction velocity did not diminish at ferulic acid concentrations above 0.2 mM. An attempt to assay the ferulic oxidation by A_f and C_n at 360 nm failed due to the low absorbance of their products at this wavelength in the present study.

A Lineweaver-Burk plot of the data from Fig. 22 and 23 shows an $S_{0.5}$ value of 0.29 mM and 0.19 mM ferulic acid for isoperoxidases A_f and C_n, respectively. The Hill slopes of the both isoperoxidases against ferulic acid are 1.0 when calculated from the above data. The $S_{0.5}$ value of 0.29 mM for ferulic acid oxidation of isoperoxidase A_f in the present study is similar to that of isoperoxidase C_4 reported by Pickering (45). However, isoperoxidase C_n seems to have a higher affinity for ferulic acid when compared to other isoperoxidases from the same culture (Table 9).

The data presented by Mäder *et al.* (Table 9) for ferulic acid oxidation of three groups of isoperoxidase, fractions G_I, G_{II} and G_{III} from *Nicotiana tabacum* var. Burley, indicate much lower $S_{0.5}$ values for ferulic acid oxidation of three groups of isoenzymes. Furthermore, $S_{0.5}$ values for ferulic acid for anodic groups (G_I and G_{II}) are higher than for cathodic groups (G_{III}). Since their isoenzyme groups contain more than one isoperoxidase, direct comparison of $S_{0.5}$ values of isoperoxidase A_f and C_n to their results is possible.

Esculetin (6,7 dihydroxycoumarin)

Esculetin has been reported to be present in tobacco leaf (79). In contrast to scopoletin or ferulic acid, esculetin inhibited the guaiacol assay of both isoperoxidases A_f and C_n (Chapter V).

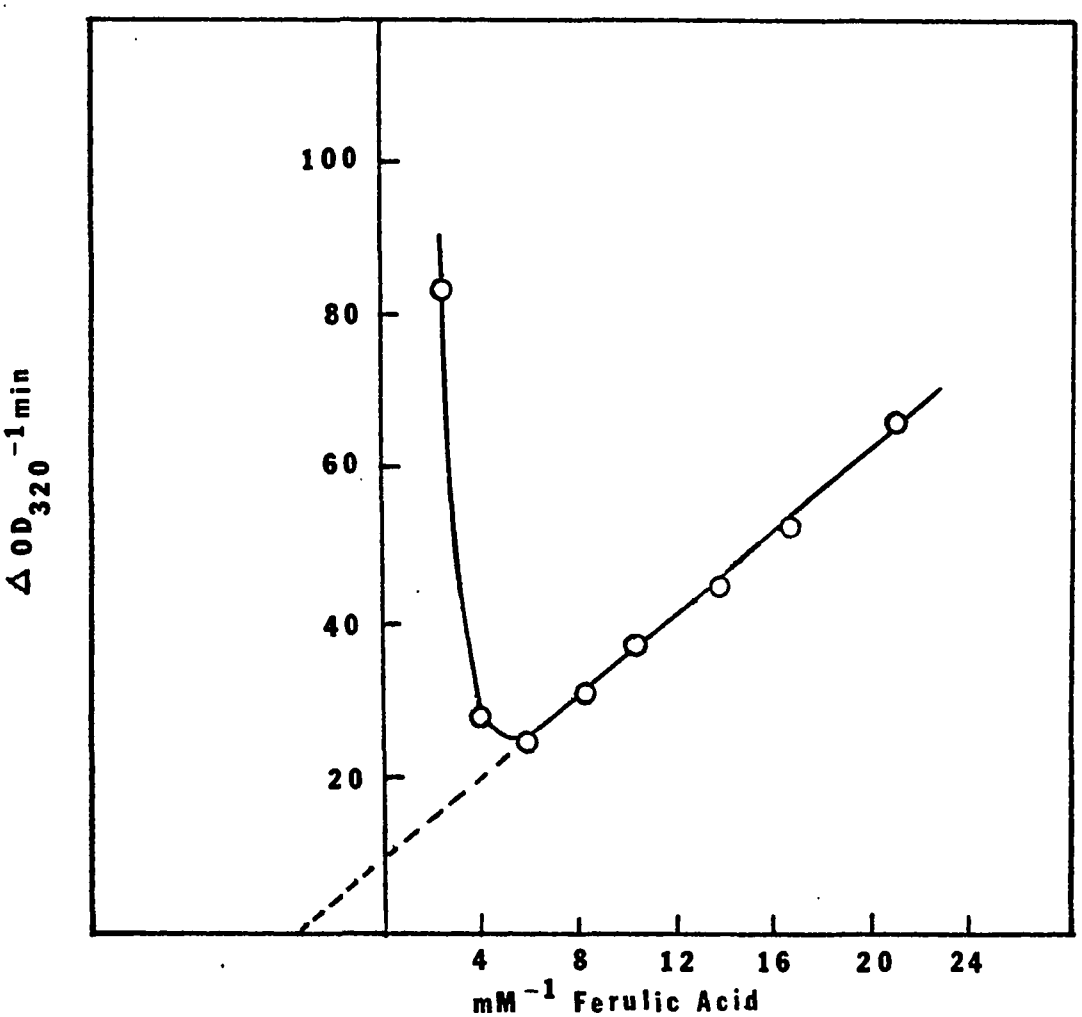


Figure 22. DOUBLE RECIPROCAL PLOT OF ACTIVITY VS. FERULIC ACID CONCENTRATION FOR ISOPEROXIDASE A_f. Assays were run at 5 mM H_2O_2 in 40 mM sodium citrate buffer (pH 5.5).

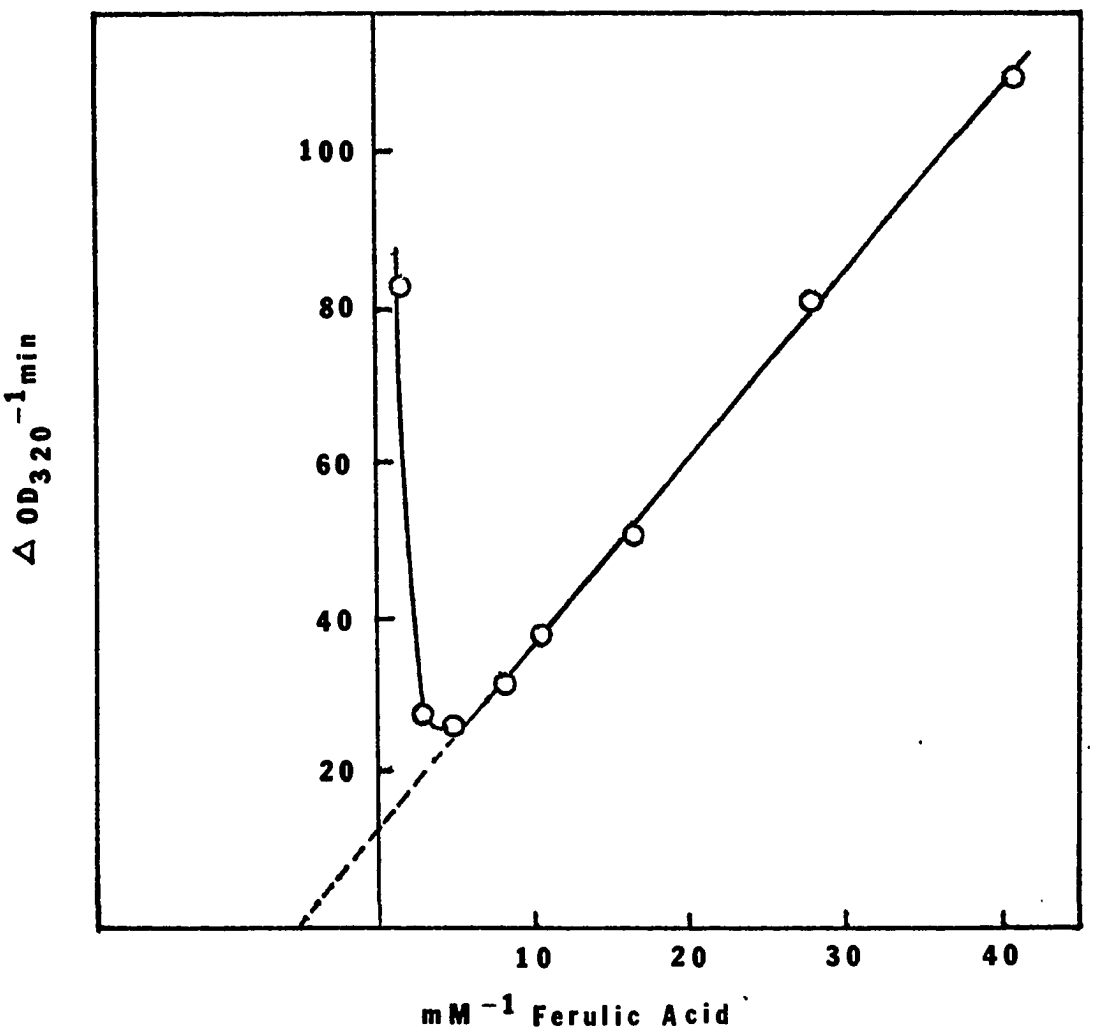


Figure 23. DOUBLE RECIPROCAL PLOT OF ACTIVITY VS. FERULIC ACID CONCENTRATION FOR ISOPEROXIDASE C_n. Assays were run at 5 mM H_2O_2 in 40 mM sodium citrate buffer (pH 5.0).

When isoperoxidase A_f or C_n was added to a mixture of 1.2 mM esculetin, 5 mM H_2C_2, and 40 mM phosphate buffer (pH 7.5), the formation of a yellow product was observed. The yellow product absorbed between 450 - 470 nm while esculetin absorbed negligibly at this wavelength. The absorption spectrum of the esculetin reaction mixture for isoperoxidases A_f or C_n was similar to that of isoperoxidase A_3 reported by Reigh (44). The increase in absorbance at 469 nm was used to investigate esculetin oxidation catalyzed by isoperoxidases A_f and C_n. The pH optimum for esculetin oxidation for both isoperoxidases was 7.5 (Fig. 24 and 25). Figures 26 and 27 show the substrate saturation curve for esculetin oxidation of isoperoxidase A_f and C_n, respectively. A Lineweaver-Burk plot of these data is linear and yields $S_{0.5}$ for esculetin equal to 0.31 mM and 1.25 mM for isoperoxidase A_f and C_n, respectively (Fig. 28 and 29). The Hill slopes for both isoperoxidases are 1.0 when calculated from Fig. 28 and 29. The $S_{0.5}$ value and pH optimum for esculetin oxidation of isoperoxidase A_f indicate further the probable identity between isoperoxidase A_f and isoperoxidase A_3 from tobacco callus culture reported by Reigh (44). Isoperoxidase A_3 exhibited a pH optimum of 7.5 and a $S_{0.5}$ value of 0.31 mM against esculetin oxidation.

However, the two isoperoxidases A_f and C_n behaved quite differently in their oxidation of esculetin. Isoperoxidase A_f shows a much higher affinity for esculetin than isoperoxidase C_n (Table 9).

Chlorogenic Acid (3-O-caffeoylquinic acid)

Chlorogenic acid along with scopoletin and ferulic acid have been identified in tobacco tissue cultures (48, 80). When isoperoxidase A_f or C_n was added to a mixture of 1.2 mM chlorogenic acid, 5 mM H_2O_2 and 40 mM citrate buffer (pH 5.0), a greenish-yellow color formed. The absorption spectra of the reacted mixture had a broad absorption peak around 400 nm

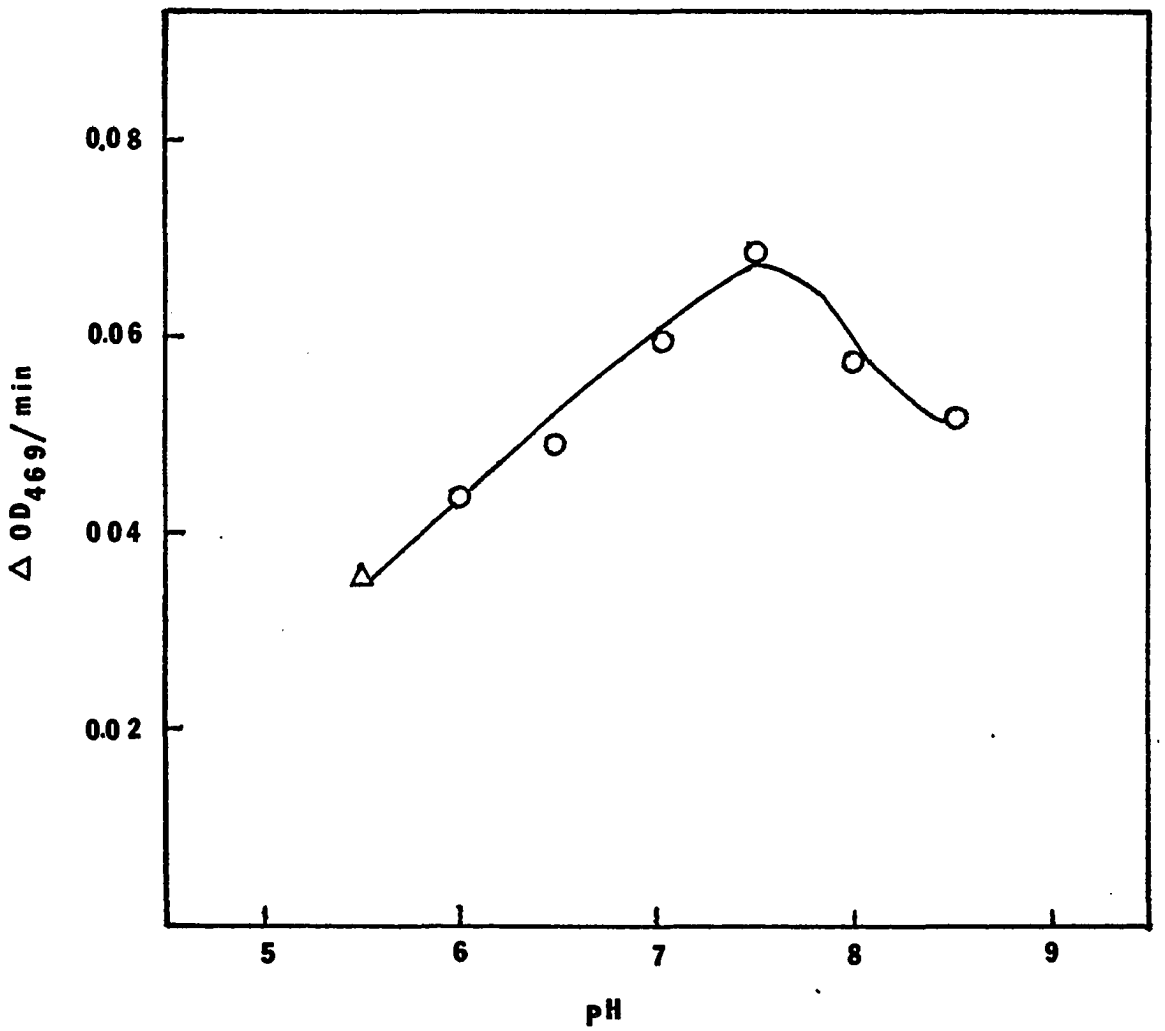


Figure 24. EFFECT OF pH ON THE ESCULETIN OXIDATION BY ISOPEROXIDASE A_f. Assays were run at 1.2 mM esculetin and 5 mM H_2O_2 in 40 mM buffer. Δ - citrate, o - phosphate.

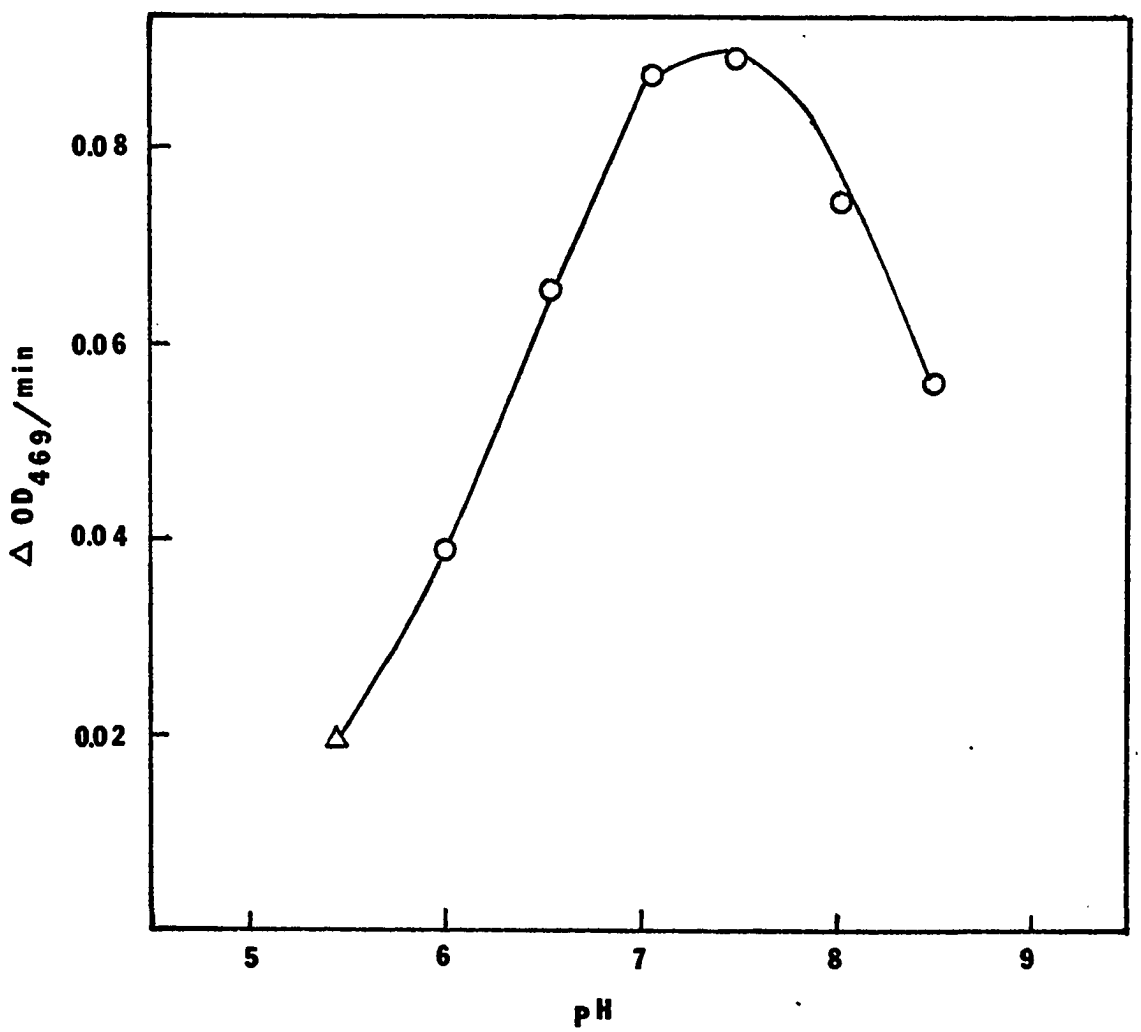


Figure 25. EFFECT OF pH ON THE ESCULETIN OXIDATION BY ISOPEROXIDASE C_n. Assays were run at 1.2 mM esculetin and 5 mM H_2O_2 in 40 mM buffer. Δ - citrate, o - phosphate.

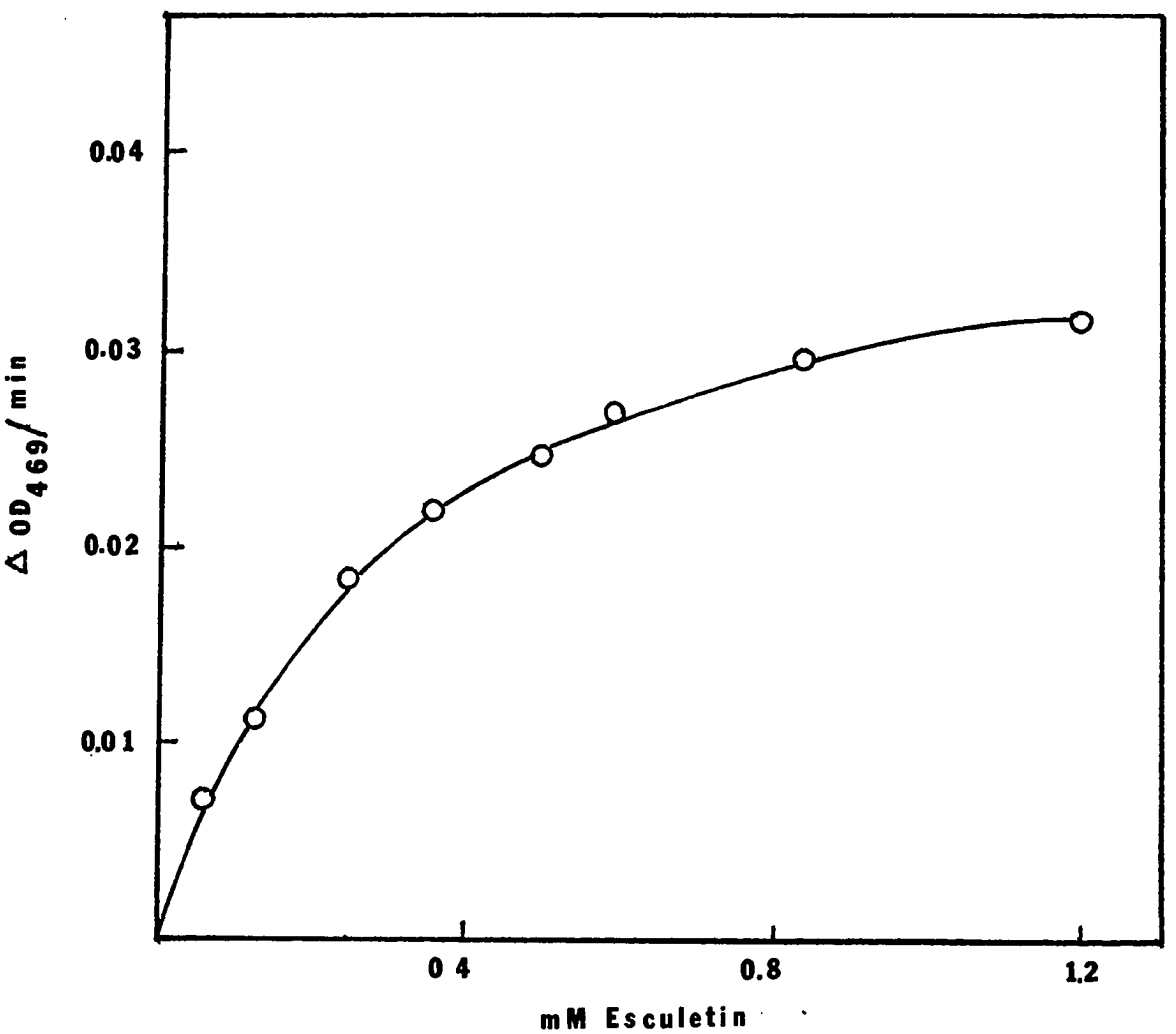


Figure 26. ESCULETIN SATURATION CURVE FOR ISOPEROXIDASE A_f. Assays were run at 5 mM H_2O_2 in 40 mM phosphate buffer (pH 7.5).

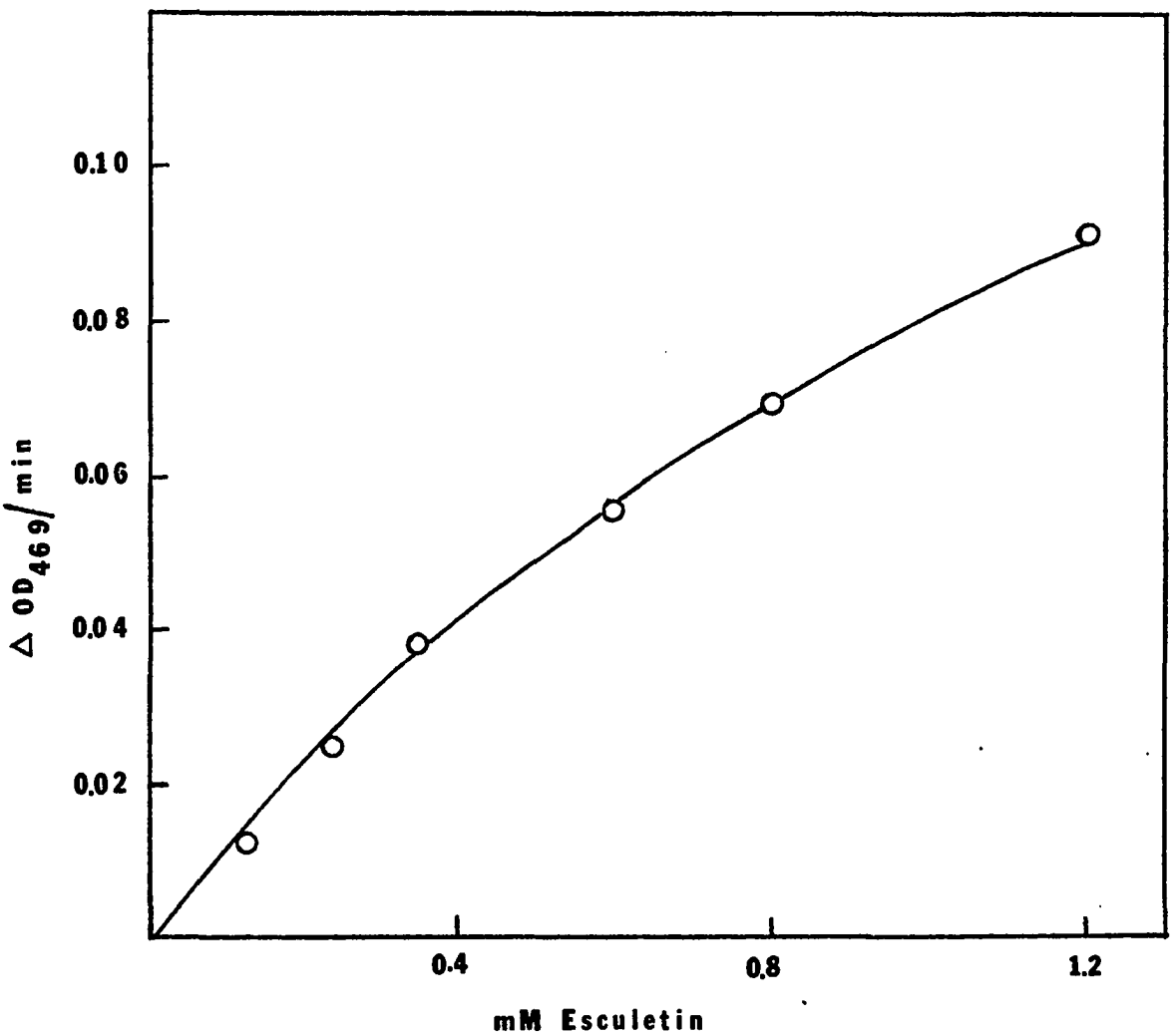


Figure 27. ESCULETIN SATURATION CURVE FOR ISOPEROXIDASE C_n. Assays were run at 5 mM H_2O_2 in 40 mM phosphate buffer (pH 7.5).

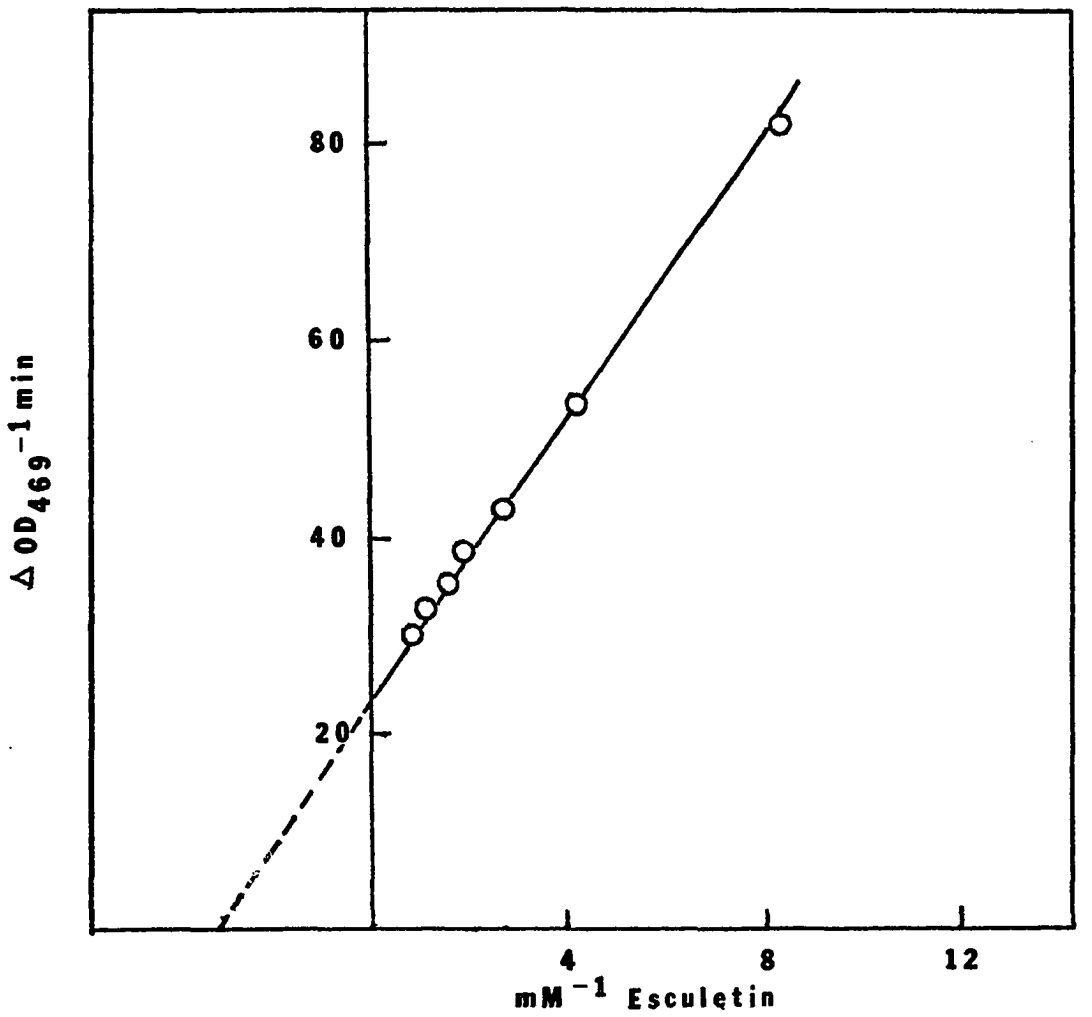


Figure 28. DOUBLE RECIPROCAL PLOT OF ACTIVITY VS. ESCULETIN CONCENTRATION FOR ISOPEROXIDASE A_f. Assays were run at 5 mM H_2O_2 in 40 mM phosphate buffer (pH 7.5).

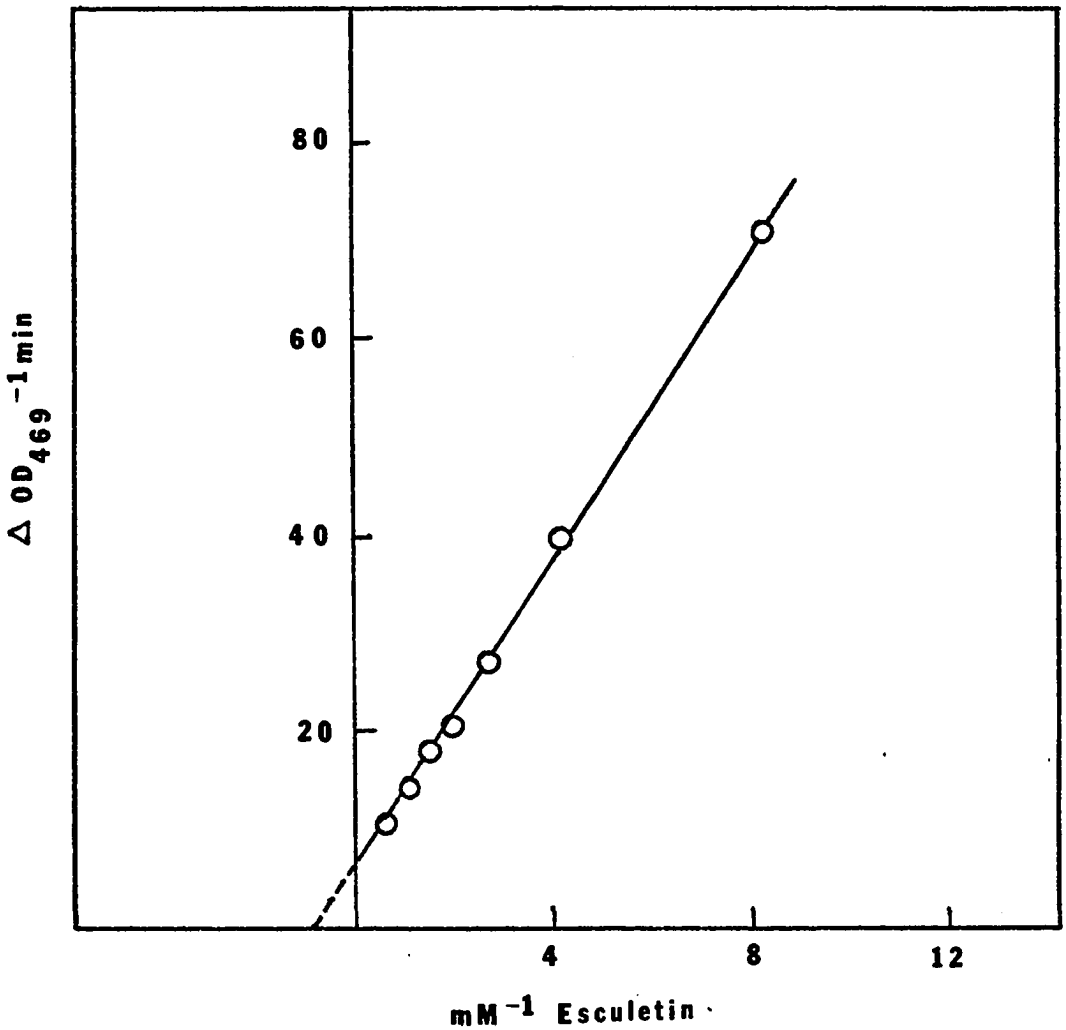


Figure 29. DOUBLE RECIPROCAL PLOT OF ACTIVITY VS. ESCULETIN CONCENTRATION FOR ISOPEROXIDASE C_n. Assays were run at 5 mM H_2O_2 in 40 mM phosphate buffer (pH 7.5).

(Fig. 30). The increase in absorbance at 400 nm was thus used to assay chlorogenic acid oxidation by isoperoxidases A_f and C_n. Since a linear rate was not observed at high substrate concentration, the slope of the tangent line of each assay curve from zero time was used as an initial velocity. The pH optimum for chlorogenic acid oxidation was determined to be 5.0 and 4.5 for isoperoxidases A_f and C_n, respectively, as shown in Fig. 31 and 32. The dependence of initial velocity on chlorogenic acid concentration of isoperoxidases A_f and C_n is indicated on Fig. 33 and 34. The Lineweaver-Burk plot of these data is linear, and the $S_{0.5}$ values of 0.26 mM and 1.10 mM were obtained for isoperoxidases A_f and C_n (Fig. 35 and 36). Hill slopes for both isoperoxidases were determined to be 1.0, using Lineweaver-Burk plots of both isoenzymes.

Comparative Substrate Utilization of Isoperoxidases A_f and C_n

In the investigation of the specificity of isoperoxidases A_f and C_n for naturally occurring compounds, four phenolic compounds which affected the guaiacol oxidizing ability of the enzymes were examined as possible substrates. These are scopoletin, ferulic acid, esculetin and chlorogenic acid. Since extinction coefficients for the various products are not known, it is impossible to determine the rates of oxidation of the different substrates quantitatively, but it can be demonstrated that one isoperoxidase probably utilizes a particular substrate better than another isoperoxidase. This can be done by comparing the oxidation rates of these substrates to the guaiacol oxidation rate for the various isoperoxidases. All peroxidase preparations were adjusted to obtain a uniform guaiacol oxidation rate (ΔOD 470/min = 0.1 for isoperoxidases A_f and C_n). The velocity obtained for the other four substrates was then compared to guaiacol according to the method of Reigh (44);

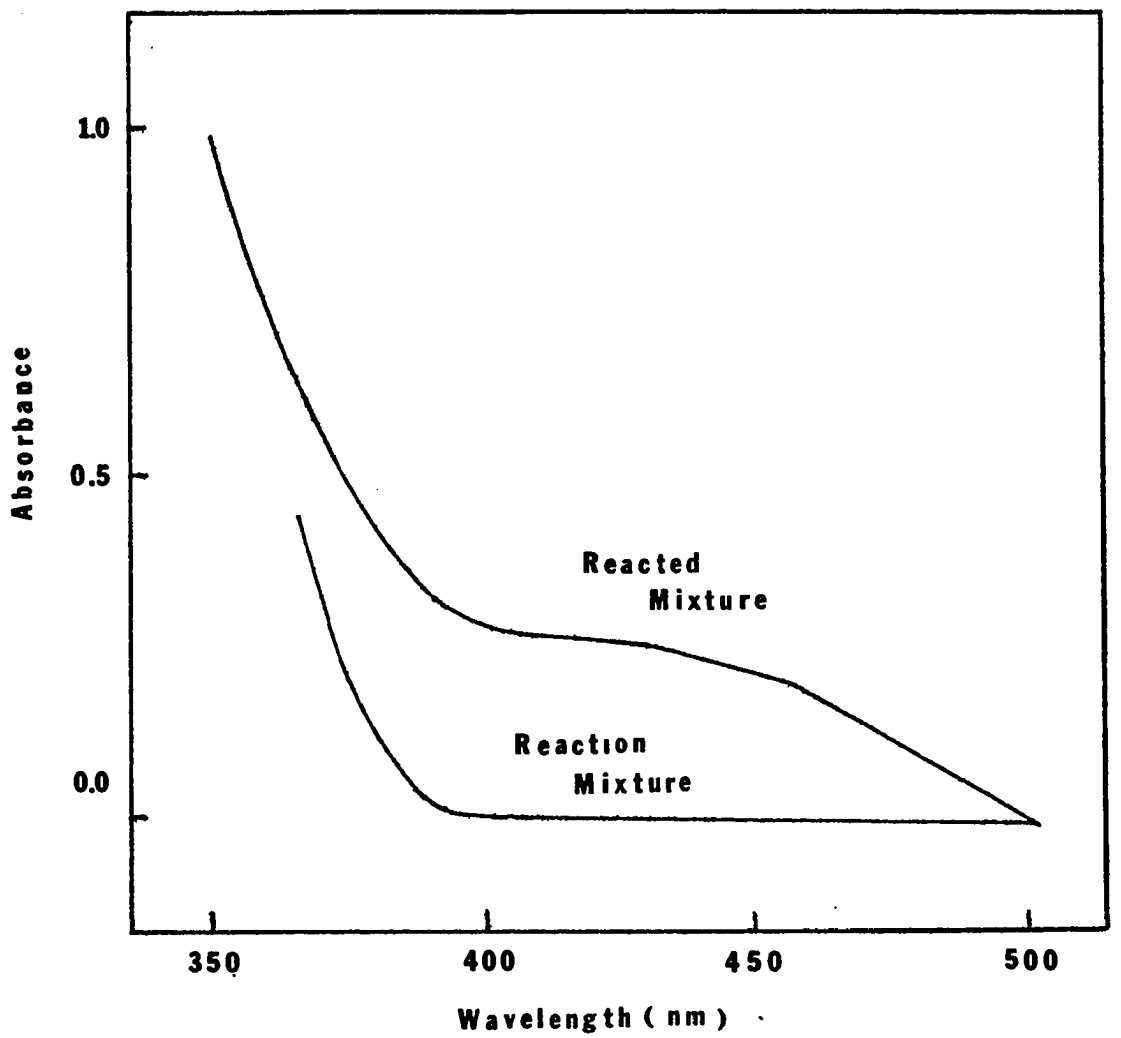


Figure 30. ABSORPTION SPECTRA OF CHLOROGENIC ACID REACTION MIXTURES. Assay solutions contained 1.2 mM chlorogenic acid and 5 mM H_2O_2 in 40 mM sodium citrate (pH 5.0).

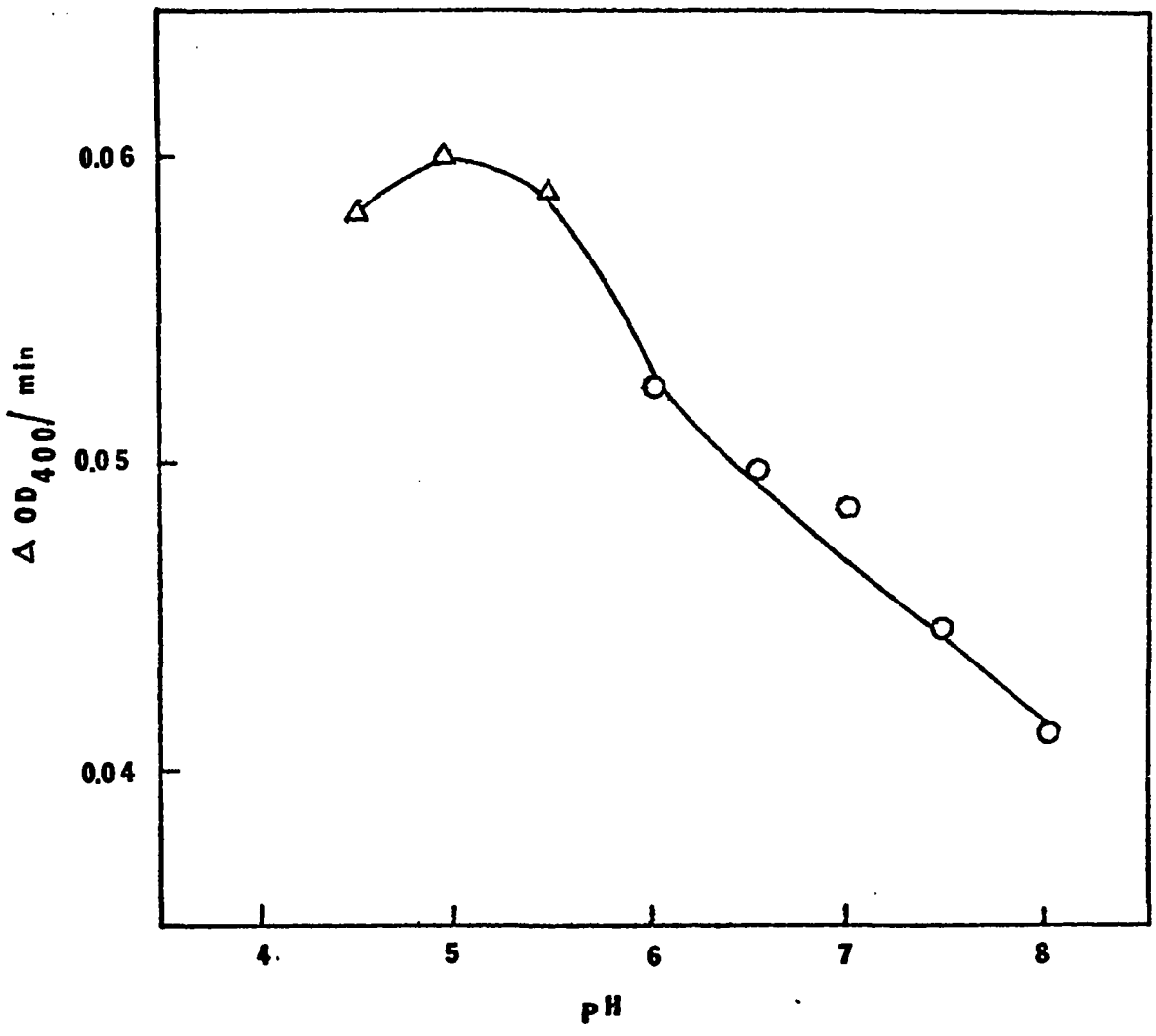


Figure 31. EFFECT OF pH ON THE CHLOROGENIC ACID OXIDATION BY ISOPEROXIDASE A_f. Assays were run at 1.2 mM chlorogenic acid and 5 mM H_2O_2 in 40 mM buffer. Δ - citrate, o - phosphate.

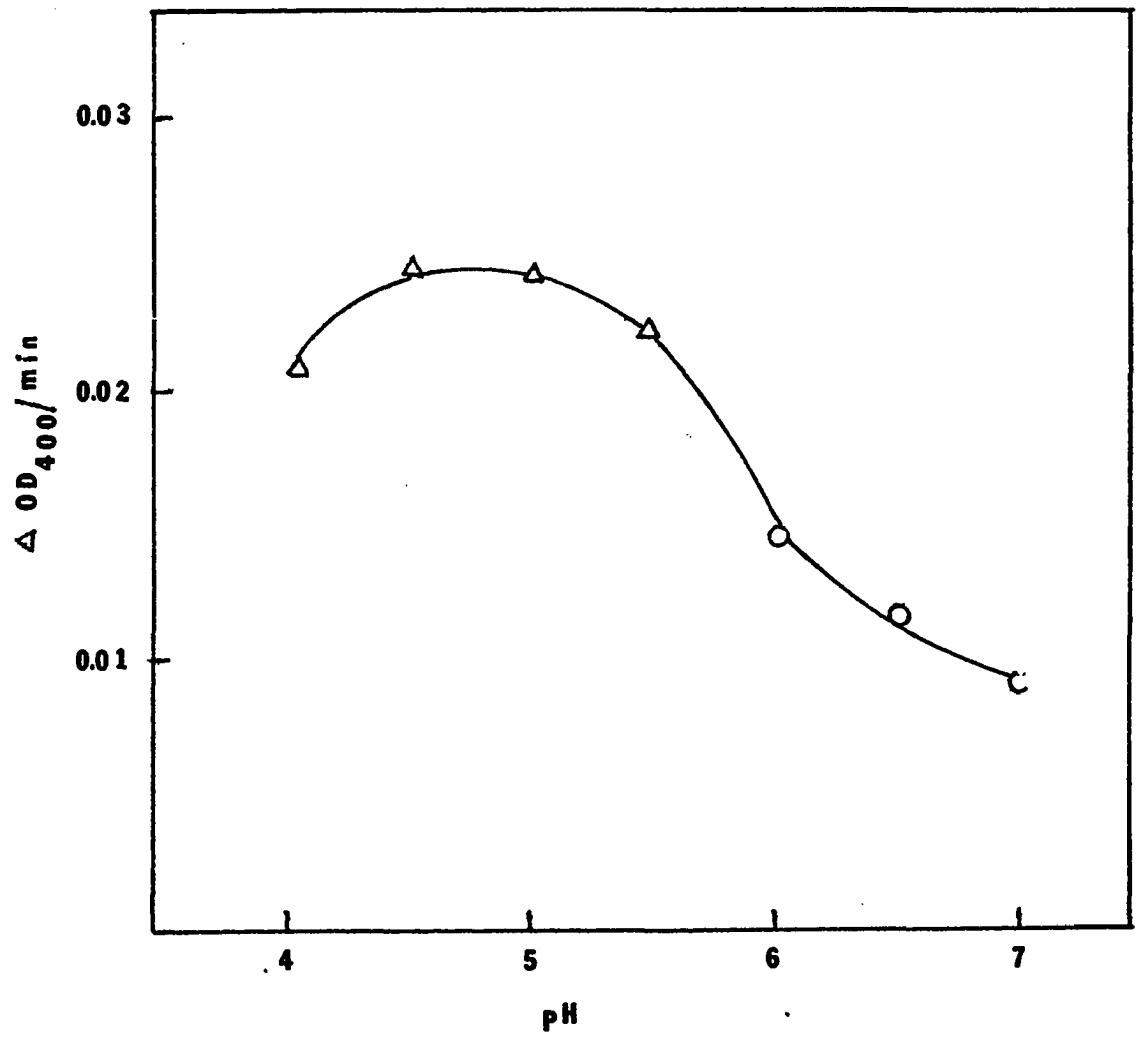


Figure 32. EFFECT OF pH ON THE CHLOROGENIC ACID OXIDATION BY ISOPEROXIDASE C_n. Assays were run at 1.2 mM chlorogenic acid and 5 mM H_2O_2 in 40 mM buffer. Δ - citrate, o - phosphate.

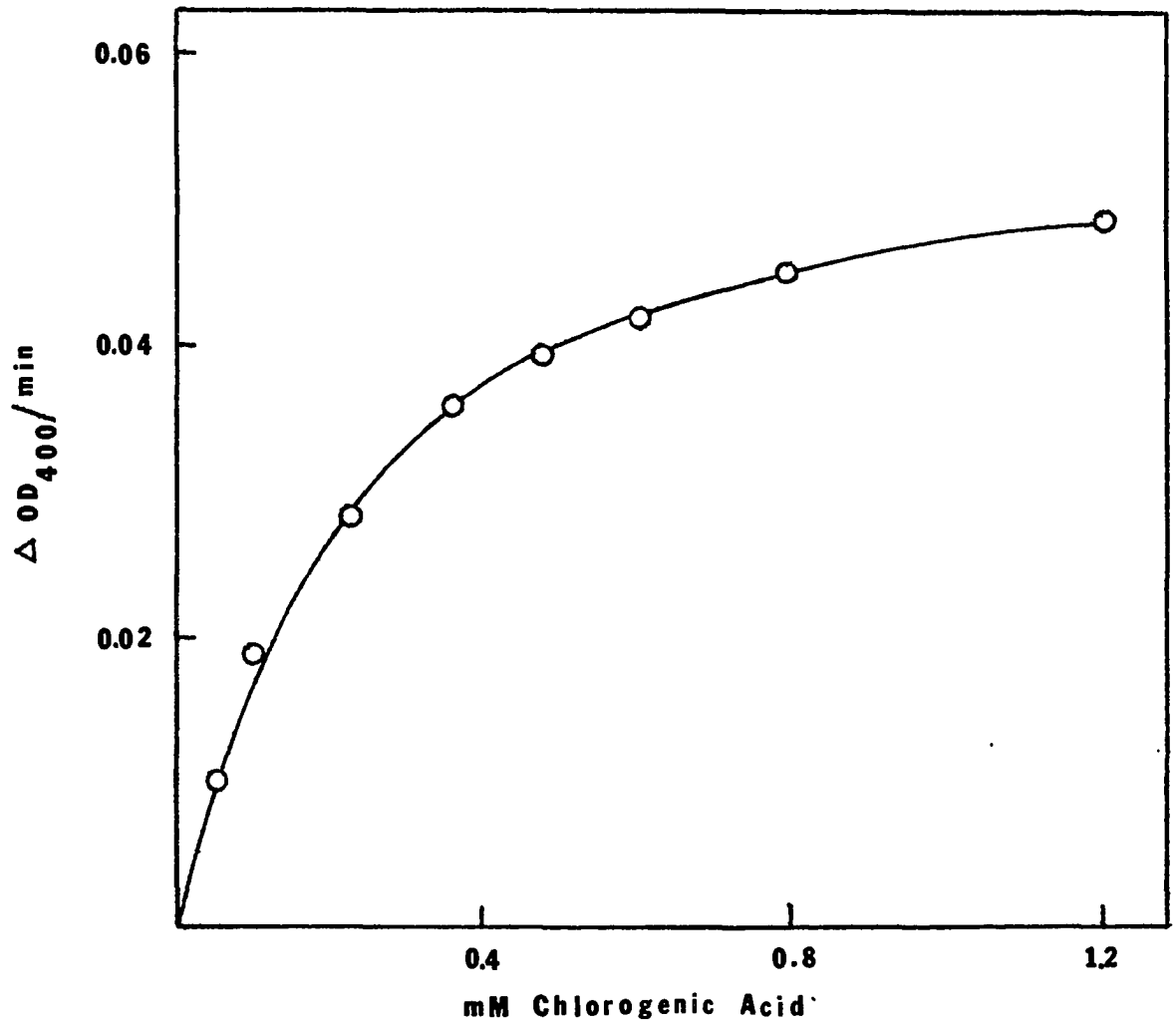


Figure 33. CHLOROGENIC ACID SATURATION CURVE FOR ISOPEROXIDASE A_f. Assays were run at 1.2 mM chlorogenic acid and 5 mM H_2O_2 in 40 mM sodium citrate (pH 5.0).

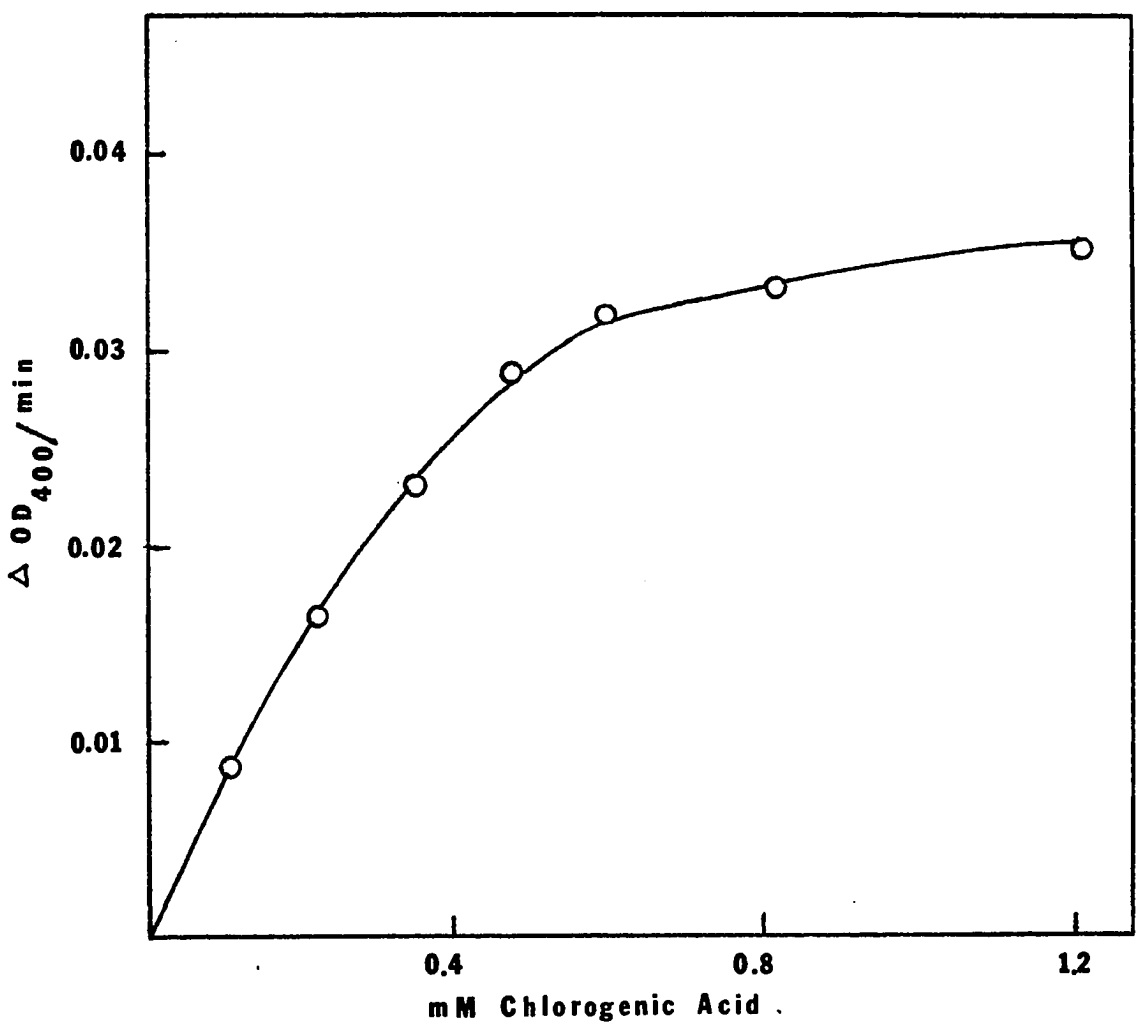


Figure 34. CHLOROGENIC ACID SATURATION CURVE FOR ISOPEROXIDASE C_n. Assays were run at 1.2 mM chlorogenic acid and 5 mM H_2O_2 in 40 mM sodium citrate (pH 4.5).

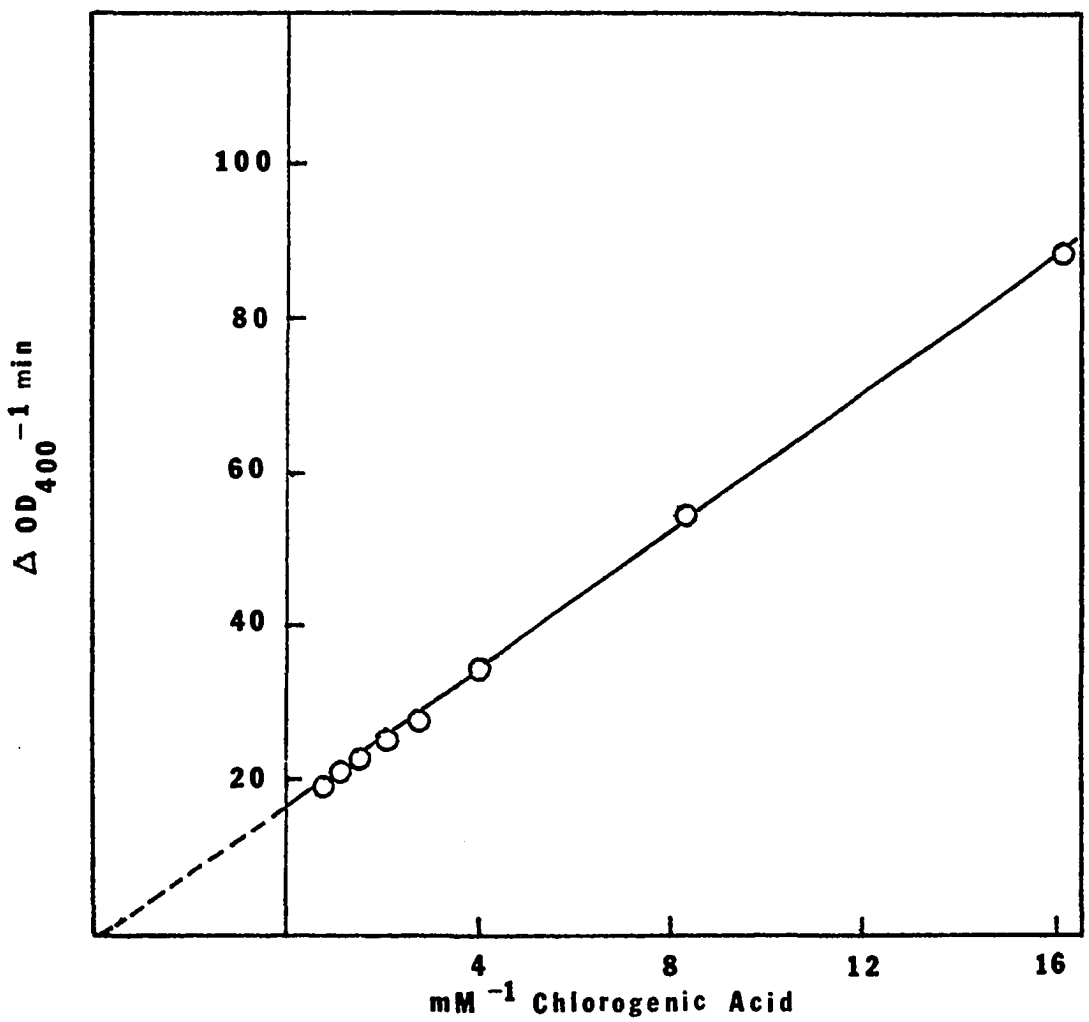


Figure 35. DOUBLE RECIPROCAL PLOT OF ACTIVITY VS. CHLOROGENIC ACID CONCENTRATION FOR ISOPEROXIDASE A_f. Assays were run at 1.2 mM chlorogenic acid and 5 mM H_2O_2 in 40 mM sodium citrate (pH 5.0).

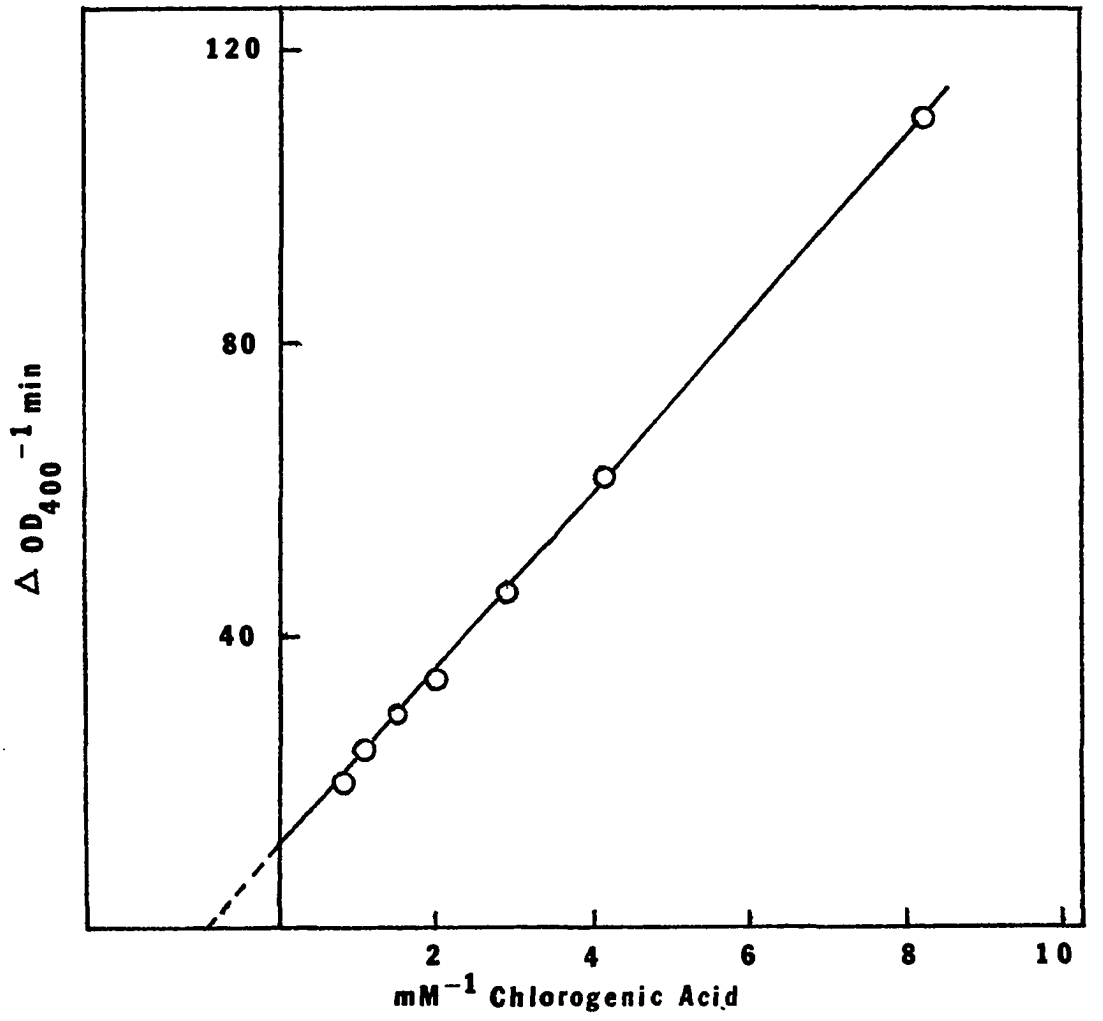


Figure 36. DOUBLE RECIPROCAL PLOT OF ACTIVITY VS. CHLOROGENIC ACID CONCENTRATION FOR ISOPEROXIDASE C_n. Assays were run at 1.2 mM chlorogenic acid and 5 mM H_2O_2 in 40 mM sodium citrate (pH 4.5).

$$\text{Activity Ratio} = \frac{\Delta OD/min \text{ Natural Compound}}{\Delta OD/min \text{ Guaiacol}}$$

Typical assay conditions and wavelength are listed in Table 10. The pH was maintained at the pH optima for the activity of isoperoxidases A_f and C_n with each of the five substrates. Table 11 shows the oxidation ratios for various substrates with various peroxidase preparations. Although any attempt to draw quantitative conclusions from these data is premature, it is apparent that isoperoxidases A_f and C_n are quite different from each other with respect to their substrate preference. It appears that isoperoxidase A_f utilizes scopoletin much more readily than isoperoxidase C_n. A_f also utilizes esculetin and chlorogenic acid better than C_n. Reigh (44) reported that an anodic isoperoxidase A_3 isolated from W-38 tobacco tissue culture is 100 -150 times more active in catalyzing the oxidation of scopoletin than either isoperoxidase A_1 or A_2 from the same source. A_1 and A_2 are isoperoxidases isolated from tobacco tissue culture W-38 by Powell (46). As indicated in Table 11, the substrate oxidation ratios of isoperoxidase A_f against scopoletin and chlorogenic acid are the same as those of isoperoxidase A_3 reported by Reigh (44). Reigh used 310 nm for assaying ferulic acid oxidation of isoperoxidase A_3. However, the different oxidation ratio against ferulic acid between isoperoxidase A_f and A_3 cannot be explained by the different wavelength used between the two studies, since the oxidation ratio of isoperoxidase A_f against ferulic acid was the same whether the oxidation was followed at 310 nm or 320 nm. It is plausible that an alteration of either isoenzyme during purification or a contamination of isoperoxidase A_f or A_3 with other isoperoxidases may have caused the difference in ferulic acid and esculetin oxidation ratios between the two isoenzymes. In constrast to A_f, isoperoxidase C_n is not capable of

TABLE 10

ASSAY CONDITIONS FOR COMPARISON EXPERIMENTS OF ISOPEROXIDASES A_f AND C_n

Substrate	H_2O_2	Buffer	Activity
1.2 mM Esculetin	5 mM	40 mM pH 7.5	ΔOD_{469}/ min
1.2 mM Scopoletin	5 mM	40 mM pH 5.0	ΔOD_{450}/ min
1.2 mM Chlorogenic Acid	5 mM	40 mM pH 4.5	ΔOD_{400}/ min
0.2 mM Ferulic acid	5 mM	40 mM pH 5.0	ΔOD_{320}/ min
15 mM Guaiacol	5 mM	40 mM pH 6.5	ΔOD_{470}/ min

TABLE 11

RELATIVE SUBSTRATE OXIDATION RATIOS FOR VARIOUS PEROXIDASE PREPARATIONS FROM TOBACCO TISSUE CULTURE

	Scopoletin	Ferulic acid	Chlorogenic acid	Esculetin
A_f	4.0	0.98	2.0	1.7
C_n	0.0	1.2	0.32	0.09
* A_1	0.03	2.8	0.63	0.35
* A_2	0.02	2.0	0.95	0.33
** A_3	4.0-5.0	3.5-5.0	1.0-2.0	0.39-0.48
+ C_4	0.01	2.20	0.46	0.63

* W-38 strain : Data were supplied by Powell (46).

** W-38 Strain : Data were supplied by Reigh (44).

+ WR-132 strain : Data were supplied by Pickering(45).

catalyzing the oxidation of scopoletin to any significant degree. On the other hand, ferulic acid is apparently the preferred substrate, among those tested, of isoperoxidase C_n. Pickering (45) isolated two cathodic isoperoxidases C_3 and C_4 from tobacco suspension culture WR-132 grown under normal conditions. Although all of the above phenolic compounds tested were evidently oxidized by isoperoxidases C_3 and C_4 in the presence of hydrogen peroxide, ferulic acid was the preferred substrate of those investigated, based on Km value and substrate activity ratios. Isoperoxidase C_n seems to be rather similar to isoperoxidase C_3 and C_4 in terms of substrate oxidation ratios when tested against four naturally-occurring phenolic compour

CHAPTER VII

FINGERPRINTING STUDY

The existence of multiple forms of peroxidase has been reported for many plants (13). The studies of individual isoperoxidases from many different plants indicate that physical, kinetic properties and substrate preferences of isoperoxidases from a single source may vary significantly (14,16,17,18,71). Although there is a considerable amount of information concerning physico-chemical and catalytic properties, information concerning structural differences among the multiple forms of peroxidase is limited.

Shih et al. (15), on the basis of tryptic peptide map experiments, showed that five horseradish peroxidase isoenzymes can be segregated into at least three distinct groups. They further indicated that there may be many points of difference in primary structure among the three groups. Welinder and Mazza (20) studied in detail the tryptic peptide patterns of five peroxidases from turnip and horseradish. The study showed that turnip peroxidases P_1 and P_2 are almost identical whereas the maps of other peroxidases are very different. They further reported that there are two highly homologous sequences containing histidine in all five examined peroxidases.

In the present study, peptide mapping of trypsin digests of eight isoperoxidases from tobacco suspension culture WR-132 (C_n, C_3, C_4, A_c and A_f) and tobacco callus culture W-38 (A_1, A_2 and A_3) was carried out in an attempt to clarify the structural relationship of these isoperoxidases.

It was also hoped that the derepression of isoperoxidase A_f in total darkness described earlier in Chapter III could be proved by comparing the trypsin peptide map of isoperoxidase A_f to be identical to isoperoxidase A_3 from tobacco callus culture W-38.

MATERIALS AND METHODS

Preparation of Enzymes

Isoperoxidases A_f, A_c and C_n were isolated from tobacco suspension culture WR-132 as described in Table 4, Chapter 4. Isoperoxidases C_3 and C_4 from the same source were purified by CM-cellulose column chromatography of 20 - 90% $(NH_4)_2SO_4$ fractionated media or cell extracts of normal grown cells according to the method of Pickering (45), with slight modification. Isoperoxidase C_4 was eluted by washing the column with 20 mM phosphate buffer (pH 6.0), and isoperoxidase C_3 was obtained by subsequent elution with 45 mM phosphate buffer (pH 6.0). Isoperoxidases A_1 and A_2 of tobacco callus culture were gifts from Dr. B. Powell. Isoperoxidase A_3 was isolated by $(NH_4)_2SO_4$ fractionation (20 - 90%) of tobacco callus culture W-38 cell extracts followed by DEAE-cellulose column chromatography (44). The elution was stepwise with phosphate buffer, pH 6.0 (5 → 15 → 40 → 60 mM). The isoperoxidase A_3 was obtained by eluting a DEAE-cellulose column with 40 mM phosphate buffer pH 6.0. All of the isoperoxidases were further purified by subjecting each isoenzyme fraction to Sephadex G-150 filtration (90 X 2.5 column).

Two-dimensional Chromatography and High Voltage Electrophoresis

Two-dimensional paper chromatography and high voltage electrophoresis were performed according to the methods of Helinski and Yanofsky (63) with slight modification. Descending chromatography was done using a solvent

of secondary butanol : formic acid (90%) : water (70:10:20) for approximately 15 hours with methyl red as a indicator. High voltage electrophoresis was performed using the apparatus manufactured by Savant for 1 hour at 2,400 V. The solvent consisted of pyridine : acetic acid: water (1:10:300), pH 3.5. Quinine sulfate was used as an indicator. The chromatogram was developed by spraying with 0.5% ninhydrin in acetone, and heating at 100°C for 20 minutes.

Preparation of Samples for Chromatography and Electrophoresis

For the preparation of samples, 5 mg of each isoperoxidase was dissolved in 1 ml of 0.05 M ammonium carbonate buffer, pH 8.3. The vials containing isoperoxidases were then immersed in a boiling water bath for 30 seconds. Eighty µg of trypsin (Sigma) in 0.5 ml of the above buffer was added to each sample and incubated at 30°C in a water bath for 1½ hours. The reaction mixture was placed in a boiling water bath for 5 min to stop the reaction and centrifuged at 27,000 X g for 15 minutes. The resulting supernatant fluid was collected and lyophilized. The lyophilized sample was dissolved in 0.1 ml deionized water and then spotted on Whatman 3 MM filter paper.

RESULTS AND DISCUSSION

The results of the peptide pattern studies are shown in Fig. 37 through Fig. 44 and the number of overlapping tryptic peptides of each isoperoxidase is summarized in Table 12. Matching or overlapping peptides were counted by overlapping the origin of each tryptic peptide pattern (Fig. 37 Fig. 44) after drawing 1 cm square lines from the origin. If any two tryptic peptides fall on the same 1 cm square line and at least touch each other, then they were defined as the matching or overlapping peptides. Comparison

of the peptide maps among three cathodic isoperoxidases (C_n, C_4 and C_3) indicates that isoperoxidase C_3 appears to be more closely related to the isoperoxidase C_4 than isoperoxidase C_n since almost half of the C_4 tryptic peptides (22 among 45 tryptic peptides) could possibly match those of C_3 (Fig. 37, 38 and 39. Table 12) whereas only 15 overlapping peptides were found in comparison of isoperoxidases C_3 and C_n. This might be expected since C_3 and C_4 migrate so close together to the cathode upon electrophoresis. Approximately 15 tryptic peptides of C_n overlapped with those of C_3 and C_4 (Table 12). A composite tryptic peptides map of cathodic isoperoxidases is indicated in Fig. 45. It appears that there are approximately 9 to 10 homologous tryptic peptides present in all three cathodic isoperoxidases C_3, C_4 and C_n. These homologous peptides are circled in Fig. 45 and homologous peptides which are possibly present in all isoperoxidases (including anodics) tested are represented as T_1, T_2 and T_3.

As far as anodic isoperoxidases are concerned, the peptide patterns of isoperoxidases A_1 and A_2 from tobacco callus culture W-38 were very similar (Table 12), though the molecular weights and subunit structure of the two isoperoxidases are dissimilar (Table 7, Chapter 4). Approximately half of the peptides (35 peptides) of both isoperoxidases overlapped each other. The peptide maps of isoperoxidases A_c and A_f, as shown in Fig. 42 and 43, indicated some similarities of both isoperoxidases to isoperoxidases A_1 and A_2. As expected, isoperoxidase A_c has more overlapping peptides than isoperoxidases A_f with isoperoxidases A_1 and A_2 (Table 12). Many overlapping peptides were also found in a comparison of isoperoxidases A_c and A_f. Half of the A_f tryptic peptides (22 of 43 peptides) match those of A_c. Fig. 46 represents the composite tryptic peptide map of anodic isoperoxidases. Eight homologous tryptic peptides, which are present in all anodic isoperoxidases

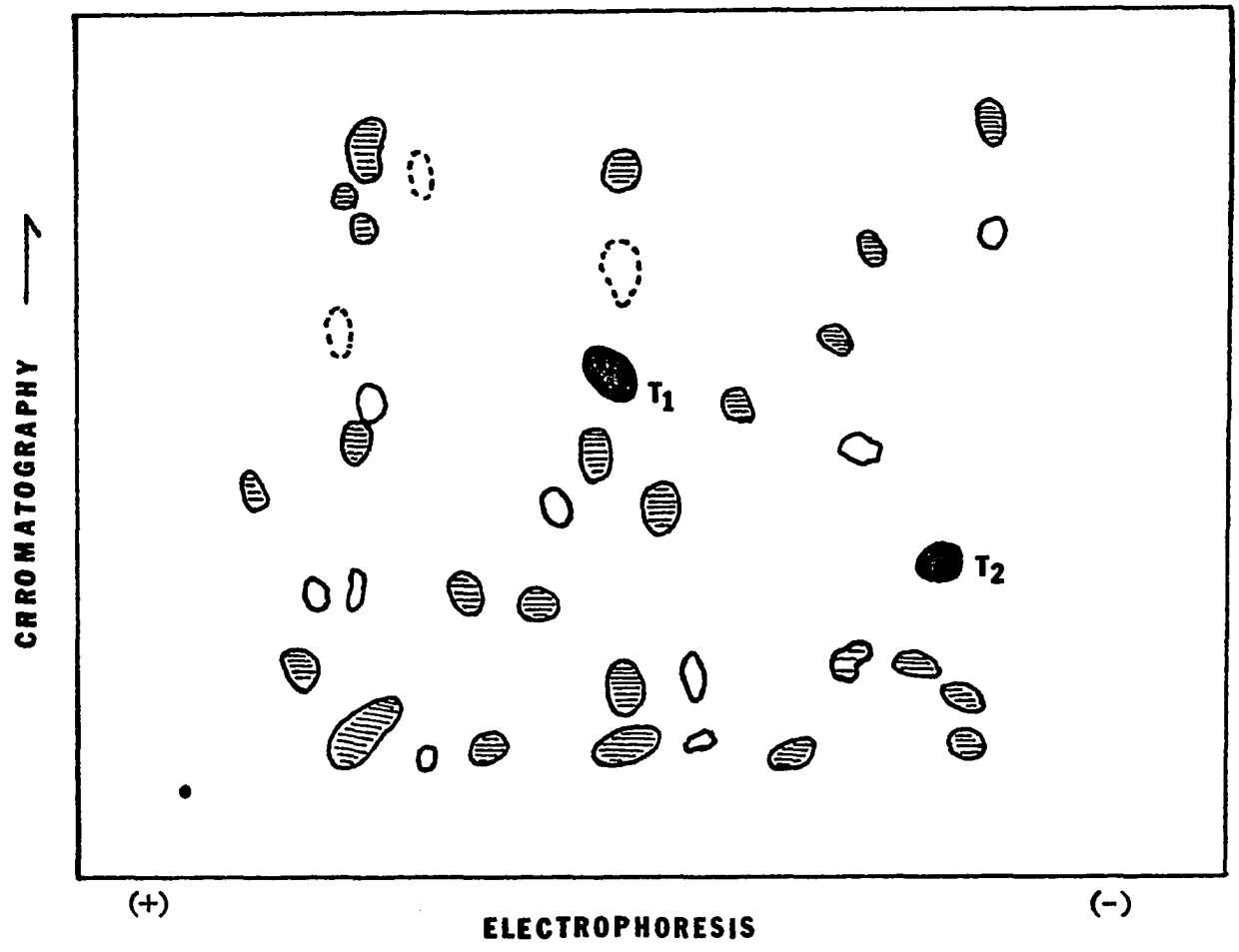


Figure 37. A TRYPSIN PEPTIDE PATTERN OF ISOPEROXIDASE C_n.

Major ninhydrin spots.

Medium intensity ninhydrin spots.

Barely visible ninhydrin spots.

Origin

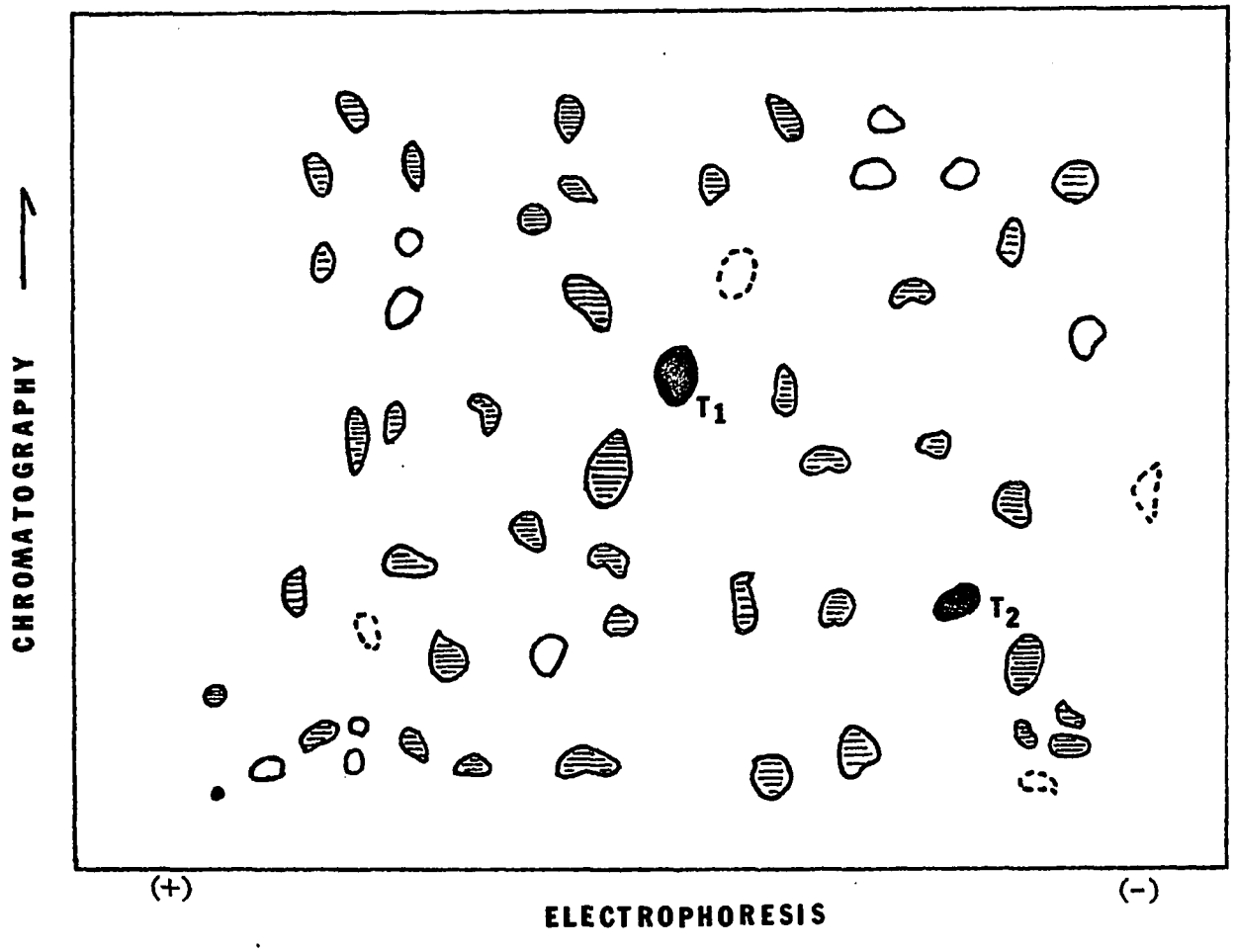


Figure 38. A TRYPSIN PEPTIDE PATTERN OF ISOPEROXIDASE C_3.

Major ninhydrin spots.

Medium intensity ninhydrin spots.

Barely visible ninhydrin spots.

Origin

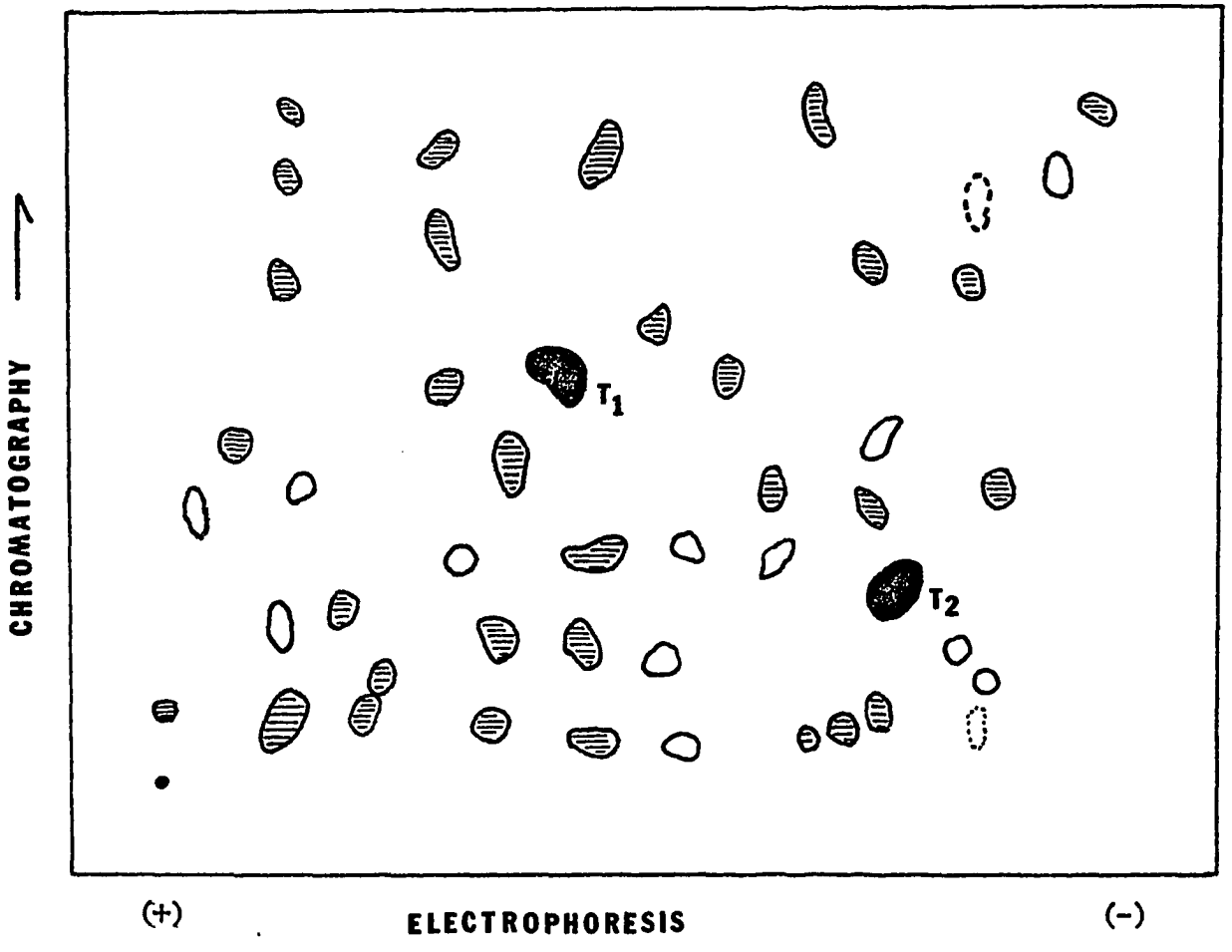


Figure 39. A TRYPSIN PEPTIDE PATTERN OF ISOPEROXIDASE C_4.

Major ninhydrin spots.

Medium intensity ninhydrin spots.

Barely visible ninhydrin spots.

Origin

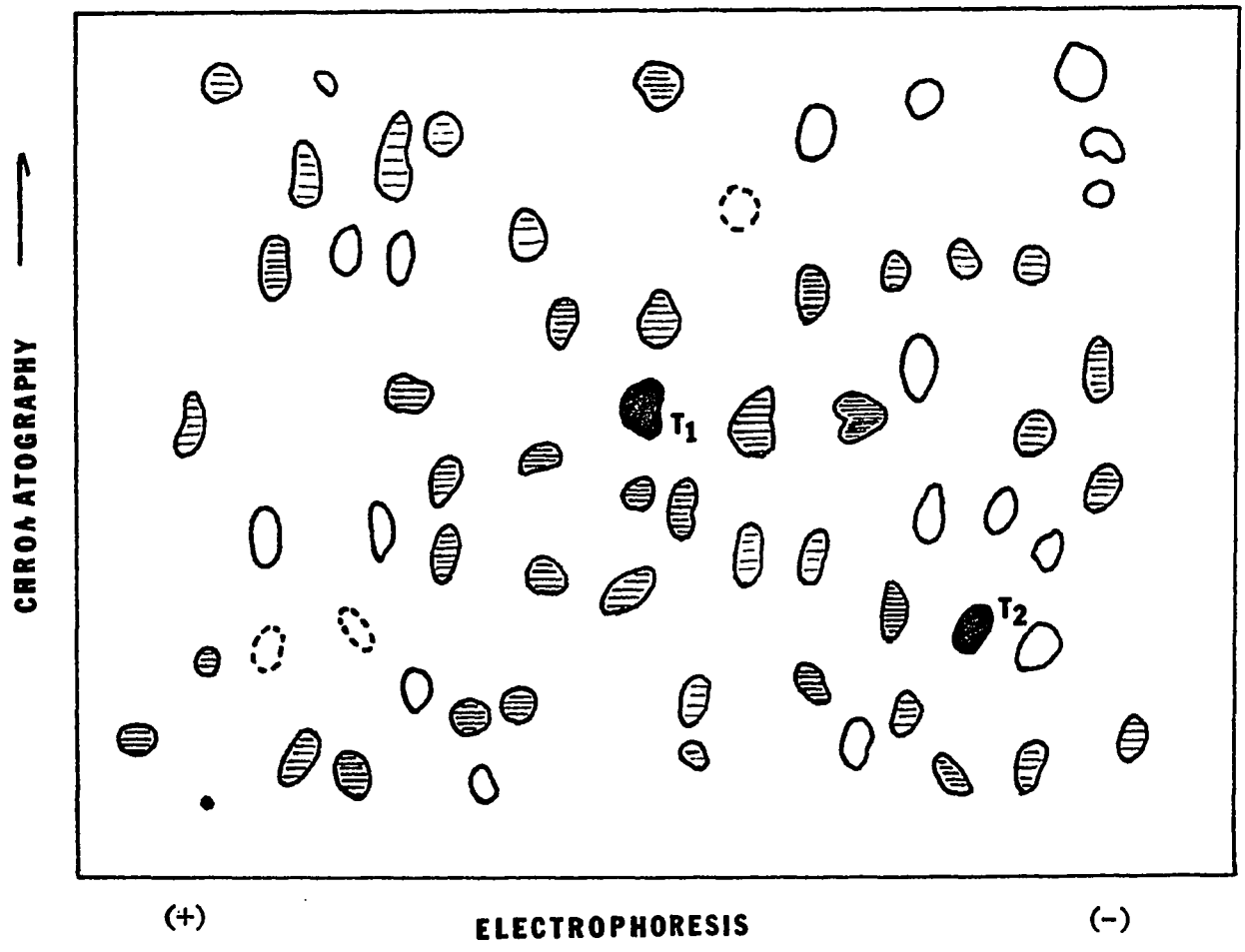


Figure 40. A TRYPSIN PEPTIDE PATTERN OF ISOPEROXIDASE A_1.

- Major ninhydrin spots.
- Medium intensity ninhydrin spots.
- Barely visible ninhydrin spots.
- Origin

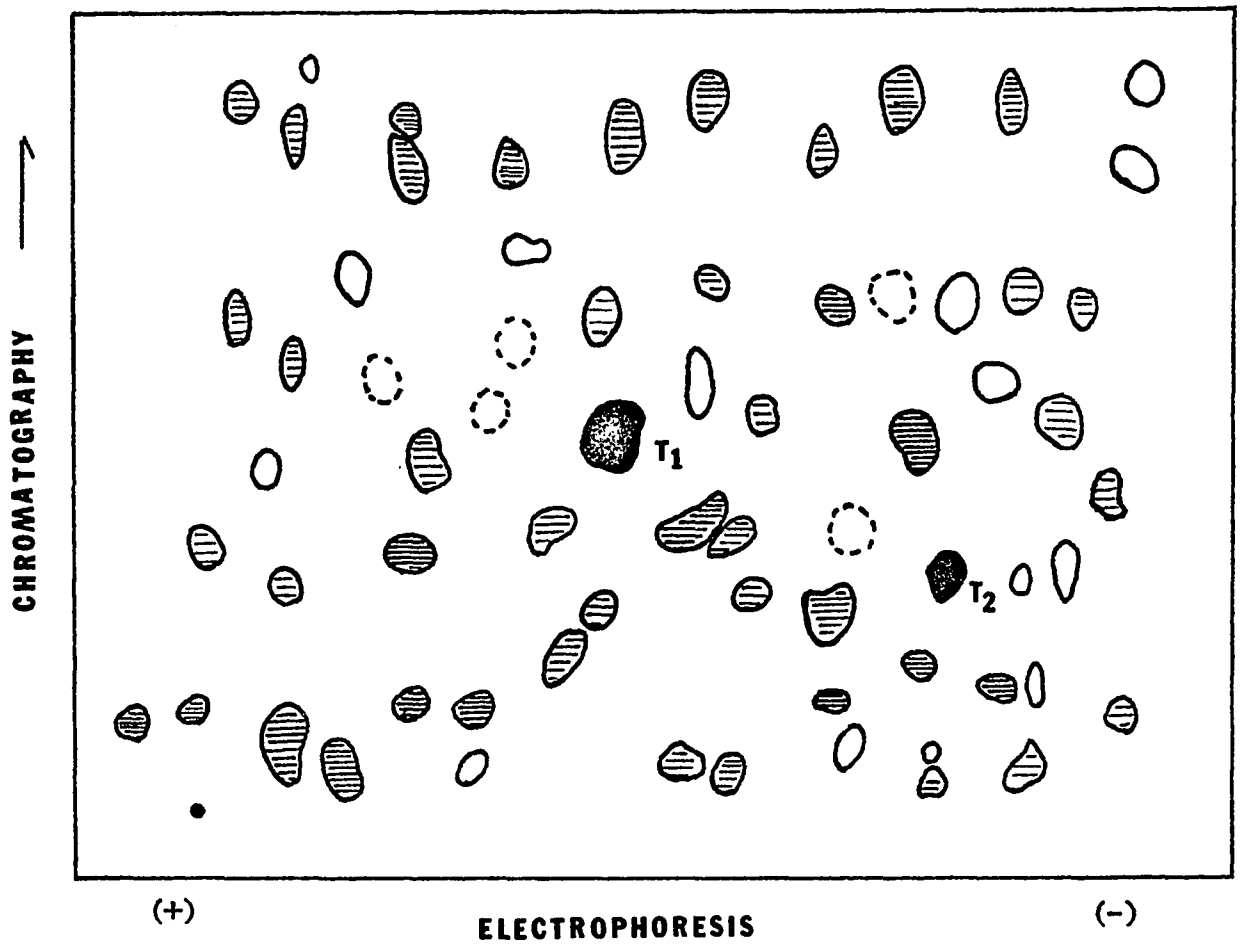


Figure 41. A TRYPSIN PEPTIDE PATTERN OF ISOPEROXIDASE A_2.

- Major ninhydrin spots.
- Medium intensity ninhydrin spots.
- Barely visible ninhydrin spots.
- Origin

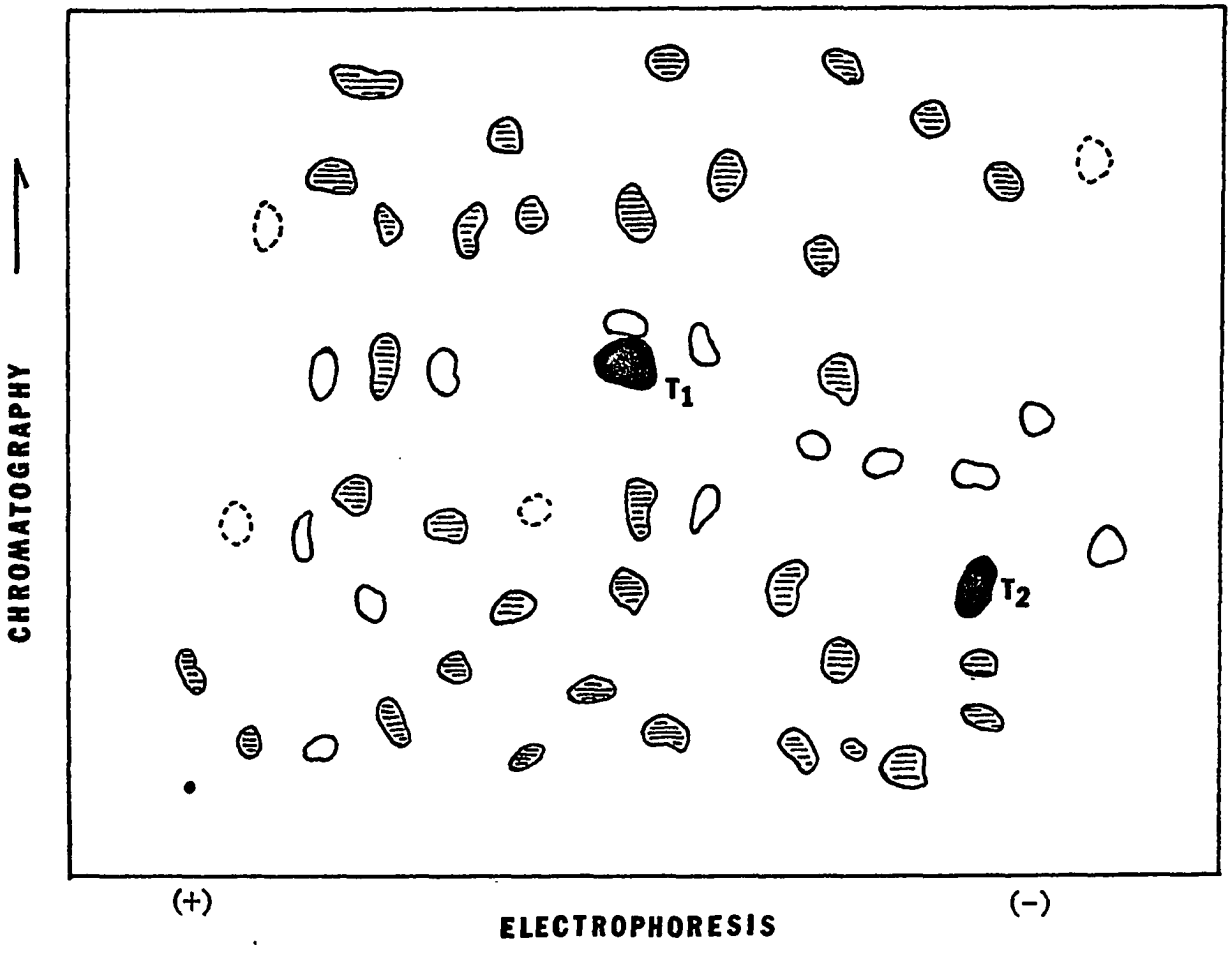


Figure 42. A TRYPSIN PEPTIDE PATTERN OF ISOPEROXIDASE A_c.

Major ninhydrin spots.

Medium intensity ninhydrin spots.

Barely visible ninhydrin spots.

Origin

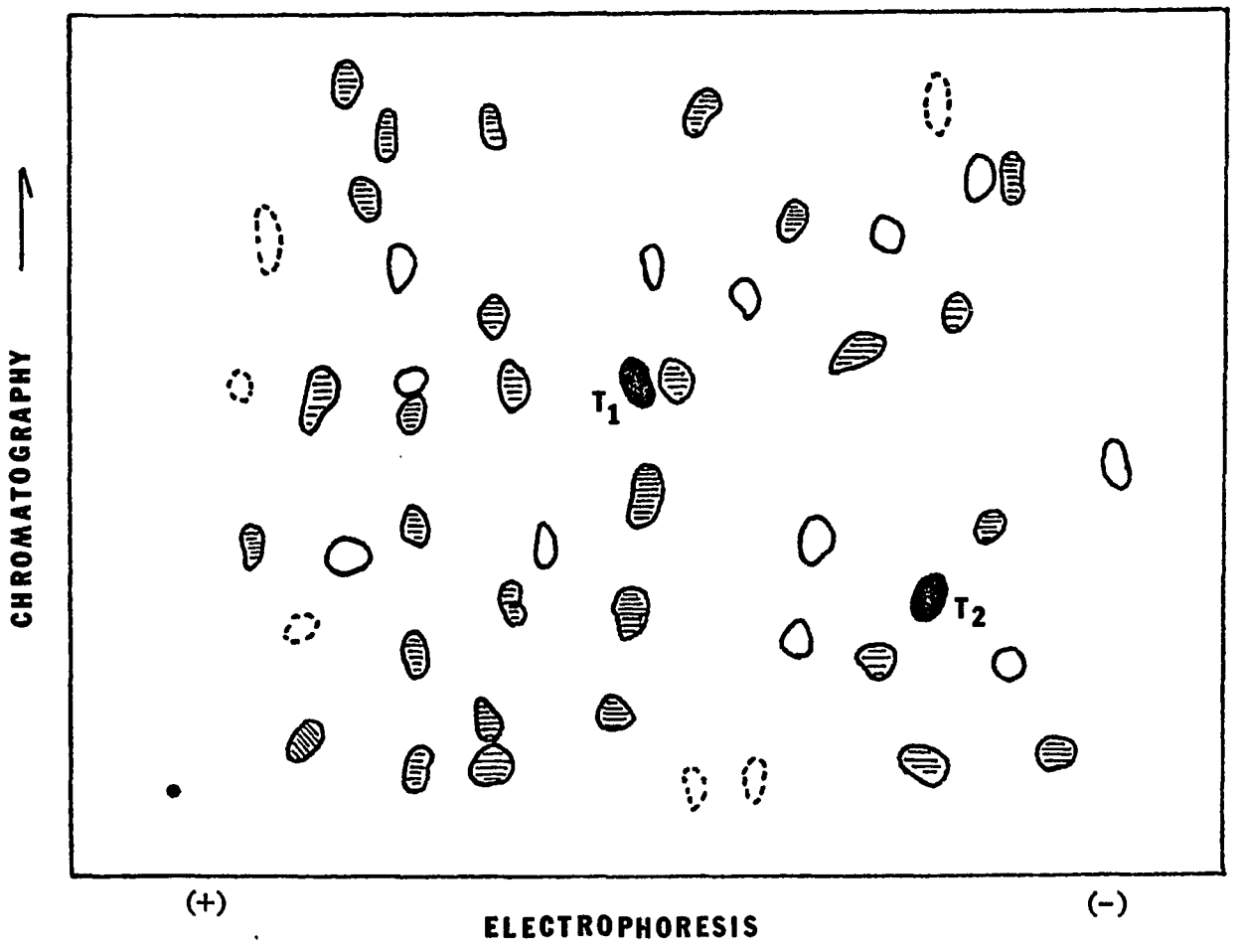


Figure 43. A TRYPSIN PEPTIDE PATTERN OF ISOPEROXIDASE A_f.

- Major ninhydrin spots.
- Medium intensity ninhydrin spots.
- Barely visible ninhydrin spots.
- Origin

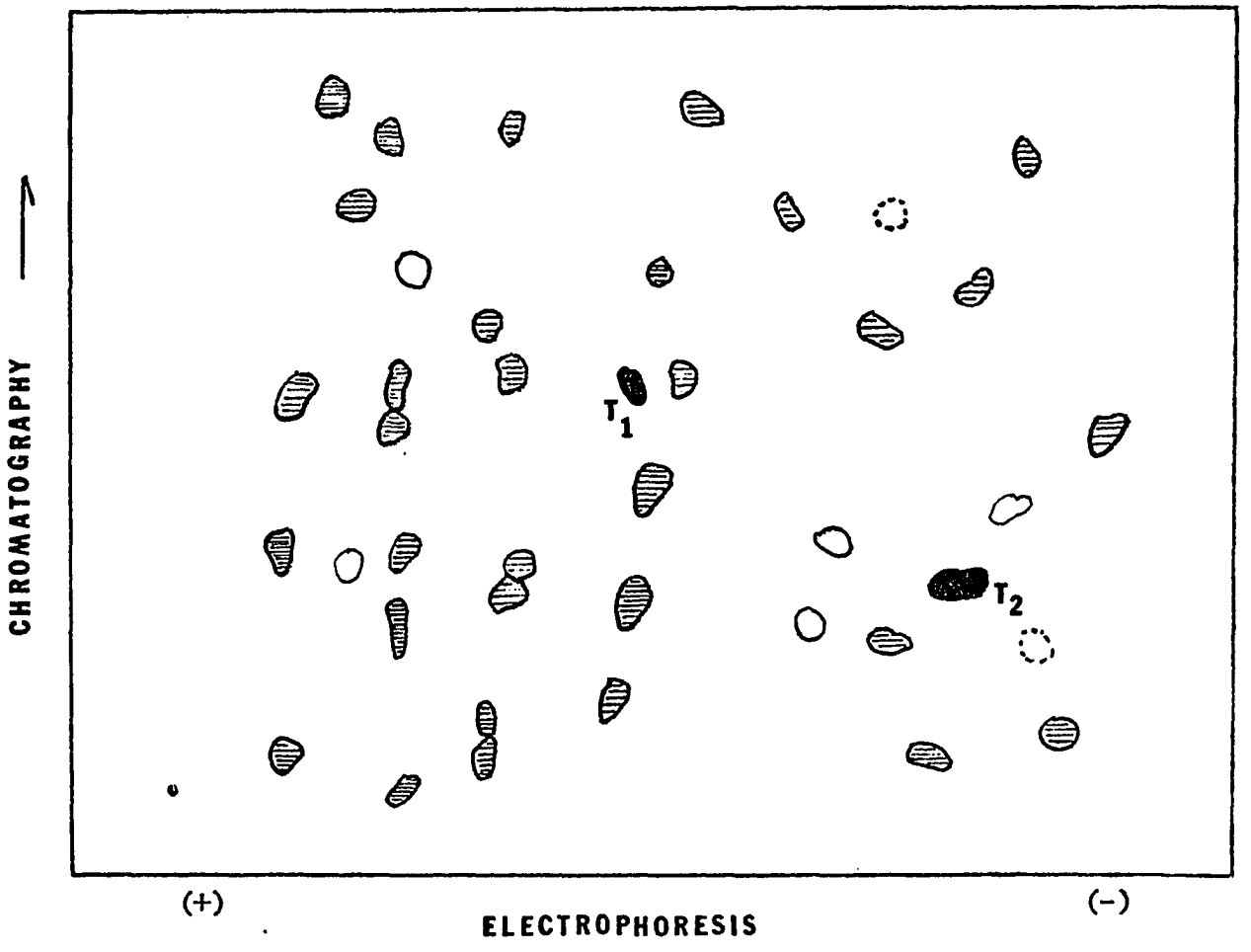


Figure 44. A TRYPSIN PEPTIDE PATTERN OF ISOPEROXIDASE A_3.

- Major ninhydrin spots.
- Medium intensity ninhydrin spots.
- Barely visible ninhydrin spots.
- Origin

TABLE 12

COMPARISON OF OVERLAPPING TRYPTIC PEPTIDES OF

VARIOUS ISOPEROXIDASES

Isoperoxidases	C_n(35)	C_4(45)	C_3(52)	A_1^*(63)	A_2^*(63)	A_c(49)	A_f(43)
C_n(35)	-	15$^+$	15	16	20	17	15
C_4(45)	15	-	22	18	23	20	10
C_3(52)	15	22	-	24	23	20	18
A_1^*(63)	16	18	24	-	35	24	18
A_2^*(63)	20	23	23	35	-	22	17
A_c(49)	17	20	20	24	22	-	22
A_f(43)	15	10	18	18	17	22	-

* : Isoperoxidases from W-38 tobacco tissue culture.
( ) : Number of tryptic peptides of isoperoxidases. The figures represent major and medium intensity peptides.
+ : Possible overlapping tryptic peptides among different isoperoxidases. Overlapping peptides were counted by overlapping origin of each tryptic peptide pattern (Fig.7 - Fig.14) after drawing 1 cm square line from origin.
note: Isoperoxidase A_3 was not included here because it was identical to A_f.

Figure 45. COMPOSITE TRYPSIN PEPTIDE MAP OF CATHODIC ISOPEROXIDASES C_n, C_4 AND C_3. All major and medium intensity trypsin peptides of C_n, C_4 and C_3 are included. Round circles represent the trypsin peptides which are present in all cathodic isoperoxidases examined. T_1, T_2, T_3 are peptides which are present in all cathodic and anodic isoperoxidases.

$n = C_n$ $3 = C_3$ $4 = C_4$

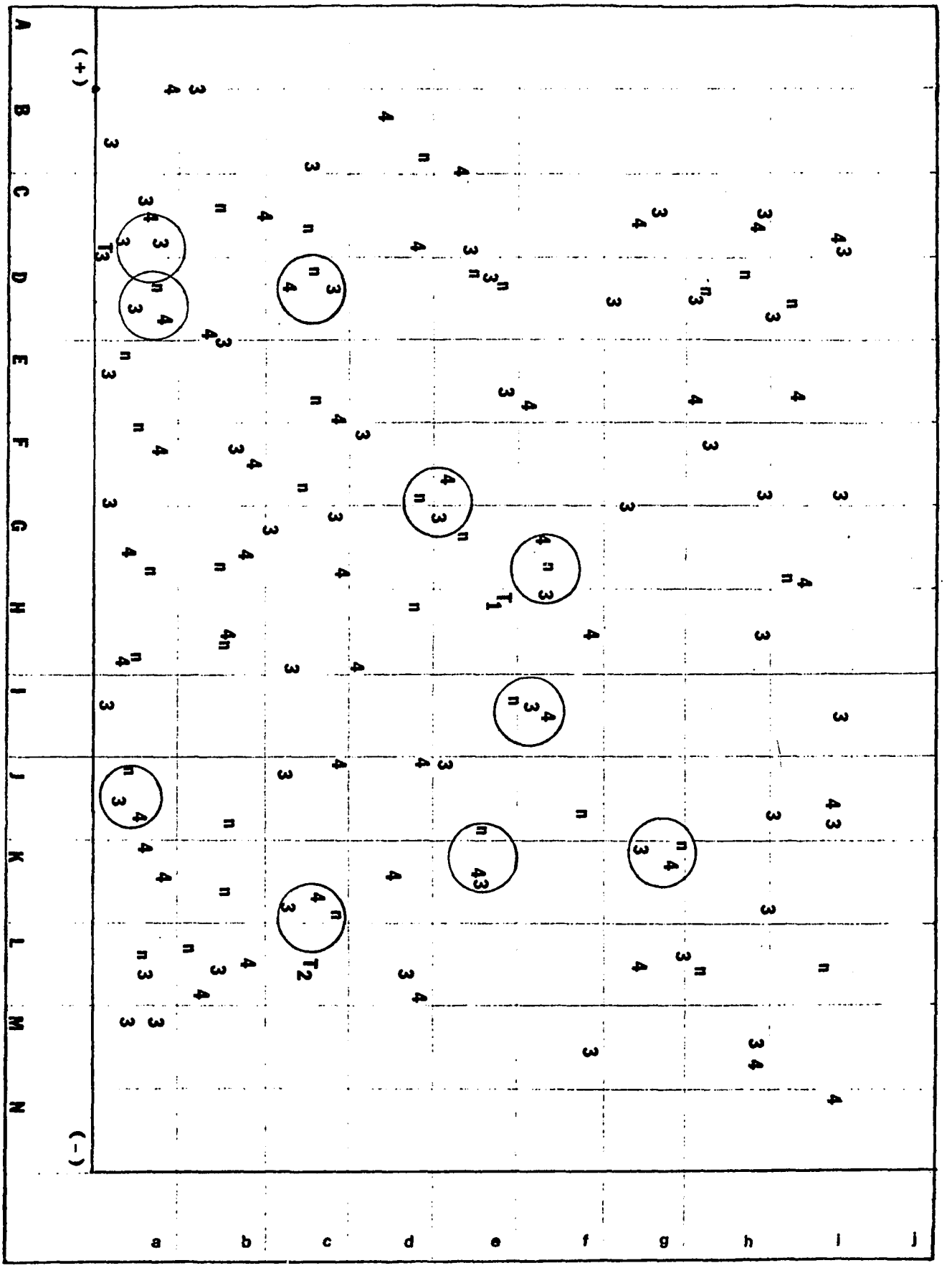

A
B
C
D
E
F
G
H
I
J
K
L
M
N
(+)
(-)
a
b
c
d
e
f
g
h
i
j
T1
T2
T3
CHROMATOGRAPHY →

Figure 46. COMPOSITE TRYPSIN PEPTIDE MAP OF ANODIC ISOPEROXIDASES A_1, A_2, A_c AND A_f. All major and medium intensity trypsin peptides of A_1, A_2, A_c and A_f are included. Round circles represent the trypsin peptides which are present in all anodic isoperoxidases examined. T_1, T_2, T_3 are peptides which are present in all cathodic and anodic isoperoxidases.

$1 = A_1$ $2 = A_2$ $c = A_c$ $f = A_f$

A
B
C
D
E
F
G
H
I
J
K
L
M
N
(+)
(-)
a
b
c
d
e
f
g
h
i
j
CHROMATOGRAPHY
T1
T2
T3

examined, are indicated as circles, and three homologous peptides of all isoperoxidases (including cathodics) are numbered as T_1, T_2 and T_3.

The tryptic peptide map of isoperoxidase A_f (Fig. 43) appeared indistinguishable from that of isoperoxidase A_3 (Fig. 44) from tobacco callus culture W-38 indicating that the two isoenzyme may well be the same. All major peptides and medium intensity peptides of A_f could also be found in the fingerprints of isoperoxidase A_3. The essentially identical peptide maps of A_f and A_3 strongly support the idea that isoperoxidase A_f is really a derepressed isoenzyme of tobacco suspension culture WR-132 grown in total darkness.

Comparisons of peptide maps between cathodic and anodic isoperoxidases showed that although no isoperoxidases so far examined are identical, except A_f and A_3, all six isoperoxidases C_n, C_4, C_3, A_1, A_2 and A_c from tobacco tissue cultures are not dramatically dissimilar in certain portions of the sequence, since many matching peptides have been found among these isoperoxidases, despite their different electrophoretic movement. In particular, the peptide maps of isoperoxidase A_1, A_2 and A_c were considerably similar to those of isoperoxidases C_3 and C_4 (Table 12). As expected from electrophoresis data, the peptide maps of the isoperoxidase $A_f(A_3)$ showed the least similarities to those of cathodic isoperoxidases (Table 12). In contrast to the other anodic isoperoxidases, however, isoperoxidase A_f appears to be more closely related to isoperoxidases C_n and C_3 than to isoperoxidase C_4.

Welinder and Mazza (20) studied the similarities and differences of five peroxidases from turnip and horseradish by the fingerprinting technique. From their peptide mapping studies, they found two highly homologous sequences containing histidine present in all five examined peroxidases. On the basis of this finding, they suggested that two histidine sequences of

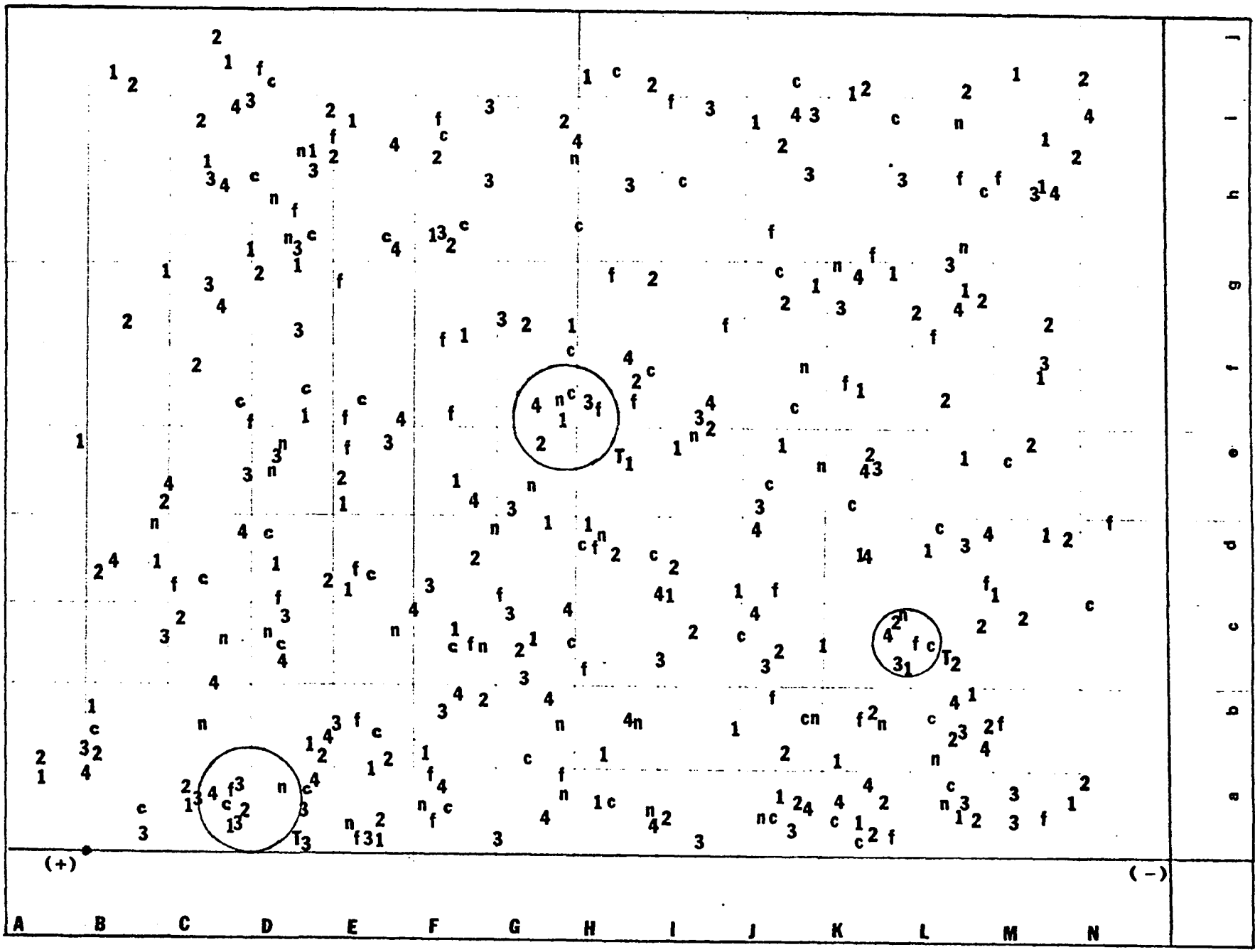
peroxidases are essential to peroxidase activity and probably are intimately linked with the heme. Fig. 47 indicates the composite tryptic peptides map of all tobacco tissue culture isoperoxidases examined (excluding A_3). As mentioned earlier, there are three highly homologous peptides, tentatively designated as T_1, T_2 and T_3, present in all of our isoperoxidases. Among those three peptides, T_1 and T_2 were large and strongly stained by ninhydrin (Fig. 37 to Fig. 44) in all isoperoxidases. T_3 peptides were also strongly stained by ninhydrin. However, the size of the peptides, as judged from the area of ninhydrin staining, varied considerably, depending upon the isoperoxidase preparation. Although it is premature, without detail analysis of the peptides, to conclude that the two homologous peptides are analogous to the homologous sequences proposed by Welinder et al. (20), it is probable that some portion of the peptides which include active sites has been conserved among all isoperoxidases from tobacco tissue cultures.

Shih et al. (15), on the basis of tryptic peptide maps, segregated horseradish peroxidase isoenzymes into three distinct groups: an anodic group, consisting of peroxidase A_1 and A_2, a second anionic group consisting only of peroxidase A_3, and a cationic group consisting of peroxidases B and C. The three groups of isoenzymes have distinctly different catalytic properties, while isoenzymes within a group possess very similar catalytic properties (71). In the present study, no obvious segregation of isoperoxidases into certain groups could be found since all isoperoxidases appeared to be different despite some homology in certain portions of the sequence among the different isoperoxidases. It is possible that isoperoxidases A_f and C_n are processed from other isoperoxidases by phosphorylation, glycosylation, or methylation of ε-amino groups of lysine. The many differences observed in the tryptic maps of the different isoperoxidases from tobacco tissue cultures, however, makes the above possibilities unlikely.

Figure 47. COMPOSITE TRYPSIN PEPTIDE MAP OF CATHODIC AND ANODIC ISOPEROXIDASES. All major and medium intensity trypsin peptides of C_n, C_4, C_3, A_1, A_2, A_c and A_f are included. Round circles, T_1, T_2, T_3, represent the trypsin peptides which are present in both cathodic and anodic isoperoxidases examined.

n = C_n 3 = C_3 4 = C_4

1 = A_1 2 = A_2 c = A_c f = A_f


CHROMATOGRAPHY
a b c d e f g h i j
A B C D E F G H I J K L M N
(+)
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T3

CHAPTER VIII

SUMMARY

When tobacco suspension cultures, WR-132, were transferred from a dim light (10 lux) growth condition to complete darkness, both quantitative and qualitative changes of the isoperoxidase pattern were observed in the medium and cells of dark-grown cultures. Most new isoperoxidases released into the medium of the dark cells were also present in the crude extracts of dark cells at approximately the same passage. The isoperoxidase pattern of the medium and cells becomes similar to that of tobacco callus culture W-38, when WR-132 tobacco culture reaches the fourth and fifth passage in total darkness. Since crude extracts of tobacco callus culture W-38 exhibit the whole array of anodic and cathodic isoperoxidases, derepression of isoperoxidases in the dark-grown tobacco suspension culture WR-132 appears to occur.

The transitory appearance of many new isoperoxidases at the 4th and 5th passages of dark grown cells and the reversion of the color (grayish white) of dark grown cells to normal color (yellow) after the 6th passage probably indicate that some tobacco cells survived the effect(s) of darkness and continuously grow while others, which produce the new isoperoxidases, die at the 4th and 5th passage.

The two major isoperoxidases, A_f and C_n, which are produced from

tobacco suspension cultures grown in total darkness have distinct physical properties, such as molecular weights and presence or absence of carbohydrate. The catalytic properties of both isoenzymes against synthetic and naturally-occurring phenolic compounds, namely guaiacol, scopoletin, ferulic acid, esculetin and chlorogenic acid also differ considerably. However, the physical and catalytic properties of isoperoxidase A_f and isoperoxidase A_3 from tobacco callus culture W-38 appear to be the same. The identical nature of isoperoxidase A_f and A_3 was further proved by the trypsin peptide mapping study.

Based upon all data, such as an isoperoxidase pattern study, physical and kinetic studies, and a fingerprinting study, it is proposed that derepression of isoperoxidases occurs in tobacco suspension culture WR-132 grown in total darkness.

REFERENCES

1. Galston, W. and P. J. Davies. 1969. Hormonal regulation in higher plants. *Science.* 163:1288-1297.

2. Stafford, H. A. and A. W. Galston. 1970. Ontogeny and hormonal control of polyphenoloxidase isozymes in tobacco pith. *Plant physiol.* 46:763-767.

3. Galston, A. W., J. Bonner, and R. S. Baker. 1953. Flavoprotein and peroxidase as components of the indoleacetic acid oxidase system of peas. *Arch. Biochem. Biophys.* 42:456-470.

4. Akazawa, T. and E. E. Conn. 1958. The oxidation of reduced pyridine nucleotides by peroxidase. *J. Biol. Chem.* 232:403-415.

5. Yang, S. F. 1967. Biosynthesis of ethylene. Ethylene formation from methional by horseradish peroxidase. *Arch. Biochem. Biophys.* 122:481-487.

6. Wong, E. and J. M. Wilson. 1976. Products of the peroxidase-catalyzed oxidation of 4,2',4'-trihydroxychalcone. *Phytochem.* 15:1325-1332.

7. Hill, J. M. 1970. The oxidation of pyridoxal and related compounds by pea-seedling extracts or systems containing peroxidase. *Phytochem.* 9:725-734.

8. Andreae, W. A. 1952. Effect of scopoletin on indoleacetic acid metabolism. *Nature.* 170:82-83.

9. Gelinas, D. A. 1973. Proposed model for the peroxidase catalyzed oxidation of indole-3-acetic acid in the presence of the inhibitor ferulic acid. *Plant Physiol.* 51:967-972.

10. Reigh, D. L., S. H. Wender and E. C. Smith. 1973. Scopoletin: A substrate for an isoperoxidase from *Nicotiana tabacum* tissue culture W-38. *Phytochem.* 12:1265-1268.

11. Pickering, J. W., B. L. Powell, S. H. Wender and E. C. Smith. 1973. Ferulic acid: A substrate for two isoperoxidases from *Nicotiana tabacum* tissue cultures. *Phytochem.* 12:2639-2643.

12. Siegel, S. M. 1955. The biochemistry of lignin formation. *Physiol. Plantarum.* 8:20-32.

13. Shannon, L. M. 1968. Plant isoenzymes. *Ann. Rev. Plant Physiol.* 19:187-210.

14. Shannon, L. M., E. Kay, and J. Y. Lew. 1966. Peroxidase Isozymes from Horseradish Roots. I. Isolation and Physical Properties. *J. Biol. Chem.* 241:2166-2172.

15. Shih, J. H. C., L. M. Shannon, E. Kay, and J. Y. Lew. 1971. Peroxidase Isoenzymes from Horseradish Roots. IV. Structural Relationships. *J. Biol. Chem.* 246:4546-4551.

16. Macnicol, P. K. 1966. Peroxidases of the Alaska pea (*Pisum sativum* L.). *Arch. Biochem. Biophys.* 117:347-356.

17. Kamel, M. Y. and A. M. Ghazy. 1973. Peroxidases of *Solanum Melongena* leaves. *Phytochem.* 12:1281-1285.

18. Powell, B. L., J. W. Pickering, S. H. Wender and E. C. Smith. 1975. Isoperoxidases from tobacco tissue cultures. *Phytochem.* 14:1715-1717.

19. Srivastava, O. P. and R. B. van Huystee. 1977. IAA oxidase and polyphenol oxidase activities of peanut peroxidase isozymes. *Phytochem.* 16:1527-1530.

20. Welinder, K. G. and G. Mazza. 1975. Similarities and differences of five peroxidases from turnip and horseradish. *Eur. J. Biochem.* 57:415-424.

21. Welinder, K. G. 1976. Covalent structure of the glycoprotein horseradish peroxidase. *FEBS Lett.* 72(1):19-23.

22. Kucherenko, V. P. 1976. Change in the activity of peroxidase and isoperoxidases during aging of plants. *Ukr. Bot. Zh.* 33(6):631-632. *Chemical Abstract.* 1977. Vol. 86(15):239.

23. Lee, T. T. 1971. Promotion of indoleacetic acid oxidase isoenzymes in tobacco callus cultures by indoleacetic acid. *Plant Physiol.* 48:56-59.

24. Lee, T. T. 1971. Increase of indoleacetic acid oxidase isoenzymes by gibberellic acid in tobacco callus cultures. *Can. J. Bot.* 49:687-693.

25. Lee, T. T. 1971. Cytokinin-controlled indoleacetic acid oxidase isoenzymes in tobacco callus cultures. *Plant Physiol.* 47:181-185.

26. Birecka, H. and M. O. Garraway. 1978. Corn leaf isoperoxidase reaction to mechanical injury and infection with *Helminthosporium maydis*. *Plant Physiol.* 61:561-566.

27. Vance, C. P. and R. T. Sherwood. 1976. Regulation of lignin formation in Reed Canarygrass in relation to disease resistance. *Plant Physiol.* 57:915-919.

28. Galston, A. W., S. Lavee and B. Z. Siegel. 1968. The induction and repression of peroxidase isozymes by 3-indoleacetic acid. *In*: F. Wightman and G. Setterfield, eds., Biochemistry and Physiology of Plant Growth Substances. Runge. Press. Ottawa 445-472.

29. Lee, T. T. 1974. Cytokinin control in subcellular localization of indoleacetic acid oxidase and peroxidase. *Phytochem.* 13:2445-2453.

30. Schafer, P. L. 1971. Studies on the effect of calcium ion concentration on growth, lignification and peroxidase activity in tobacco tissue. Ph.D dissertation, University of Oklahoma, Norman.

31. Schafer, P., S. H. Wender and E. C. Smith. 1971. Effect of scopoletin on two anodic isoperoxidases isolated from tobacco tissue culture W-38. *Plant Physiol.* 48:232-233.

32. Penel, C. and H. Greppin. 1975. The balance between acid and basic peroxidases and its photoperiodic control in spinach leaves. *Plant Sci. Lett.* 5:41-48.

33. De Jong, D. W., A. C. Olson, K. M. Hawker and E. F. Jansen. 1968. Effect of cultivation temperature on peroxidase isozyme of plant cells grown in suspension. *Plant Physiol.* 43:841-844.

34. McCown, B. H., D. D. McCown, G. E. Beck and T. C. Hall. 1970. Isozyme complements of *Dianthus* callus cultures : Influence of light and temperature. *Amer. J. Bot.* 57:148-152.

35. Leu, S-L. K., S. H. Wender and E. C. Smith. 1975. Effect of darkness on isoperoxidases in tobacco tissue cultures. *Phytochem.* 14:2551-2554.

36. Legrand, B., G. Thomas, P. Claude and G. Hubert. 1976. Light and hormonal control of phenolic inhibitors of peroxidase in *Cichorium intybus* L. *Plant Biochem. J.* 3(2):119-127.

37. Legrand, B. 1977. Action de la lumière sur les peroxydases et sur la teneur en composés phénoliques de tissus de feuilles de *Cichorium intybus* L. cultivés *in vitro*. *Biologia Plantarum.* 19(1):27-33.

38. Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiol. Plant.* 15:473-497.

39. Brewer, G., Jr. 1970. An introduction to isozyme techniques. Academic Press, Inc., New York. pp.16-39.

40. Graham, R. C., U. Lundholm and M. J. Karnovsky. 1965. Cytochemical demonstration of peroxidase activity with 3-amino-9-ethyl carbazole. *J. Histochem. Cytochem.* 13:150.

41. Ornstein, L. and B. J. Davis. 1962. Disc electrophoresis, Part II. Distillation Products Industries. Rochester, New York.

42. Weber, K., J. R. Pringle and M. Osborn. 1972. Measurement of molecular weights by electrophoresis on SDS-acrylamide gels. *In* : C. H. W. Hirs and Tamasheff, eds., Methods in Enzymology, Academic Press, Inc., London, vol.26, pp.3-27.

43. Lance, C. 1955. Sur la détermination de l'activité peroxydase des extraits brûts de tissus végétaux. *Rev. Gen. Bot.* 62:609.

44. Reigh, D. L. 1973. Kinetic and physical properties of isoperoxidase A_3 from tobacco tissue culture W-38. Ph.D dissertation, University of Oklahoma, Norman.

45. Pickering, J. W. 1974. Part I. Characterization of two isoperoxidases from tobacco tissue culture WR-132. Part II. Effect of some phenolic compounds on enzyme activities in rat lung. Ph.D dissertation. University of Oklahoma, Norman.

46. Powell, B. L. 1974. Isolation and characterization of two anodic isoperoxidases from tobacco tissue culture W-38. Ph.D dissertation. University of Oklahoma, Norman.

47. Lowry, O. H., N. J. Rosebrough, A. L. Farr and R. J. Randall. 1951. Protein measurement with Folin phenol reagent. *J. Biol. Chem.* 193:265-275.

48. Leu, S-L. K. 1974. Effect of light on the growth, peroxidase isozymes and phenolic compounds in *Nicotiana tabacum* tissue culture WR-132. M.S. thesis. University of Oklahoma, Norman.

49. Izvorska, N. and N. Bakurdzhieva. 1975. Cytological modifications and changes in peroxidase and IAA oxidase activity in peas callus tissues following gamma-irradiation. *Fiziol. Rast.* 1(3):36-43 *Chemical Abstracts.* 1976. 84(20):202.

50. Gladilovich, B. R. and G. G. Antonova. 1974. Boron content and enzyme activities of white-headed cabbage infected with anbury clubroot. *Zap. Leningr. S-kh. Inst.* 239:98-102. *Chemical Abstracts.* 1977. 86(13):252.

51. Galston, A. W. and L. Y. Dalberg. 1954. The adaptive formation and physiological significance of indoleacetic acid oxidase. *Am. J. Botany.* 41:373-380.

52. Van Overbeck, J. 1935. The growth hormone and the dwarf type of growth in corn. *Proc. Natl. Acad. Sci.* U. S. 21:292-299.

53. Palmieri, S., M. Odoardi, G. P. Soressi and F. Salamini. 1978. Indoleacetic acid oxidase activity in two high-peroxidase tomato mutants. *Physiol. Plant.* 42:85-90.

54. Andrews, P. 1964. Estimation of the molecular weights of proteins by Sephadex gel-filtration. *Biochem. J.* 91:222-233.

55. Glossman, H. and D. N. Neville Jr. 1971. Glycoproteins of cell surfaces. *J. Biol. Chem.* 246:6339-6346.

56. McGuckin, W. F. and B. F. McKenzie. 1958. Periodic acid-fuchsin-sulfite-staining method for evaluation of glycoproteins. *Clin. Chem.* 4:476-483.

57. Roe, J. H. 1955. The determination of sugar in blood and spinal fluid with anthrone reagent. *J. Biol. Chem.* 212:335-343.

58. Boas, N. F. 1953. Method for the determination of hexosamines in tissues. *J. Biol. Chem.* 204:553-563.

59. Baar, S. 1954. Quantitative estimation of glucose by paper partition chromatography. *Biochem. J.* 58:175-176.

60. Morita, Y. and K. Kameda. 1959. Studies on phyto-peroxidase. Part XII. Amino Acid Composition of Japanese-radish peroxidase a. *Bull. Agr. Chem. Soc.* Japan. 23(1):28-33.

61. Mazza, G., C. Job and M. Bouchet. 1973. Chemical composition and hydrodynamic characteristics of turnip peroxidases. *Biochem. Biophys. Acta.* 322:218-223.

62. Shimizu, K. and Y. Morita. 1966. Studies on phyto-peroxidase. Part XVIII. Chemical composition of Japanese-radish peroxidase c. *Agr. Biol. Chem.* 30(2):149-154.

63. Helinski, D. R. and C. Yanofsky. 1962. Peptide studies on the wild type A protein of the tryptophan synthetase of *Escherichia coli*. *Biochem. Biophy. Acta.* 63:10-19.

64. Penon, P., J-P. Cecchini, R. Miassod, J. Richard, M. Teissere and M. Teissere and M-H Pinna. 1970. Peroxidases associated with lentil root ribosomes. *Phytochem.* 9:73-86.

65. Murphy, M. J. and C. Oh Eocha. 1973. Peroxidase from the red alga *Cystoclonium purpureim*. *Phytochem.* 12:55-59.

66. Srivastava, O. P. and R. B. van Huystee. 1977. Spectral and molecular properties of peanut peroxidase isozymes. *Phytochem.* 16:1657-1659.

67. Morita, Y. and K. Kameda. 1957. Crystallization of Japanese radish peroxidase a. *Bull. Res. Inst. Food. Sci.* Kyoto Univ. 12:14-23.

68. Morita, Y. C. Yoshida and Y. Maeda. 1971. Phytoperoxidase XXV. Properties and structures of peroxidase isoenzymes of Japanese-radish. *Agr. Biol. Chem.* 35:1074-1083.

69. Rücker, W. and B. J. Radola. 1971. Isoelectric patterns of peroxidase isoenzymes from tobacco cultures. *Planta.* 99:192-198.

70. Shinshi, H. and M. Noguchi. 1975. Properties of peroxidases from tobacco cell suspension culture. *Phytochem.* 14:2141-2144.

71. Kay, E., L. M. Shannon and J. Y. Lew. 1967. Peroxidase isozymes from horseradish roots. II. Catalytic properties. *J. Biol. Chem.* 242:2470-2473.

72. Mäder, M. and M. Bopp. 1977. Über die physiologische bedeutung der peroxidase-isoenzym-gruppen des Tabaks anhand einiger biochemischer. II. pH-Optima, Michaelis-Constants, Maximal Oxidation-Rates. *Z. Pflanzenphysiol.* Bd. 82.S:247-260.

73. Goldacre, P. L., A. W. Galston and R. L. Weintraub. 1953. The effect of substituted phenols on the activity of indole-acetic acid oxidase of peas. *Arch. Biochem. Biophys.* 43:358-373.

74. Sirois, J. C. and R. W. Miller. 1972. The mechanism of the scopoletin-induced inhibition of the peroxidase catalyzed degradation of indole-3-acetic acid. *Plant Physiol.* 49:1012-1018.

75. Lineweaver, H. and D. Burk. 1934. The determination of enzyme dissociation constants. *J. Am. Chem. Soc.* 56:658-665.

76. Hill, A. V. 1913. The combination of haemoglobin with oxygen and carbon monoxide. *Biochem. J.* 7:471-480.

77. Atkinson, D. E., J. A. Hathaway and E. C. Smith. 1965. Kinetics of regulatory enzymes. *J. Biol. Chem.* 240:2682-2690.

78. Koshland, D. E., Jr. 1970. The molecular basis for enzyme regulation. *In* : The Enzymes. 3rd edn. (Paul D. Boyer, ed) 1:342-297.

79. Stedman, R. L. 1967. The chemical composition of tobacco and tobacco smoke. *Chem. Rev.* 68:153-207.

80. Steck, W. 1968. Metabolism of cinnamic acid in plants : Chlorogenic acid formation. *Phytochem.* 1:1711-1717.