

Summer 1971

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MERRILL C. HOYLE

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71-19,435

HOYLE, Merrill C., 1931-
CHARACTERISTICS AND PURIFICATION OF
INDOLEACETIC ACID OXIDASE IN YELLOW BIRCH.

University of New Hampshire, Ph.D., 1971
Biochemistry

University Microfilms, A XEROX Company, Ann Arbor, Michigan

CHARACTERISTICS AND PURIFICATION
OF INDOLEACETIC ACID
OXIDASE IN YELLOW
BIRCH

by

MERRILL C. HOYLE

B.S., University of Massachusetts, 1959

M.S., University of Massachusetts, 1961

A THESIS

Submitted to the University of New Hampshire
In Partial Fulfillment of
The Requirements for the Degree of

Doctor of Philosophy
Graduate School
Plant Science Department
July, 1970

This thesis has been examined and approved.

Douglas M. Routley

Thesis director, ~~Douglas~~ Routley, Prof. of Plant Science

Lincoln Peirce

Lincoln Peirce, Prof. of Plant Science

Russell Eggert

Russell Eggert, Prof. of Horticulture

Richard Schreiber

Richard Schreiber, Prof. of Botany

Harold Hocker, Jr.

Harold Hocker, Assoc. Prof. of Forest Resources

July 20, 1970

Date

ACKNOWLEDGEMENTS

The author wishes to acknowledge the assistance and consultation received from the following people:

Douglas Routley for consultation and guidance given throughout the course of these investigations.

Members of my committee for their constructive criticism and suggestions.

William Leak for his interest in this work.

Nancy Pease and Don Thompson for their assistance in the laboratory.

The support of the Northeastern Forest Experiment Station, U. S. Forest Service, U.S.D.A., is gratefully acknowledged.

Carmen L. Couture for conscientious typing.

TABLE OF CONTENTS

LIST OF TABLES.....	vi
LIST OF ILLUSTRATIONS.....	vii
ABSTRACT.....	ix
I. INTRODUCTION.....	1
II. LITERATURE REVIEW.....	3
1. Distribution of IAA Oxidase in the Plant.....	3
2. Distribution of IAA Oxidase in the Cell.....	4
3. Extraction of IAA Oxidase.....	4
4. Evaluating IAA Oxidase Activity.....	6
5. Physical Identity of IAA Oxidase Activity.....	13
III. METHODS AND MATERIALS.....	17
1. Leaf Samples.....	17
2. Enzyme Extraction.....	17
3. Ultrafiltration.....	19
4. Gel Chromatography.....	19
5. IAA Oxidase Assays.....	20
6. Peroxidase Assay.....	23
7. Total Protein.....	23
IV. RESULTS.....	24
1. Extraction.....	24
2. Measuring Enzyme Activity.....	24
3. Purification and Fractionation.....	55
V. DISCUSSION.....	70
1. Extraction Problems.....	70
2. Cofactor Requirements.....	71
3. Locus of IAA Oxidase Activity.....	78

LITERATURE CITED..... 81
APPENDIX..... 87

LIST OF TABLES

I.	ACIDITY OF VARIOUS AQUEOUS EXTRACTS FROM YELLOW BIRCH LEAVES.....	26
II.	EFFECTS OF DCP AND Mn^{2+} ON IAA OXIDASE ACTIVITY OF BIRCH LEAF EXTRACTS TREATED WITH PVP AND PARTIALLY PURIFIED.....	30
III.	EFFECTS OF p-COUMARIC ACID ON IAA OXIDASE ACTIVITY IN BIRCH LEAF EXTRACTS.....	41
IV.	EFFECT OF SUBSTRATE LEVELS ON IAA OXIDASE ACTIVITY OF BIRCH ENZYME.....	49
V.	COMPARISON OF IAA OXIDASE AND PEROXIDASE IN CRUDE EXTRACTS AFTER TWO MONTHS IN COLD ($4^{\circ}C.$) STORAGE.....	52
VI.	FLOW SHEET FOR ULTRAMEMBRANE FILTRATION OF BIRCH LEAF EXTRACTS.....	60
VII.	ULTRAFILTRATION DATA FOR SAMPLE BEFORE CHROMATOGRAPHY ON BIO-GEL, P-100.....	62
VIII.	MEMBRANE ULTRAFILTER PURIFICATION OF CRUDE BIRCH EXTRACT FOR CHROMATOGRAPHY ON SE-SEPHADEX COLUMN.....	66

LIST OF FIGURES

1.	EFFECT OF PVP ON IAA OXIDASE ACTIVITY OF BIRCH LEAF EXTRACTS.....	27
2.	SALKOWSKI ASSAY OF BIRCH ENZYME TREATED WITH PVP....	29
3.	EFFECT OF DCP AND Mn^{2+} ON IAA OXIDASE ACTIVITY OF COMMERCIAL (N.B.Co.) HORSERADISH PEROXIDASE.....	32
4.	EFFECT OF HYDROGEN PEROXIDE ON IAA OXIDASE ACTIVITY OF BIRCH LEAF EXTRACTS.....	34
5.	RELATIVE CONTRIBUTION OF HYDROGEN PEROXIDE AND p-COUMARIC ACID TO IAA OXIDASE FROM BIRCH LEAVES....	35
6.	EFFECTS OF p-COUMARIC ACID ON CRUDE EXTRACTS OF BIRCH ENZYME (SEEDLINGS).....	37
7.	EFFECT OF p-COUMARIC ACID LEVELS ON IAA OXIDASE ACTIVITY OF BIRCH ENZYME AFTER DIALYSIS (SEEDLINGS).	38
8.	EFFECT OF p-COUMARIC ACID ON CRUDE EXTRACTS OF BIRCH ENZYME (SAPLINGS).....	39
9.	EFFECT OF p-COUMARIC ACID LEVELS ON IAA OXIDASE ACTIVITY OF BIRCH ENZYME AFTER DIALYSIS (SAPLINGS)..	40
10.	TESTING FOR COMPETITIVE INHIBITION BY p-COUMARIC ACID.....	42
11.	EFFECT OF p-COUMARIC ACID ON IAA OXIDASE ACTIVITY OF COMMERCIAL HRPO (N.B.Co.).....	44
12.	EFFECT OF CATALASE ON IAA OXIDASE ACTIVITY OF HRPO (N.B.Co.).....	45
13a.	EFFECT OF pH ON IAA OXIDASE ACTIVITY AND PEROXIDASE ACTIVITY IN BIRCH LEAF EXTRACTS.....	46
13b.	EFFECT OF pH ON IAA OXIDASE ACTIVITY OF HRPO (N.B.Co.).....	47
14.	EFFECT OF ENZYME CONCENTRATION ON INITIAL VELOCITY OF IAA OXIDASE ACTIVITY IN BIRCH LEAF EXTRACTS.....	50
15.	EFFECT OF ACETATE BUFFER STRENGTH ON IAA OXIDASE ACTIVITY IN BIRCH LEAF EXTRACTS.....	51
16.	THERMAL INACTIVATION OF BIRCH LEAF ENZYMES.....	54

17.	REACTION SPECTRA FOR BIRCH ENZYME SHOWING APPEARANCE OF PRODUCT (3-METHYLENEOXINDOLE).....	56
18.	IAA OXIDASE ACTIVITY IN AMMONIUM SULFATE FRACTIONS OF BIRCH LEAF EXTRACTS.....	57
19.	DESALTING OF BIRCH LEAF EXTRACT ON SEPHADEX G-25.....	59
20.	GEL FILTRATION OF ULTRAFILTERED EXTRACT FROM BIRCH LEAVES (BIO-GEL, p-100).....	63
21.	CHROMATOGRAPHY OF BIRCH LEAF EXTRACT ON SE-SEPHADEX, AFTER ULTRAFILTRATION.....	67
22.	CHROMATOGRAPHY OF COMMERCIAL HORSERADISH PEROXIDASE (N.B.Co.) ON SE-SEPHADEX.....	68
23.	OXYGEN CATHODE STANDARD CURVE.....	88
24.	FOLIN-LOWRY PROTEIN STANDARD CURVE.....	89
25.	SALKOWSKI STANDARD CURVE.....	90
26.	ASSAY OF IAA OXIDASE ACTIVITY IN BIRCH LEAF EXTRACTS WITH DIMETHYLAMINOCINNAMALDEHYDE (DMACA).....	91

ABSTRACT

CHARACTERISTICS AND PURIFICATION OF
INDOLEACETIC ACID OXIDASE IN
YELLOW BIRCH

by

MERRILL C. HOYLE

An hypothesis was developed to offer a biochemical explanation for the malady known as "birch dieback." Briefly, the hypothesis states that Mn^{2+} stimulation of IAA oxidase systems in yellow birch leaves is primarily responsible for "dieback." The main assumptions in the hypothesis were: (1) that indoleacetic acid (IAA) is the primary hormone controlling plant growth; and (2) that the level of IAA present at any given time in a particular tissue is determined by the enzymatic activity of IAA oxidase.

This work was conducted to examine some basic questions about IAA oxidase in yellow birch leaves so that valid physiological experiments can be designed to test several points relative to the above hypothesis. The present objectives were to establish the presence of IAA oxidase in yellow birch leaves, to determine the cofactor requirements of the enzyme, and to learn if IAA oxidase activity was another catalytic function of a peroxidase, or was, in fact, due to a separate enzyme.

Yellow birch leaves were a potent source of IAA oxidase activity. Birch leaf enzyme could oxidize from 3.2 to 5.0 mg IAA per gram of fresh tissue in ten minutes at maximum velocity, and was therefore equal to pineapple enzyme activity. IAA oxidase activity was proportional to enzyme concentration, and the activity was maximum at about pH 3.6. Strong polyphenol inhibition was removed by treatment with polyvinylpyrrolidone (PVP), and partial inhibition from larger molecules (polymers) was overcome by heat treatment.

The usual cofactors, 2,4 dichlorophenol (DCP) and Mn^{2+} , had little or no effect on IAA oxidase activity of birch enzyme. But H_2O_2 and p-coumaric acid were strong promoters of birch enzyme. The level of p-coumaric required for maximum velocity, increased as purification increased. Commercially purified horseradish peroxidase (HRPO) displayed maximum IAA oxidase activity at pH 3.0, was unaffected by DCP or Mn^{2+} , was inhibited by p-coumaric, and was unaffected by catalase. Based on these results and those reported in the literature the idea is discussed that cofactor requirements of IAA oxidase may be a peculiarity of in vitro assays and are not truly in vivo requirements. The end product of birch IAA oxidase activity was identified as 3-methyleneoxindole.

Both IAA oxidase activity and peroxidase activity moved together during purification on ultrafilter membranes, gel filtration, and gel chromatography on SE-Sephadex. These results were interpreted to mean that both types of enzyme

activity are common to the same protein molecule. Gel chromatography results with commercial HRPO completely substantiated the results with birch enzyme.

INTRODUCTION

This work was undertaken to examine the possible involvement of indoleacetic acid (IAA) oxidase in the malady known as "birch dieback" (25).

IAA is generally recognized as the universal plant growth hormone. The hormone is needed for both cell proliferation and cell elongation. Generally only minute quantities of IAA (i.e., a few micrograms per kilogram of plant tissue) are present in growing tissues at any given time due to its destruction by the oxidative enzyme (12). The activity of IAA oxidase is usually activated by monophenols and Mn^{2+} , and is inhibited by numerous polyphenols (64).

Recent papers by Morgan et al. (40,41,42) and Taylor et al. (62) showed that IAA oxidase activity in cotton leaves was greatly increased by toxic levels of Mn^{2+} . The resulting auxin deficiency was accompanied by the following symptoms: necrotic spots scattered over the leaves; death of apical meristem; elongation of axillary buds and subsequent death; and repetition of this pattern as other dormant buds developed.

Given that "birch dieback" symptoms are similar to those described for Mn^{2+} toxicity in tobacco, and given that high levels of Mn^{2+} have been found in mature yellow birch trees and in seedlings (27,28), we can hypothesize that Mn^{2+} stimulation of IAA oxidase systems in birch is primarily responsible for dieback.

However, before studying the in vivo behavior of IAA

oxidase activity, it is necessary to examine several basic questions about the operational requirements of the enzyme system. First, of course, we must establish that IAA oxidase activity is present in yellow birch leaves, but more importantly, we need to determine the quantities and types of cofactors that are required for meaningful assays. Further we need to acquire a more precise understanding about the actual physical nature of the enzyme. At present, two schools of thought prevail: one says that IAA oxidase activity is simply another function of the common peroxidase (which normally acts upon polyphenolic substrates), i.e., that there are probably two active sites on the peroxidase protein (54); while the other contends that the two types of activity can be separated, and therefore each is a distinct entity (52). In the work reported here, our objectives were to: (1) establish the presence of IAA oxidase activity in yellow birch leaves; (2) work out meaningful assay systems to measure IAA oxidase activity in vitro; and (3) attempt purification of the crude enzyme extracts in order to clarify our basic understanding about the physical and chemical properties of IAA oxidase.

LITERATURE REVIEW

Since the first clear demonstration of IAA oxidase activity in etiolated pea seedlings by Tang and Bonner (61), research on this enzyme has proliferated greatly. The earlier work has been adequately reviewed by Galston and Hillman (15) and Hare (24), and the more recent work on IAA oxidase has been discussed in several symposia (67,68). The entire scope of IAA oxidase research is too broad to be covered here. Rather our discussion of IAA oxidase activity will be limited to papers that are pertinent to the objectives stated for the present work.

Distribution of IAA Oxidase in the Plant

In general, the ability of plant extracts to oxidize IAA increases from growing tips to basal segments of roots and stems (i.e., from younger tissue to older tissue (24), but there are exceptions. Greater IAA oxidase activity with age has been reported for carrot (29), tobacco (40), and pea epicotyl (14). Contrast to this, reports by Sequeira (51) that he could detect little or no IAA oxidase activity in tobacco stems and leaves while Meudt (38) reported less activity in older leaves than in younger leaves of tobacco. The pineapple enzyme also had higher activity in the stem tip with lowest amounts in leaves and roots (20).

These somewhat anomalous results are probably due, in part, to the inverse relationship between IAA oxidase activity and the powerful inhibitors that are endogenous to

many plant enzyme extracts. For example, in tobacco Morgan (40) found that inhibitor activity was highest in the apex and declined basipetally (IAA oxidase activity was the inverse of this). His studies on distribution of enzyme and its inhibitor in greenhouse plants were verified in field grown plants also. However, the most conclusive work on distribution of auxin protectors (i.e., IAA oxidase inhibitors) was conducted on Japanese morning glory stems by Stonier and co-workers (55,56,57,58). Three high molecular weight inhibitors of IAA oxidase were extracted in increasing amounts going from young stem to older (basal) portions.

Distribution of IAA Oxidase in the Cell

Recently, Rautela and Payne (49) have reported that peroxidase enzymes were located mainly in the soluble fraction of cells from sugar beet leaves, whereas chloroplasts and mitochondria only had small amounts. If we accept that IAA oxidase activity is another function of peroxidase (the weight of evidence concurs), we can then assume that IAA oxidase also is found primarily in the soluble fraction of cells.

Extraction of IAA Oxidase

Enzyme assay, purification, study of cofactor requirements, and determination of isoenzymes, etc. are meaningful to the extent that extraction of the enzyme is complete. Problems with absorption of enzymatic protein to cell debris, partial denaturation of enzyme during extraction, or tanning

of proteins (i.e., bound to polyphenols) can all occur.

Consequently, the general recommendation (63) is to prepare aqueous extracts, in the cold, at or near neutral pH. These conditions, aimed primarily at preventing denaturation, have been widely used in studies of IAA oxidase (13,17,18,21, 29,41,50,59,61). Other workers chose to work simply with expressed sap (65,66).

Adsorption of soluble proteins to cell fragments has been reviewed by Newcomb (43) and more recently studied for peroxidase isoenzymes in bean leaves (1). This latter work reported that pH and ionic strength played important roles in recovery of peroxidase isoenzymes. At low ionic strength and neutral pH a large portion of cationic peroxidase was sorbed to natural leaf polymers which acted like ion-exchanges. The cationic peroxidase could be extracted by increasing ionic strength from 0.1u to 0.7u. Neutral and anionic enzyme came out with the lower ionic strength. Higher pH (above 7.0) improved yields of ionic peroxidases at the low ionic strength but not at the high ionic strength.

The problem of precipitated and inactivated enzymes due to phenolic attachment (i.e., tanning) becomes more serious with use of green tissue (this is the reason many early investigators worked solely with etiolated tissue). One can partially cope with this problem by use of acetone powders (63) whereby proteins are precipitated and most phenolics are retained in solution. Further insurance against phenol inactivation of enzymes can be gained through use of reducing

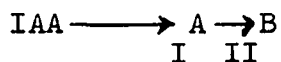
reagents (2) or insoluble, hydrogen-bonding polymers (34). Phenols (if no steric hindrance) can form hydrogen bonds with protein (probably via the peptide oxygen), or phenols can be oxidized to quinones which can polymerize and form covalent bonds to protein (by 1,4-addition of sulfhydryl groups, imino group of proline or free amino groups). Consequently, sulfhydryl reagents such as cysteine, mercaptoethanol, dithiothreitol or metabisulphite (all reducing agents) help by preventing quinone formation during extraction, and insoluble polymers such as polyvinylpyrrolidone (PVP) form hydrogen bonds with the troublesome phenols.

Evaluating IAA Oxidase Activity

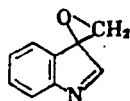
Reaction Sequence

The stoichiometry of enzymatic oxidation of IAA was determined early (61,65). Essentially the oxidation is aerobic and one mole of oxygen is consumed per mole of IAA oxidized, with the release of one mole of carbon dioxide. Studies with labeled IAA (44,60) have shown that the carboxyl carbon is lost and that the 2-carbon and the 3-carbon (on the pyrrole ring) are retained.

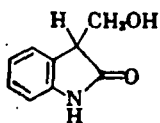
There are still some obscurities in the reaction sequence for IAA oxidation, but much detail has been worked out (44,45,46,47,48). Very simply, the pertinent reactions can be shown as:



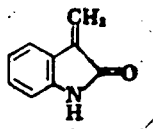
IAA to A is enzymatic and consumes one mole of oxygen with the liberation of one mole of carbon dioxide. The second step is non-enzymatic and does not involve either oxygen or hydrogen peroxide; rather it is spontaneous and acid catalyzed. The formation of A can be followed at 261nm and B can be followed at 272nm. Intermediate and final products have been identified (26). Intermediate A is most probably an epoxide with the following structure,



and intermediate B (which has been isolated) is oxindole-3-carbinol (see below) which slowly converts



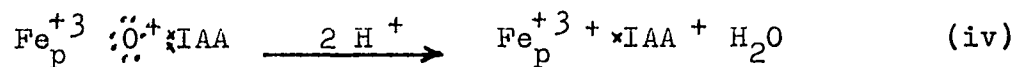
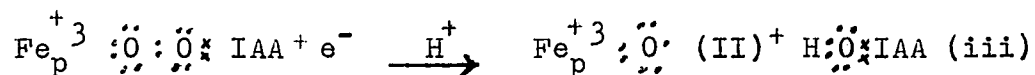
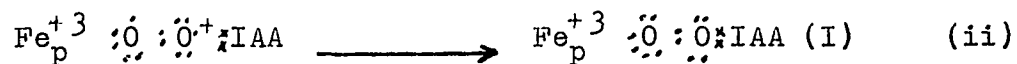
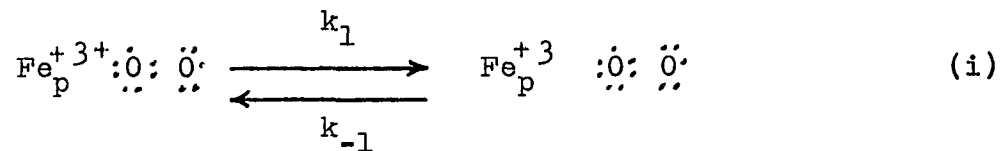
to the final product, 3-methyleneoxindole (see below), having



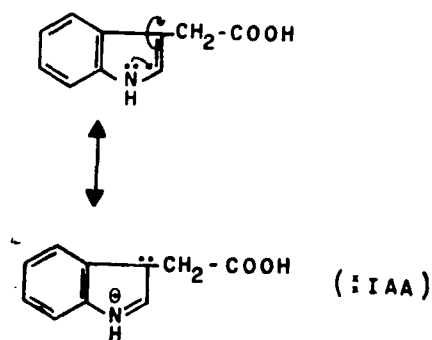
intense double peaks at 247nm and 253nm.

This reaction sequence is apparently triggered by the enzymatic production of IAA free radicals (69) and is auto-catalytic thereafter. For example, recent work by Fox and

co-workers (9,10) attempts to rationalize the oxidase function of peroxidase by the following:



In equation (i), oxygenated peroxidase is formed that further combines with IAA (which is carrying 2 excess electrons at the 3 position on the indole ring - see below) to form complex I having an absorption maximum at 410 nm.



Complex I is then converted to complex II (absorption maximum 417 nm) via a one electron reduction (the electron is provided either by a reducing group on the enzyme or an added reducing agent). Finally complex II is reduced further (this time using one of excess electrons at carbon 3 of another molecule of IAA) to yield free enzyme and an IAA free radical. This IAA free radical would then enter the reaction series of Hinman and Lang (26). Fox's system eliminates the need to produce H_2O_2 in one reaction and use it as substrate in the next as suggested by Kenten (30). The system further accounts for oxygen uptake, generation of free radicals, and shows the need for a reducing agent (such as DCP or p-coumaric). Carbon dioxide evolution occurs early in the reaction sequence with formation of the intermediate epoxide shown above (26).

Measurement

It is possible then to monitor IAA oxidase activity in several ways. Loss of substrate (IAA) has been measured with the Salkowski reagent of Tang and Bonner (61) or the improved Salkowski of Gordon and Weber (19). Problems connected with use of this reagent are: bleaching of the pink color by bright light; some polyphenols reduce the final color while other phenols give colors themselves (37). Finally, there are anomalous effects like those of Hinman and Lang (26) where Salkowski color decreased from 0.65 to 0.06 then rose again to 0.49 in four hours. They inferred

that some of the neutral indole (produced with high concentrations of IAA) either gives the Salkowski test directly or is reconverted, in the acid medium, to IAA.

Appearance of products can now be measured with dimethylaminocinnamaldehyde (DMACA) according to Meudt and Gaines (38). This reagent gave a wine-red color with IAA oxidation products in solution and produced little or no color with IAA itself. To the author's knowledge, there are no reported studies of IAA oxidase where DMACA has been used. It does seem that DMACA would also be a suitable reagent for detecting IAA oxidase bands on electrophoretograms. Because of this very problem, the power of electrophoresis has never been applied to studies of IAA oxidase.

As mentioned above, intermediates and final product formation from IAA oxidase can be followed spectrophotometrically. IAA destruction can be followed at 261 nm (44) and appearance of methyleneoxindole at 247 and 254 nm (26).

In addition, one can monitor IAA oxidation by oxygen consumption. Manometry has been widely used for this purpose in studies of IAA oxidase (40,59,61). Criticism has been leveled at use of Warburg manometers on two points: (1) this method generally requires use of overly large amounts of IAA (i.e., in excess of 2×10^{-4} M) wherein neutral indole is the principle product instead of methyleneoxindole; and (2) the method is only sensitive enough to measure the steady state velocity of IAA oxidation but not sensitive enough to detect the higher initial velocity (46).

A more sensitive and kinetic instrument for measuring oxygen uptake by enzymes is the improved oxygen cathode of Clark (4). This instrument has been used to study phenol oxidases (3) but never (to my knowledge) used with IAA oxidases.

Cofactors

Enzymatic oxidation of IAA has been reported to require one or more of the following as a necessary cofactor: 2,4-dichlorophenol (DCP), hydrogen peroxide (H_2O_2), Mn^{2+} , and infrequently p-coumaric (15,24). The activating effect of Mn^{2+} was first elucidated by Wagenknecht and Burris (65) in bean root and pea seedling extracts. A Mn^{2+} improvement of IAA oxidase activity has also been shown for carrot (29) and tobacco (40). However, Mn^{2+} is not always necessary. Purified horseradish peroxidase acting as IAA oxidase is reported both to require Mn^{2+} (30) and not to require Mn^{2+} (10,44). Neither was Mn^{2+} required for the Omphalia enzyme (47) nor citrus enzyme (18) even after dialysis.

The requirement for DCP is not quite so ambiguous. First reported by Goldacre et al. (17), the monophenol promotion of IAA oxidase was generally confirmed in later studies (24). Concentrations of 10^{-4} to 10^{-5} M were usually optimal (18). In the few cases where DCP did not improve IAA oxidase activity (20), some phenolic compound such as p-coumaric (21) or its depside, (22) was required. Para-coumaric attached to the flavanoid, kaempferol, via three glucose units has been isolated and identified as a natural

promoter also (11). However, partly purified tobacco enzyme was stimulated only by malic acid and not by DCP (33).

Whereas, other work with tobacco IAA oxidase preparations showed a definite DCP need (41,51). We should note also that electrophoretically purified peroxidase (commercial preparation) catalyzes IAA oxidation very well without either Mn^{2+} or DCP (10,26).

However, Fox and co-workers (9,10) further point out that in the absence of DCP (which acts as reducing agent or electron donor), some enzyme is destroyed (i.e., free active enzyme is not entirely regenerated from the enzyme-substrate complex as discussed previously in regard to equation iv) probably because it has to donate electrons for equation iii. But with added reducing agent (e.g., DCP or p-coumaric) enzyme-substrate complexes are made and broken much more quickly and free active enzyme is totally regenerated. This then seems to explain why DCP (or p-coumaric) is not an absolute requirement, but if used, promotes the IAA oxidative activity. Engelsma's work (7) complements the above mechanism. He has shown that p-coumaric acid (and ferulic) destruction is also coupled to IAA oxidation by horseradish peroxidase, or by an enzyme from gherkin seedlings.

The results with H_2O_2 are less ambiguous than those stated for Mn^{2+} and DCP. After extensive work with H_2O_2 on purified Omphalia enzyme, Ray (47) concluded that H_2O_2 was not a major reaction intermediate. Fox and Purves (9) have also determined that H_2O_2 is not a necessary reactant in the

IAA oxidative activity of purified horseradish peroxidase.

Physical Identity of IAA Oxidase Activity

Exactly where does IAA oxidase activity reside? This question is presently unanswerable in its specifics, but generally it is known that IAA oxidase activity "resides," in some way, with the better known peroxidase.

Presently, three hypotheses or concepts are under discussion in the literature. One considers that the two types of activity are separable and distinct enzymes; the second views the two types of activity as residing on one enzyme (peroxidase) but with two active centers: and the third calls attention to the fact of peroxidase isoenzymes where one member of the family of isoenzymes may be the primary residence of IAA activity. These can be examined briefly.

The idea of separate enzymes was put forth by Sequeira and Mineo (52). In other work, they had noted that fresh preparations (tobacco roots) lost IAA oxidase activity upon several weeks in storage; whereas, peroxidase activity was unchanged. Further they found that thermal inactivation points and pH optima for both types of activity were different. By use of SE-Sephadex and 0.1 M eluting buffer, they reported a major IAA oxidase fraction (at 5.4 elution volumes) with little or no peroxidase activity from both tobacco root extracts and commercial horseradish peroxidase preparations.

Siegel and Galston (54) have reported on experiments suggesting two active centers on peroxidase enzymes. With acidified acetone, they were able to precipitate the apoenzyme

from its heme moiety. Apoenzyme oxidized IAA in the presence but not absence of Mn^{2+} and DCP cofactors, but was devoid of peroxidase activity. Partial restoration of peroxidase activity occurred with recombination of heme and apoenzyme. (Verification of the IAA oxidase activity of apoenzyme prepared in similar fashion has been obtained in the author's laboratory also (unpublished). These workers concluded that the IAA oxidase function is possessed by the non-heme protein part of the molecule, and the peroxidase function depends on a heme-protein attachment. They further theorized that the IAA oxidase isolate reported by Sequeira and Mineo (52) may have been free apoenzyme that exists in vivo.

There are some problems with these reports however. First, other workers have shown that peroxidase and IAA oxidase activities run parallel during various steps of purification (45,59), and that thermal inactivation is the same for both (45). And more recently, Ku et al. (32) have found that horseradish peroxidase cleaved into heme and apoenzyme by acid butanone is nearly devoid of IAA oxidase activity. They therefore concluded that the heme prosthetic group is necessary for catalytic activity.

The reality of the existence of several peroxidase isoenzymes is another consideration. In most of the studies considered here, the total oxidase or peroxidase activity has been used without consideration of the multiple forms of an enzyme (i.e., isoenzymes) having slightly different properties and probably different distributions in the organism with re-

gard to time and space.

Four peroxidase isoenzymes were separated from Alaska pea extracts (36) that also contained IAA oxidase activity by use of Dowex 50 cation exchange resin. Two of the cationic species were absent from the roots while the third cationic species was absent from leaf lamina. A neutral species was present throughout the plant. The C₃ cationic species had an IAA oxidase/peroxidase ratio that was tenfold higher than the next most active species, and this was the principle species of stem and root apical regions.

Seven peroxidase isoenzymes (3 anionic, 4 cationic) were separated from horseradish roots (53) and characterized for amino acid composition, carbohydrate component, and type of heme prosthetic group. Fractions B and C (cationic) were more basic than the fractions A-1, A-2, and A-3 (anionic) due to a higher content of arginine. All isoenzymes contained about 18% carbohydrate with differences in individual sugar components, and all isoenzymes contained only protohemin IX.

The same purification techniques were tested on commercial preparations of horseradish peroxidase and it was found that all the anionic isoenzymes were missing, but that the four cationic isoenzymes were present (31). Therefore it is clear that commercial enzyme preparations would not be entirely apropos for studies desiring to locate active centers.

Finally, in considering the preceding discussion, it is evident that little is known with finality about the IAA oxidase activity of plant extracts. As Goldacre (16) has

pointed out so well: "The important task facing us is not only to resolve the descriptive biochemistry of the macerates, especially for each tissue under consideration, but to interpret the meaning of this IAA-destroying activity for the plant."

It is obvious that more penetrating study is needed about the basic chemistry of IAA oxidase activity before we can legitimately proceed to studies of in vivo activity. The complete extraction of all activity from a given tissue or organ needs to be insured, the requirement for cofactors (or perhaps the lack of any) needs further elucidation, and most necessary, the exact physical locus of IAA oxidase activity at the molecular level needs clarification.

METHODS AND MATERIALS

Leaf Samples

Seedlings for this work were obtained by germinating yellow birch seed on Perlite, wetted with distilled water, and set under a combination of CW fluorescent lamps supplemented with far-red from incandescent bulbs. Photoperiod was twenty hours, and day/night temperatures were 27°/18° C., respectively. About ten days after germination, young seedlings were planted in plastic pots filled with forest humus (known to produce seedlings with several thousand parts per million Mn^{2+} in the leaves) (28) and watered daily with distilled water. Ten weeks after planting in pots, leaves were harvested for enzyme preparation. Older basal leaves and incompletely developed leaves near the shoot apex were not used. Leaves were divided into 10g lots (fresh weight), placed in plastic bags and stored in the freezer (-12° C.). In addition, some leaves were collected during the summer of 1969 from young (10-12 feet tall) yellow birch trees growing in the wild. These leaves were weighed and stored as above.

Enzyme Extraction

Frozen, 10g leaf samples were taken for preparation of crude enzyme extracts. Leaves were cut into small pieces and homogenized in 50 ml of cold 0.05 M acetate buffer (pH 5.2) using a Sorvall Omnimixer with an ice bath. The resulting brei was centrifuged for 15 minutes at 22,000 xg at 4° C. The supernatant was saved and the brei was re-extracted

with 10 ml cold buffer, centrifuged as above for 10 minutes and the second supernatant combined with the first. Total volume of crude extract was about 60 ml.

Portions of these crude extracts were then assayed for IAA oxidase activity or partially purified as follows. Routinely, crude extracts were treated with PVP to remove phenolic compounds capable of deactivating enzymes (34). Ten ml of crude extract were shaken for twenty minutes with approximately 5ml of cold, hydrated, insoluble PVP (Polyclar AT) and centrifuged as above. Supernatants were then assayed or further purified by acetone precipitation, salt precipitation and/or dialysis.

Acetone precipitates were made at dry ice temperature after the method of Galston and Baker (13). Cold acetone was added to crude extract at the rate of 4 ml acetone per 10 ml of extract and centrifuged in the cold at 22,000 xg for 10 minutes. The protein pellet was redissolved in its original volume of extraction buffer by magnetic stirring in the cold.

Ammonium sulfate fractions of crude extracts were prepared by addition of solid reagent according to the schedule given by Green and Hughes (23). Extracts were stirred for 15 minutes in the cold (4° C.), centrifuged as above, and the pellet redissolved in its original volume of buffer.

Dialysis was carried out against a liter of distilled-deionized water, with stirring, in the cold for 24, 48, or 72 hours.

Ultrafiltration

An Amicon ultrafiltration apparatus with Diaflo membranes was used to partially separate enzyme protein from other macromolecules of higher and lower molecular weight (MW). These very thin (0.1 to 1.5 μ) membranes are mounted on a much thicker layer of microporous sponge to give a combination of good selectivity, high throughput, and non-clogging characteristics. Prevention of surface clogging is insured by use of a magnetic stirrer.

In practice, 10 ml of crude extract treated with PVP were put into the ultrafilter cell above an XM-100 membrane (i.e., cutoff value equals 100,000 M.W.) and the safety valve was installed. Nitrogen pressure was set at 18 p.s.i. and ultrafiltration continued, with magnetic stirring, until five times the starting volume had passed. This required about 12 hours. Extracting buffer was used to rebuild liquid volume inside the cell. The total filtrate was then concentrated to the initial 10 ml by ultrafiltration under 50 p.s.i. of nitrogen with a UM-20E (cutoff at 20,000 M.W.) or a UM-10 membrane (cutoff at 10,000 M.W.). The retentate, with molecules less than 100,000 M.W. but greater than 10,000 - 20,000 M.W., was taken for IAA oxidase assay or further purified.

Gel Chromatography

Desalting (8) and enzyme purifications were carried out with various chromatographic gels by means of "molecular sieving" and ion exchange. Most of this work was conducted with Ace Glass columns of 2.5 cm by 30 cm or 2.5 cm by 45 cm

dimensions. Sephadex gels of cross-linked dextran and Bio-Gels of cross-linked polyacrylamide were used.

In practice, gels were hydrated with eluting buffer by stirring for periods of time recommended by the manufacturers. Fines were decanted and columns were packed by letting the gel settle through a funnel secured to the top of the column. The column outlet was opened as the gel bed began to form, and the gel was continuously stirred in the funnel until the entire height of the gel bed was attained. Final equilibration with eluting buffer was accomplished in the cold (4° C.) by passing several volumes of buffer through the column under slight positive pressure with a kinetic clamp pump to regulate flow.

Protein samples were applied by pipet to the top of the gel bed after buffer had been eluted to this level. Sample was then allowed to drain into the top of the gel bed followed by several small washings with buffer: allowing each to drain into the bed. Buffer was then carefully added to a height of 3 cm above the gel bed and the column was attached to the buffer reservoir via the kinetic clamp pump. Column effluent was monitored at 280 nm with an ISCO dual beam optical unit and recorded on an Esterline-Angus 6 inch strip recorder. Five ml fractions were collected with a volumetric siphon and ISCO rotary fraction collector. All runs were made at 4° C.

IAA Oxidase Assays

IAA oxidase activity was evaluated primarily with the O_2 cathode (6), secondarily by the improved Salkowski method

(19), and a few tests were made with dimethylaminocinnamaldehyde (DMACA) (39).

For measurement of O_2 uptake from enzyme reaction mixtures, a Clark type O_2 cathode (4) connected to a Sargent Model XV recording polarograph was used. The electrode tip, covered with a Teflon membrane, was inserted into the side of a 2 ml capacity reaction vessel surrounded by a water jacket, and a constant 0.8 volts polarizing voltage was applied. Constant temperature was maintained with circulating water, and constant stirring in the reaction vessel was obtained with a miniature magnetic stirring bar. (Constant atmospheric pressure was assumed but, of course, did not occur in fact).

The instrument was calibrated for O_2 saturation current by bubbling O_2 through liquid in the reaction vessel, and zero O_2 current was obtained by use of Na_2SO_3 . A standard curve (See Appendix) of current vs. O_2 concentration was plotted from these data and used to calculate O_2 uptake for intermediate current changes. Initial velocities for IAA oxidase activity curves are presented in the results either as $ugO_2/min.$ or as angular degrees. This latter measurement bears a curvilinear relationship to the former measurement. Consequently, a 60° angular velocity is much more than twice the O_2 uptake of a 30° angular velocity.

Enzyme reaction mixtures varied somewhat depending upon the particular experiment. However, the following composition was initially used. IAA, 0.2 ml of 15×10^{-4} M; 2,4-DGP, 0.1 ml of 10^{-4} M; $MnCl_2$, 0.1 ml of 10^{-4} M; acetate buffer pH 3.6, 0.4 ml of 0.2 M; and enzyme, 0.6 ml for a total

volume of 1.4 ml. Later in the work, DCP and MnCl_2 were replaced by p-coumaric, 0.1 ml of 3×10^{-3} M; and H_2O_2 , 0.1 ml of 3×10^{-3} M. Most assays were run at 35° C., but other temperatures were used in special cases.

IAA solutions were prepared fresh each day by dissolution in 1 ml of 70% ethyl alcohol and made to volume with distilled deionized water. Para-coumaric acid solutions were prepared in like fashion and stored in the dark at 4° C. when not in use. Stock solutions of 0.3 M H_2O_2 were kept in the dark at 4° C. and diluted 1:100 for use.

Loss of IAA was measured with improved Salkowski reagent. In our technique, three times the usual amount of reaction mixture components (total volume 5.2 ml) was used, and one ml aliquots were taken periodically during the reaction to measure residual IAA. Two ml of Salkowski reagent was mixed with these 1 ml aliquots and read on a Beckman spectrophotometer (Model DU) at 530 nm after 30 minutes developing time. Residual IAA was calculated by difference. Absorbance was linear between 1 and 30 ug IAA per ml (See Appendix).

DMACA was tested to determine if it would form colored reaction products from activity of birch IAA oxidase. The procedure of Meudt and Gaines (39) was followed. Two ml of DMACA (1% solution dissolved in 2 M HCl) were mixed with 2 ml of enzyme reaction mixture and read at 562 nm after developing for 70 minutes in the dark.

Identification of the end product, 3-methyleneoxin-

dole, was determined by recording absorption spectra of birch IAA oxidase activity on a Beckman Model DB-G recording spectrophotometer in the UV region. Regular reaction mixtures were diluted 1:1 for this purpose.

Peroxidase Assay

Peroxidase activity in birch leaf extracts was followed by modifying the method of Sequeira and Mineo (52). The reaction mixture contained 0.1 ml of 0.02 M guaiacol, 1.0 ml of 0.4 M H₂O₂, 3.8 ml of 0.2 M acetate buffer (pH 4.0), and 0.2 ml of enzyme. Changes in absorbance at 470 nm were measured at room temperature with the Beckman DU spectrophotometer.

Total Protein

Protein was estimated by O.D. at 280 nm for column effluents. Total protein in crude and partly purified extracts (i.e., after dialysis, ammonium sulfate fractionation, or acetone precipitation) was estimated by the method of Lowry et al. (35).

RESULTS

Extraction

Both fresh leaves and their acetone powders were extracted with buffer solutions or distilled-deionized water. The homogenates of green brei were very viscous (especially from acetone powders), and attempts to squeeze the brei through cheesecloth were unsuccessful. After numerous trials, a double extraction with acid buffer combined with high speed centrifugation was adopted (as described in previous section). Unbuffered extracts usually measured around pH 5.0. So a weak buffer, near this acidity, was used routinely for all extractions and found to be satisfactory (Table I). Color of the extracts varied from light green to dark brown and varied with age of leaves extracted. Leaves from actively growing seedlings gave green extracts, but leaves from seedlings that had entered into senescence were always some degree of brown. Undoubtedly there had been some loss of chlorophyll and a degree of quinone formation.

Measuring Enzyme Activity

Effects with PVP

Assuming that phenolic compounds would be present in the extracts and would inhibit IAA oxidase activity to different degrees, the use of polyvinylpyrrolidone (PVP) was adopted. The effectiveness of PVP treatment was visibly indicated by a marked reduction in brown color of extracts from older leaves. Phenolic inhibition of IAA oxidase activity and its reduction

by PVP is shown in Figure 1 for two different samples. Clearly, the amount of phenolic contamination in different extracts was quite variable. Generally, there was no measurable oxygen utilization when PVP treatment was omitted.

Table I. Acidity of Various Aqueous Extracts from Yellow Birch Leaves.

Acidity measured with Beckman Model G glass electrode.

Sample No.	Extractant	pH of Extract
33	Distilled Water	4.88
35	"	4.92
36	"	4.95
39	"	4.95
50	"	5.10
52b	"	4.65
51a	Acetate buffer 0.05M, pH 5.2	5.25
51b	"	5.18
53a ₁	"	5.12
53a ₂	"	5.20
53a ₃	"	5.10

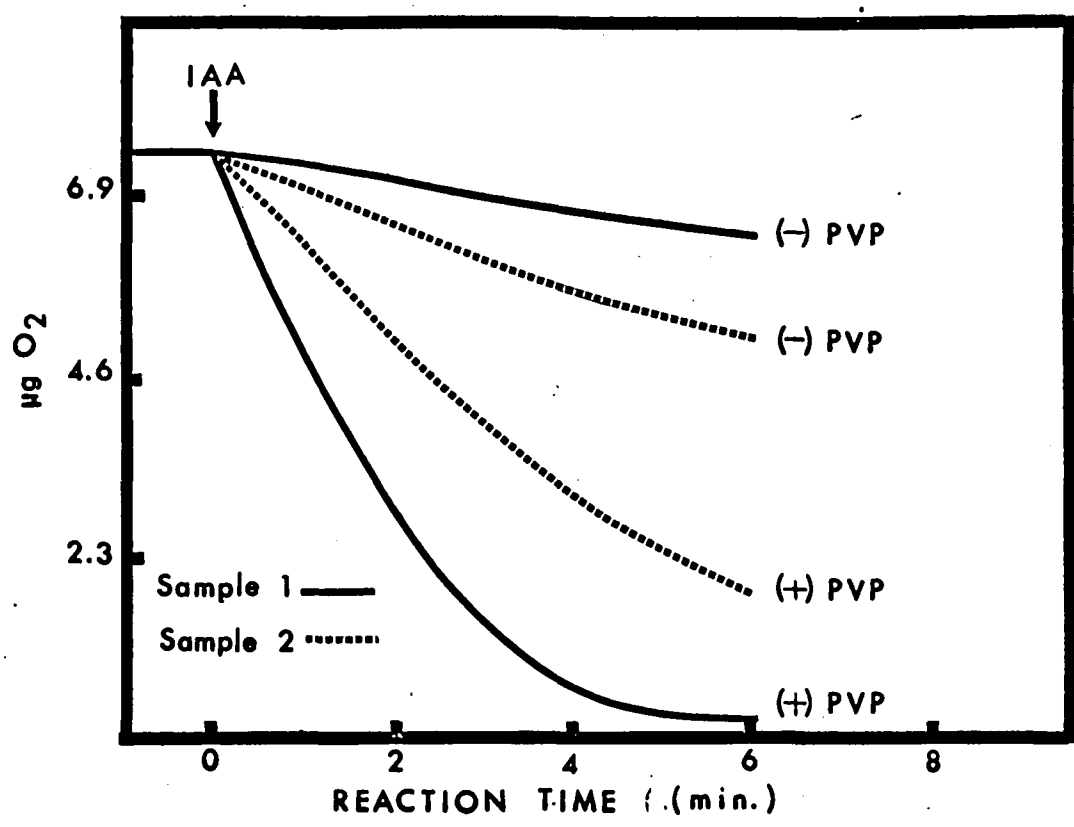


Fig. 1. Effect of PVP on IAA oxidase activity of birch leaf extracts.

Salkowski assays for residual IAA in reaction mixes with crude birch enzyme were impossible to make without PVP treatment of the enzyme. Instead of the usual pink color than develops with addition of Salkowski reagent to enzyme reaction mixtures, a brown precipitate formed and there was little or no pink color. However, by using PVP treated enzyme, the Salkowski determinations were satisfactory. Typical progress curves for IAA oxidase activity measured with Salkowski reagent are shown in Figure 2. Note, that in both Figure 1 and Figure 2, there is no lag period prior to attainment of maximum velocity.

Effects of Mn^{2+} and DCP

Numerous trials were conducted to determine the necessity of DCP and/or Mn^{2+} as cofactors for in vitro assay of IAA oxidase activity. Early in these investigations, the detection of IAA oxidase activity in birch leaves was a "hit-or-miss" situation even with PVP treatment. However, when active extracts were found, tests such as those shown in Table II were made. These results indicated that IAA oxidase activity in any given crude extract was hardly changed by the presence or absence of DCP and Mn^{2+} , whether these cofactors were omitted singly or together. When changes did occur, they usually showed slight increases in maximum velocity when DCP and Mn were omitted. Neither was there any improvement in oxidase activity when DCP and Mn^{2+} were each tested at various concentrations of 10^{-5} , 10^{-4} , and 10^{-3} molar.

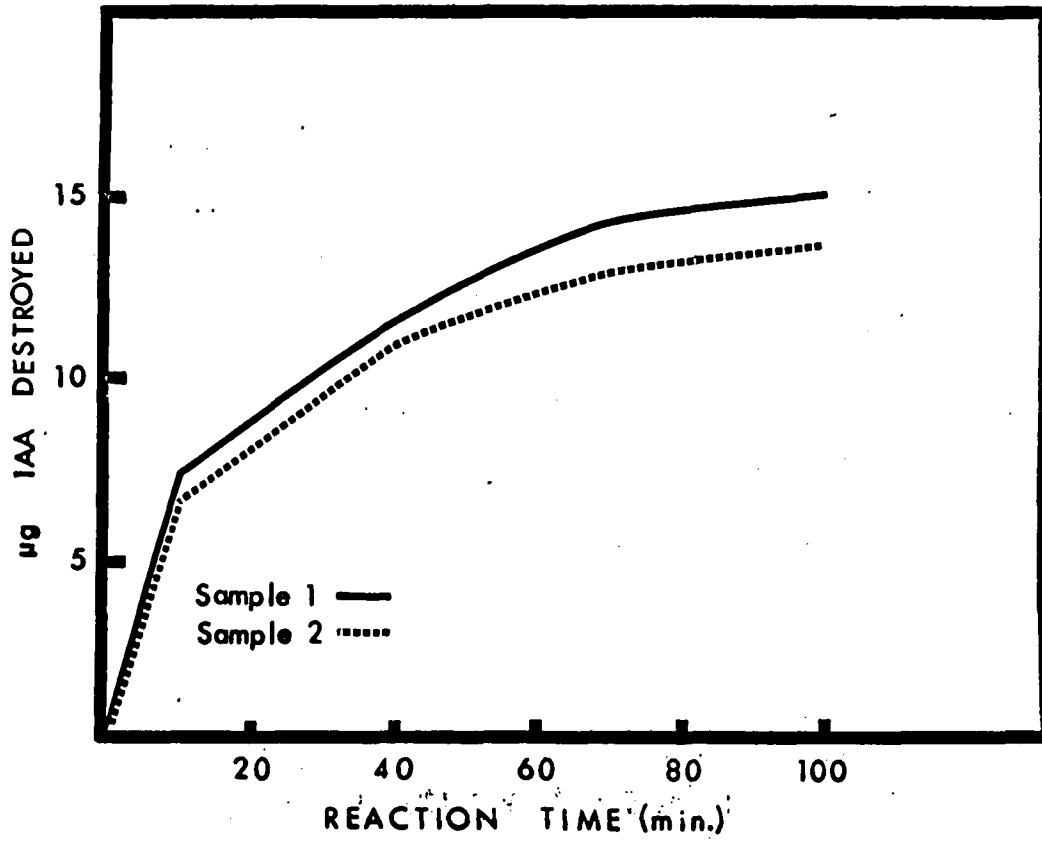


Fig. 2. Salkowski assay of birch enzyme treated with PVP.

Table II. Effects of DCP and Mn²⁺ on IAA Oxidase Activity of Birch Leaf Extracts Treated with PVP and Partially Purified.

Oxygen cathode assays at pH 4.6, temperature 30° C., amounts of cofactors are as given in "Methods and Materials." Maximum velocity is given in angular degrees.

Sample No.	Treatment	Cofactors Omitted	Maximum Velocity
40	crude	none	19
40	crude	DCP, Mn	19
40	dialyzed	none	0
40	dialyzed	DCP, Mn	0
40	35-70% ammonium sulfate ppt.	none	3
40	acetone ppt.	none	4
38	crude	none	33
38	crude	Mn	38
38	crude	DCP	40
38	acetone ppt.	none	2

Upon partial purification of birch enzyme extracts by dialysis, ammonium sulfate fractionation, or acetone precipitation practically no IAA oxidase activity could be detected (Table II). The addition of Mn^{2+} and DCP (either singly or in combination) did not restore activity to purified extracts either. Therefore it was concluded that the cofactors, DCP and Mn^{2+} , were not necessary for IAA oxidase activity of the birch leaf enzyme. Further, it was apparent that naturally occurring endogenous cofactors were lost by all of the three purification procedures. On the possibility that catalytic quantities of Mn^{2+} , or some other heavy metal ion, might be present in the extracts, a chelate (EDTA) was incubated with the reaction mixtures. The EDTA had no effect on either dialyzed or nondialyzed enzyme.

As discussed previously, highly purified commercial preparations of horseradish peroxidase (HRPO) have been assayed for IAA oxidase activity without DCP and Mn^{2+} , but some improvement generally is reported when these cofactors are included. An example of our tests made with commercial HRPO is shown in Figure 3. These and other tests showed quite clearly that highly purified HRPO does not require either DCP or Mn^{2+} for maximum IAA oxidase activity.

Effects of p-Coumaric and H_2O_2

Several compounds were tested for their ability to promote the IAA oxidase activity of birch leaf enzyme. L-malic acid was tried because of its positive effect on partially purified cotton enzyme (33), but was found to have

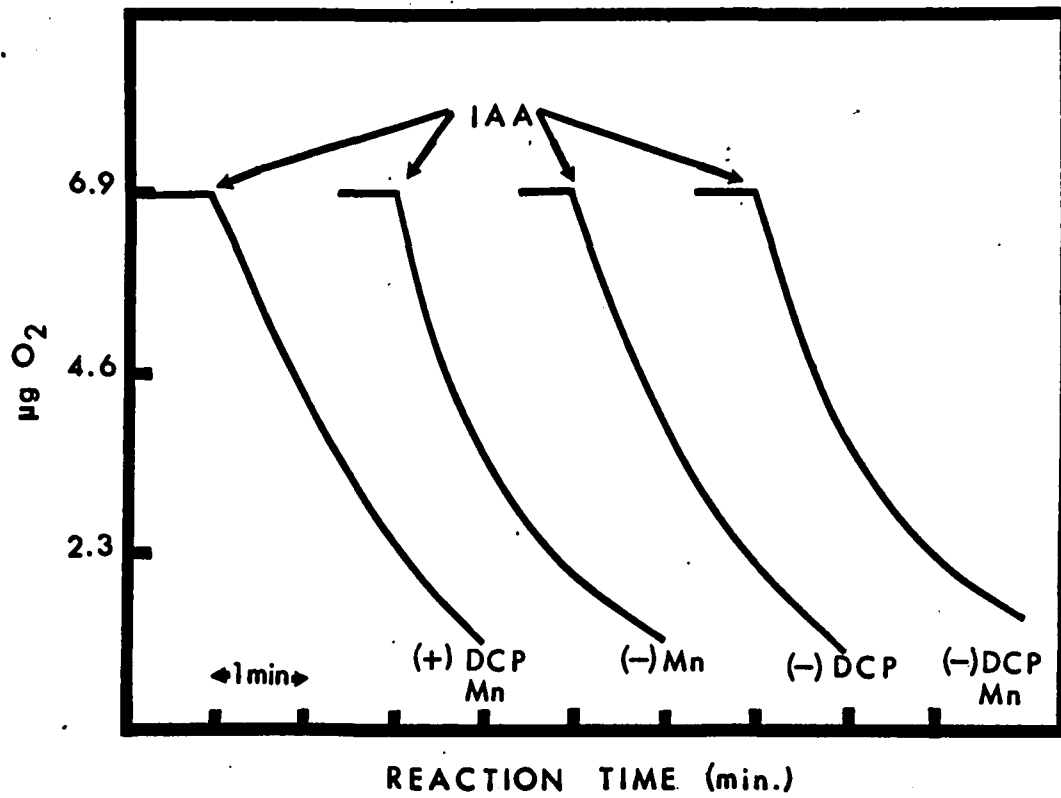


Fig. 3. Effect of DCP and Mn^{2+} on IAA oxidase activity of commercial (N.B.Co.) horse-radish peroxidase.

no effect on birch enzyme. Oxalic acid was without effect also. Salicylic acid was tested because it is close in structure to methyl salicylate (oil of wintergreen) an endogenous compound in yellow birch. Results with salicylic acid were likewise negative.

Considering the reports of stimulatory effects by H_2O_2 on Omphalia IAA oxidase (47), this compound was tested next and found to have a positive effect. In early trials with crude extracts, maximum velocity of IAA oxidase increased as H_2O_2 concentration increased from 0.1u moles to 0.4u moles. Most of the increase occurred between zero and 0.3u moles H_2O_2 ; whereas the increase from 0.3u moles to 0.4u moles was only about 4° of angular velocity at maximum. Stimulation of birch IAA oxidase activity by H_2O_2 was quite variable among different samples of crude extracts, but activity improved in all. A striking example is given in Figure 4. The angular velocity of 22° without H_2O_2 increased to 74° in the presence of 0.45u moles H_2O_2 .

The need for p-coumaric acid was finally determined in ammonium sulfate fractions of crude birch enzyme. Even in the presence of H_2O_2 , no activity could be measured in these fractions until p-coumaric acid was added. Similarly, dialyzed or acetone precipitated enzyme could not be re-activated until p-coumaric was given in addition to H_2O_2 . The relative contribution of these two compounds on birch IAA oxidase is shown in Figure 5. With IAA alone, the maximum angular velocity was only 12° , but this increased to 36°

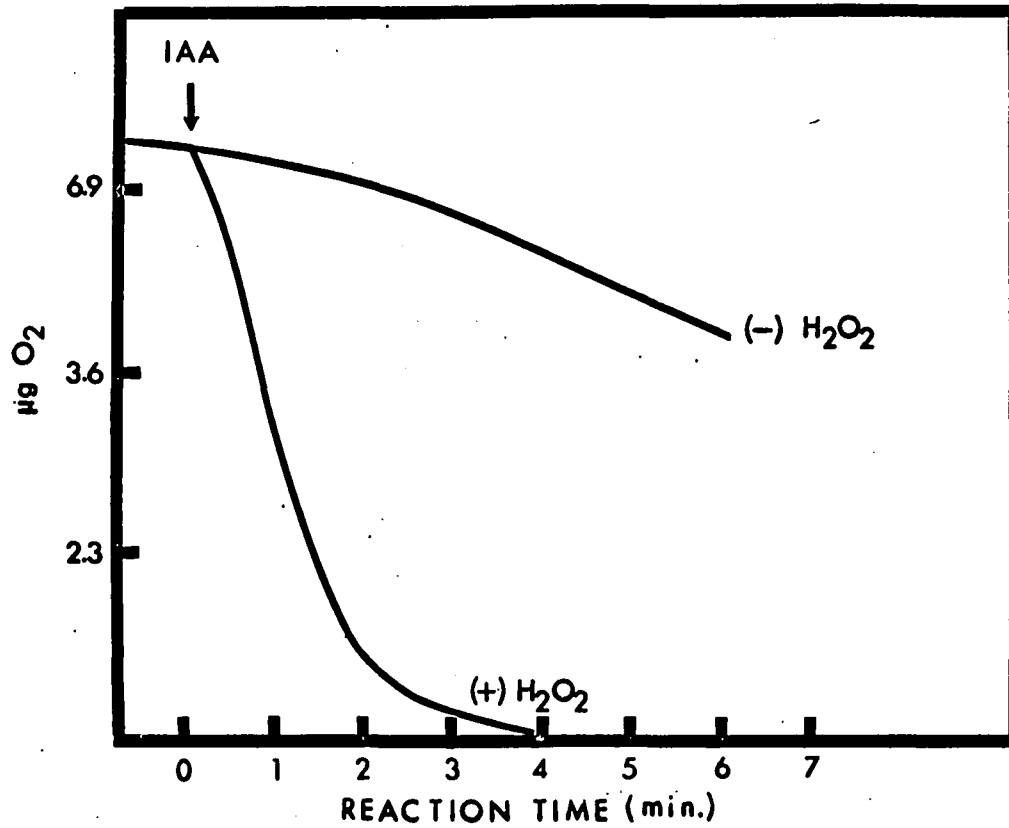


Fig. 4. Effect of hydrogen peroxide on IAA oxidase activity of birch leaf extracts.

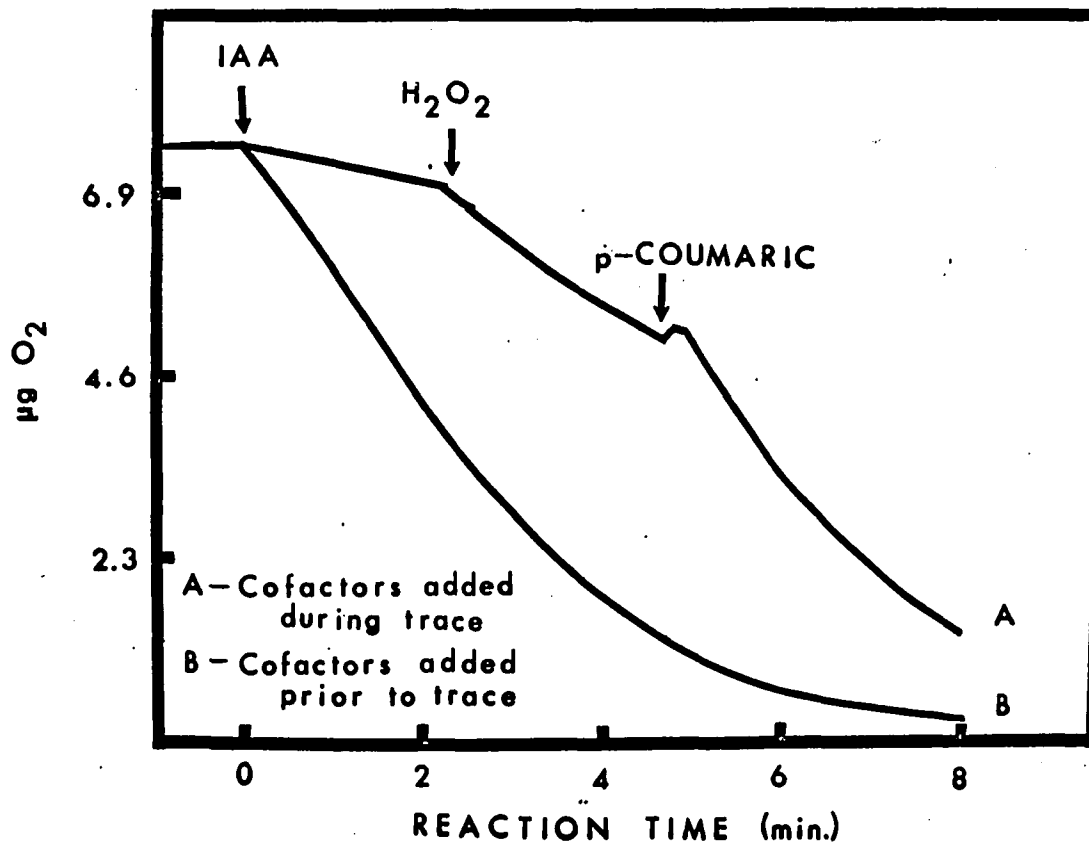


Fig. 5. Relative contribution of hydrogen peroxide and p-coumaric acid to IAA oxidase from birch leaves.

with addition of H_2O_2 and higher still to 55° when p-coumaric was added.

The endogenous levels of p-coumaric (or its natural counterpart) varied among sample extracts. This was indicated by the degree of measurable activity when p-coumaric was omitted (compare uppermost curves in Figures 6 and 8). The response in IAA oxidase activity to increasing levels of p-coumaric was also different for different extracts. The sample in Figure 6 seemed to be approaching its maximum velocity with 0.6u moles of p-coumaric; whereas the sample in Figure 8 was still responding strongly to levels of p-coumaric up to 1.35u moles.

Dialysis effectively removed the endogenous cofactor (see upper curves in Figures 7 and 9). There was little or no activity in the absence of p-coumaric, but with increasing levels of p-coumaric IAA oxidase activity in dialysates could be restored to a fraction, to the full amount, or in excess of the activity in crude extracts (Figures 7 and 9, Table III). To date, the exact amount of p-coumaric that is needed to realize maximum IAA oxidase activity in variously treated extracts is not clear. It appears to be around one micromole, plus or minus a few tenths, for our system.

Tests for competitive inhibition between IAA and p-coumaric for the active site of birch IAA oxidase are shown in Figure 10. If some degree of competitive inhibition was possible, then the velocity of IAA oxidase activity would be less when p-coumaric was added to the enzyme before IAA than when p-coumaric was added after IAA. However, the maximum

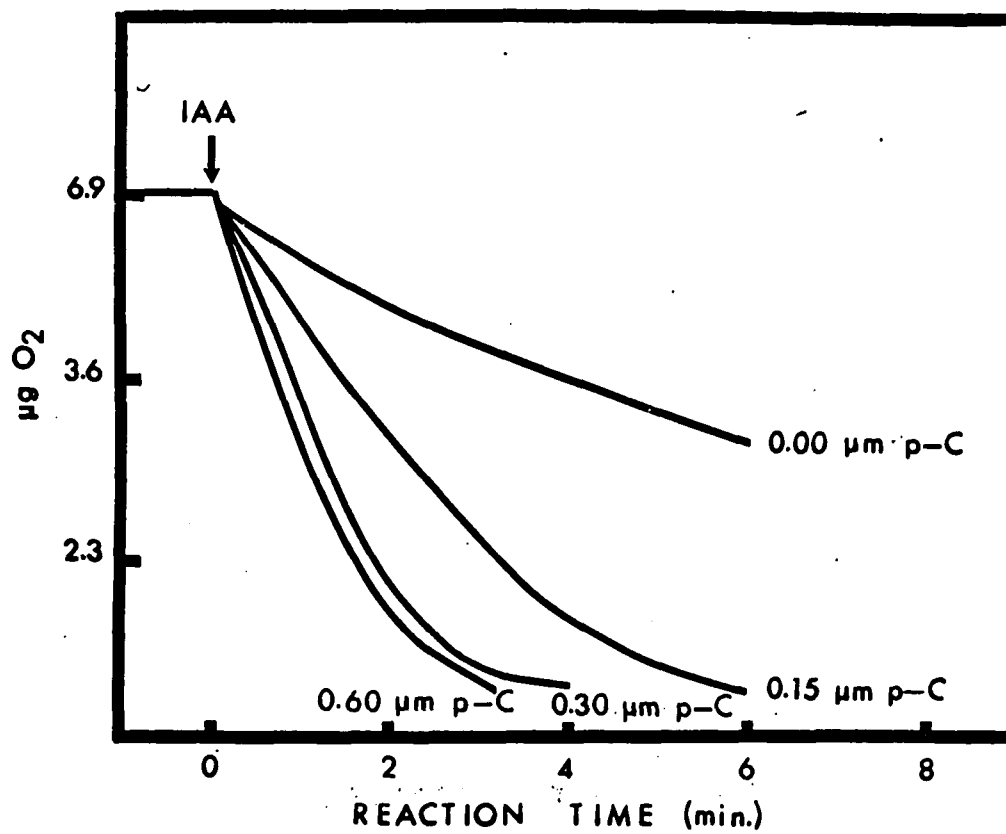


Fig. 6. Effects of p-Coumaric acid on crude extracts of birch enzyme (seedlings).

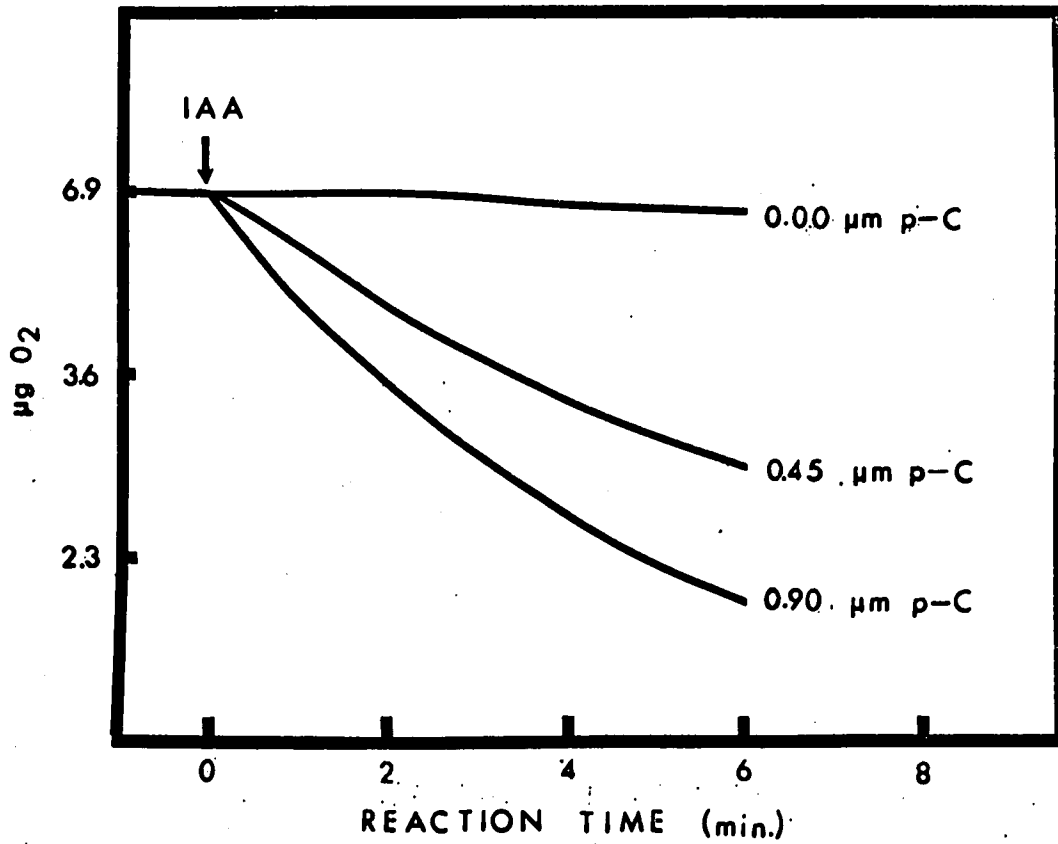


Fig. 7. Effect of p-Coumaric acid levels on IAA oxidase activity of birch enzyme after dialysis (seedlings).

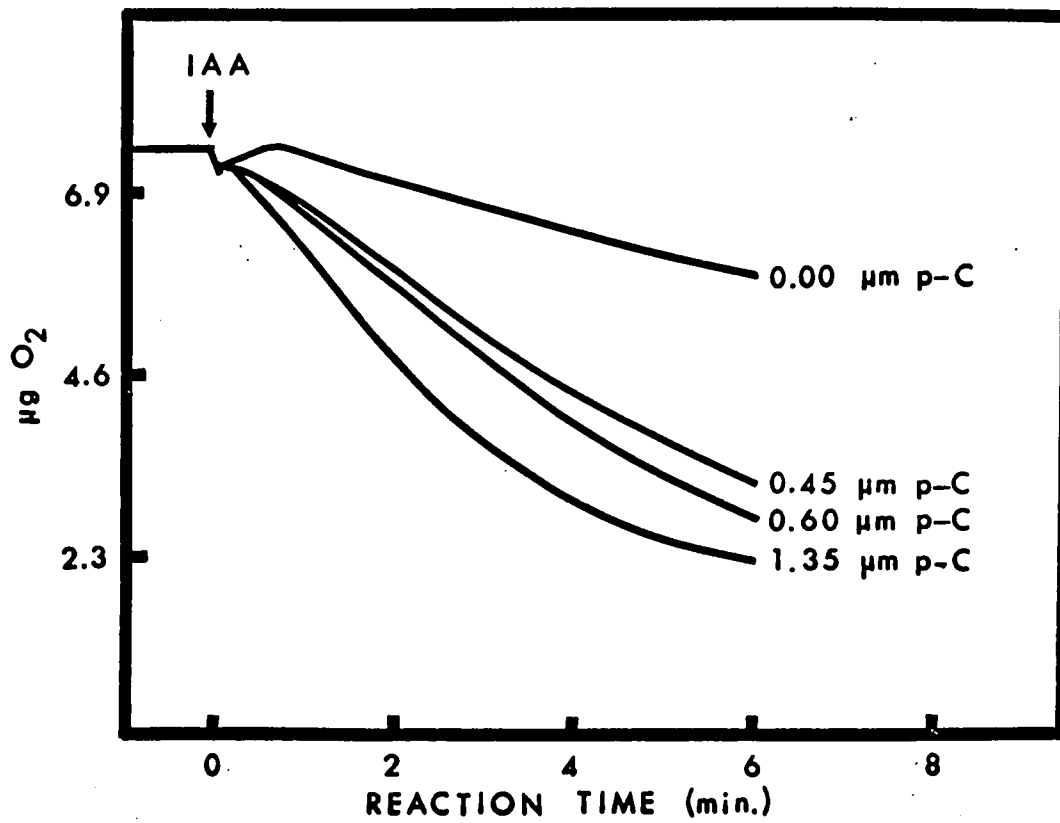


Fig. 8. Effect of p-Coumaric acid on crude extracts of birch enzyme (saplings).

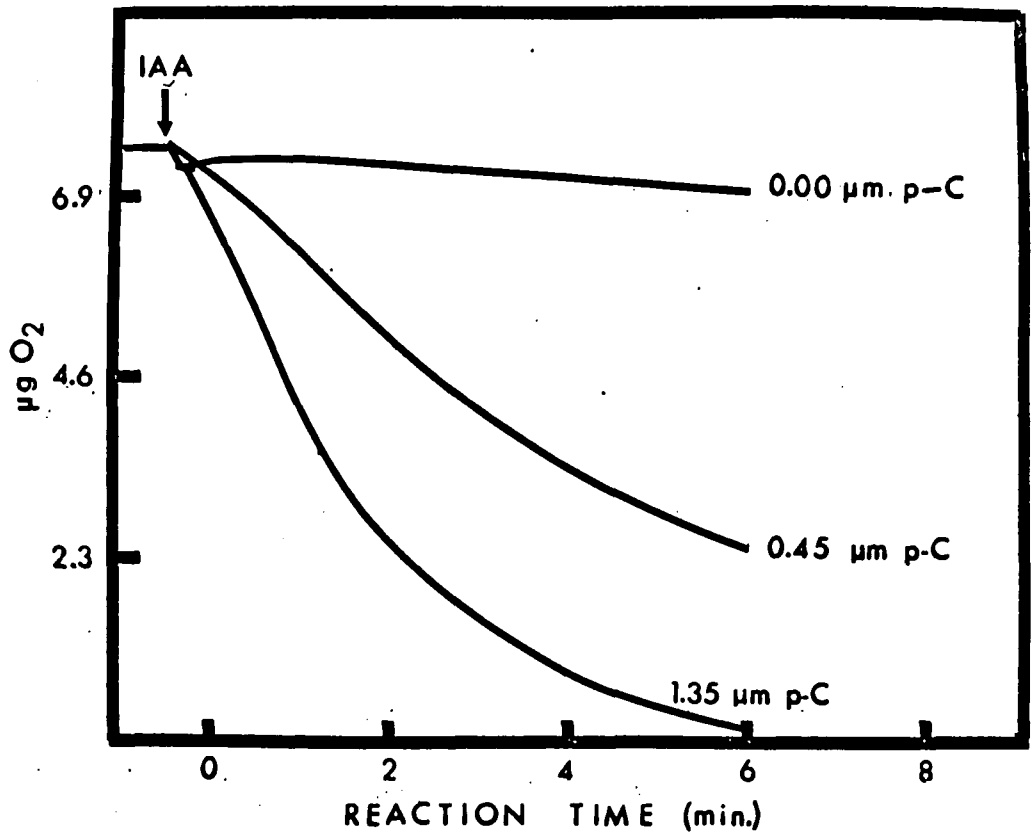


Fig. 9. Effect of p-Coumaric acid levels on IAA oxidase activity of birch enzyme after dialysis (saplings).

Table III. Effect of p-Coumaric Acid on IAA Oxidase Activity in Birch Leaf Extracts.

Oxygen cathode assays at temperature 35°, pH 3.6.

Maximum velocity given in angular degrees.

Sample No.	Type of Extract	p-Coumaric added (u moles)	Maximum Velocity°
5870G-1	crude	0.45	10
"	"	0.90	21
"	"	1.35	33
52Hd.	crude	0.45	45
"	"	1.35	52
"	dialyzed	0.45	52
"	"	1.35	62

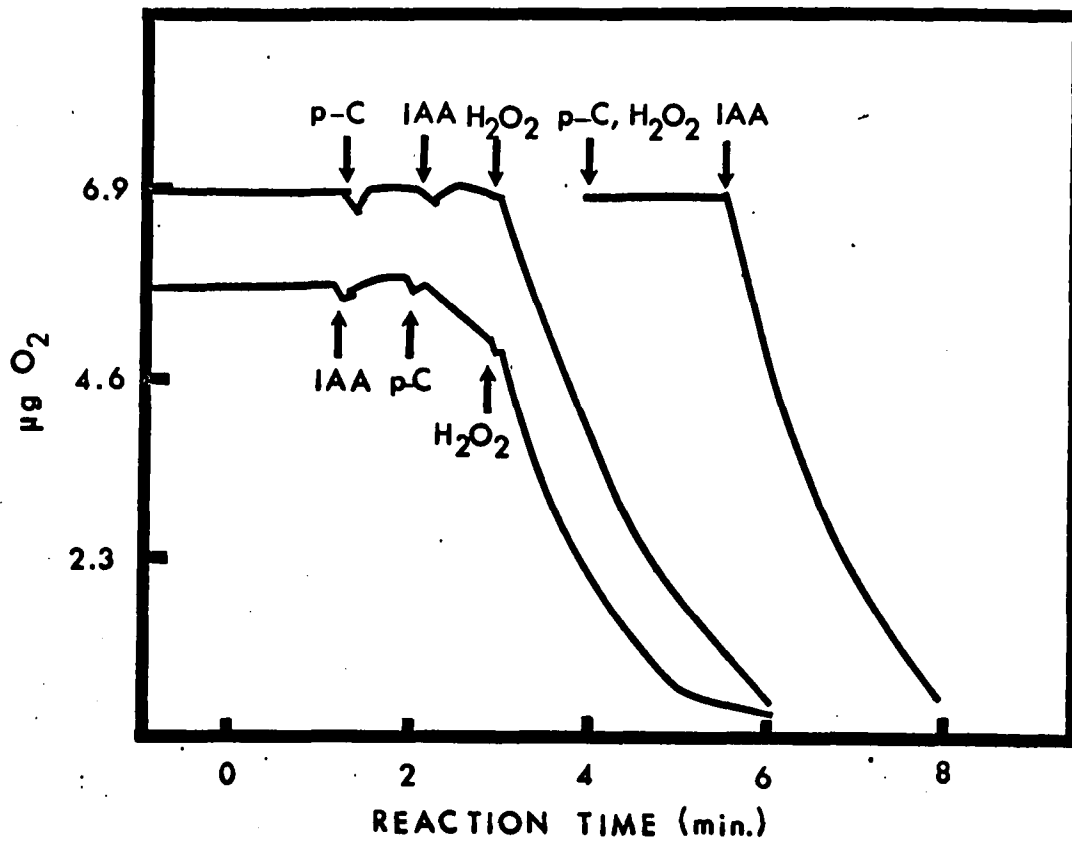


Fig. 10. Testing for competitive inhibition by p-Coumaric acid.

velocity for IAA oxidase was the same regardless of the order in which reactants were added. HRPO was inhibited by p-coumaric (Figure 11), but the nature of the inhibition has not been examined.

The IAA oxidase activity of HRPO was not affected by addition of catalase, which verifies that H_2O_2 is not a reaction intermediate for this enzyme (Figure 12). The effects of catalase on IAA oxidase activity of birch enzyme were not conclusive from the oxygen cathode data; since oxygen was produced (catalase on H_2O_2 in reaction mixtures) and consumed (IAA oxidase activity) when both enzymes were present. The resulting curve was a trace of net oxygen level. The experiments with birch enzyme did show that crude extracts being assayed soon after treatment with PVP have an active catalase inhibitor which becomes inactive after several days incubation at room temperature or by dialysis.

Effect of pH

Maximum IAA oxidase activity of birch enzyme and HRPO both occur at high acidity. Birch enzyme displayed greatest oxidase activity at pH 4.0 by static analysis with Salkowski reagent (Figure 13a), but subsequent kinetic analysis with recording oxygen cathode indicate maximum oxidase activity closer to pH 3.6. The oxidase function of HRPO operates maximally in the region pH 3.0 to pH 3.6 (Figure 13b).

Maximum peroxidase activity of birch enzyme was estimated at pH 4.0 (Figure 13a) when determined by static analysis. Closer approximations than this have not been made.

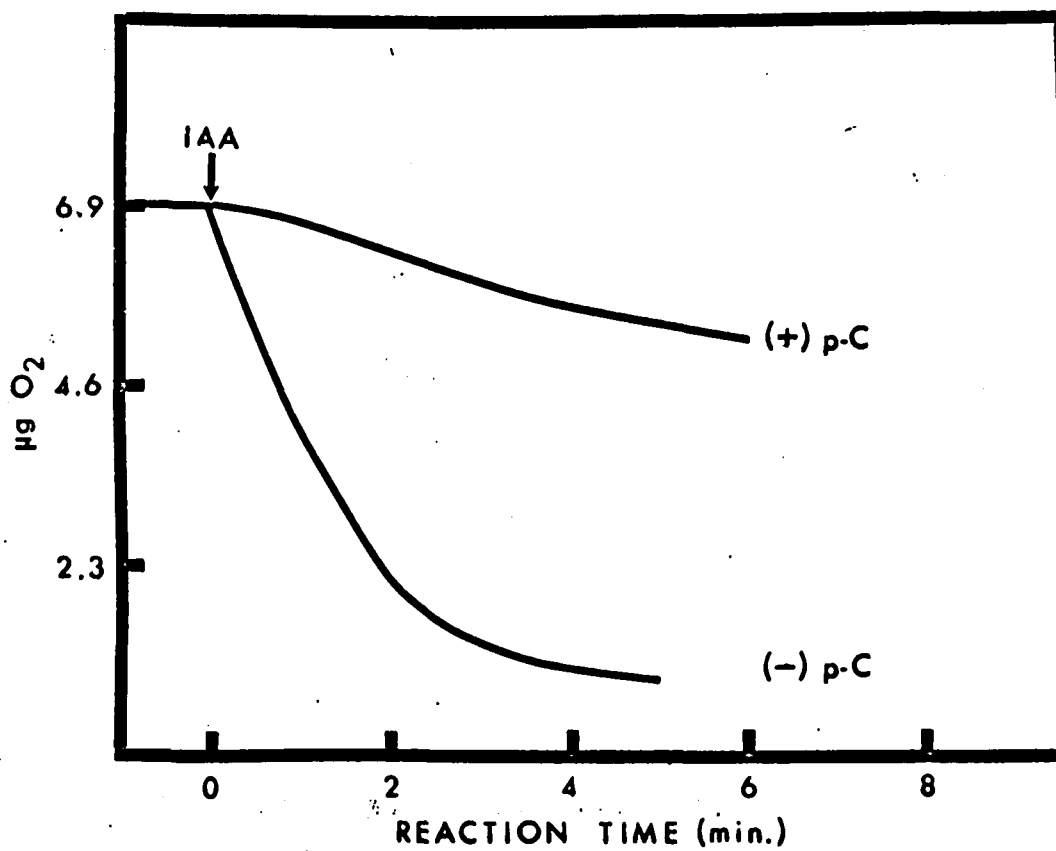


Fig. 11. Effect of p-Coumaric acid on IAA oxidase activity of commercial HRPO (N.B.Co.).

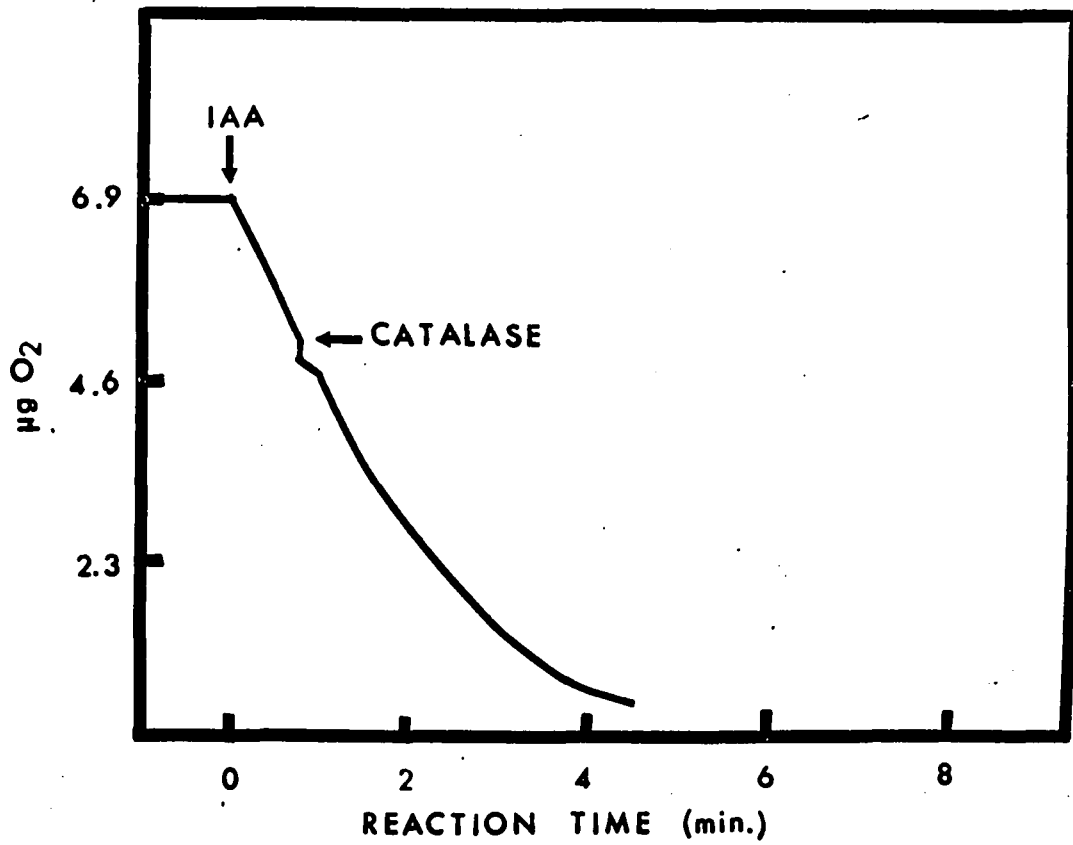


Fig. 12. Effect of catalase on IAA oxidase activity of HRPO (N.B.Co.).

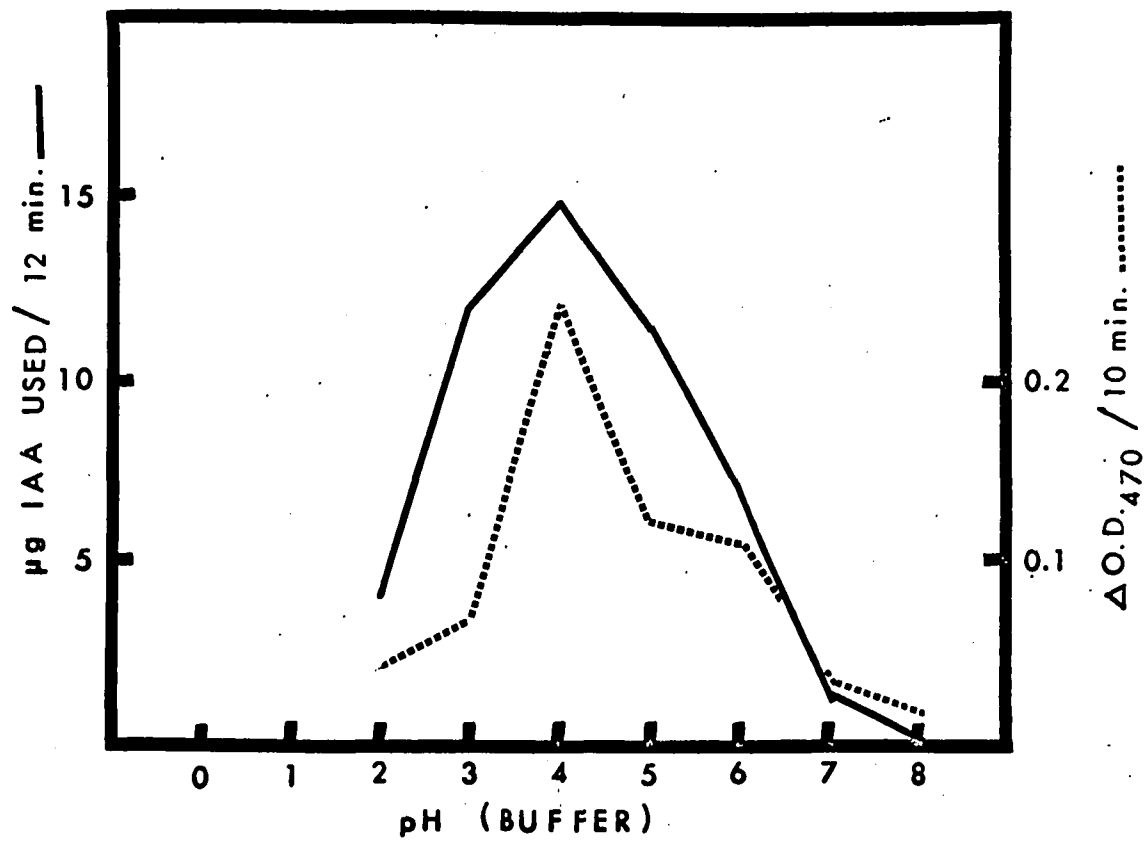


Fig. 13a. Effect of pH on IAA oxidase activity and peroxidase activity in birch leaf extracts.

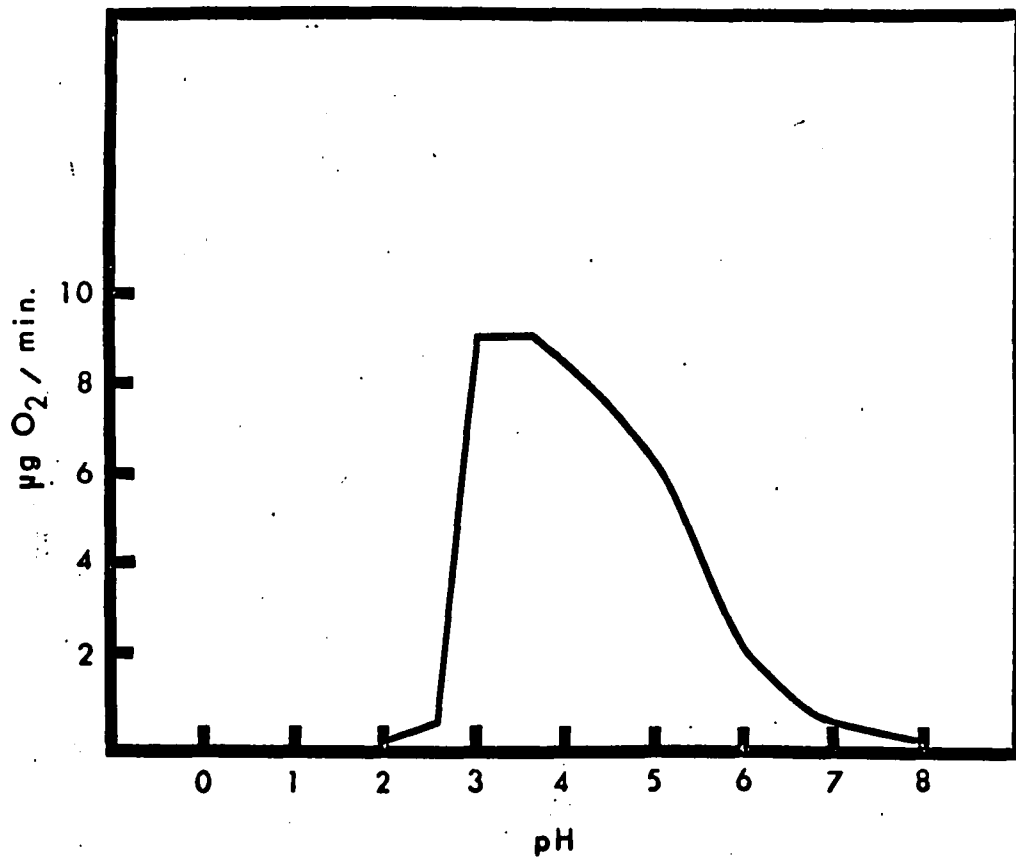


Fig. 13b. Effect of pH on IAA oxidase activity of HRPO (N.B.Co.).

Effects of Substrate, Enzyme, and Buffer

For a given level of birch enzyme, IAA oxidase activity increased with substrate up to 0.3 μ moles, and this has been used as the amount required for enzyme saturation in our standard system (Table IV). Duration of the oxidase reaction was proportional to the amount of IAA.

Maximum velocity of IAA oxidase activity in birch enzyme (Figure 14) increases with enzyme levels up to 0.6 ml in our system with 0.3 μ moles of substrate (IAA) present.

Oxidase activity did not change with buffer concentration over the range 0.1 M to 0.4 M, but there was a slight improvement with 0.6 M (Figure 15).

Effect of Storage Time

In practice, the birch leaf extracts were held in cold storage without PVP treatment. Aliquots of the stored enzyme were taken when needed, treated with PVP and assayed after a period at incubation temperature. Handled this way, aqueous extracts retained their IAA oxidase and guaiacol peroxidase activities after several months in storage (Table V). The data given suggest that peroxidase activity and oxidase activity tend to vary together, but this is not always the case. In fact, some extracts have been tested that had considerable IAA oxidase activity (i.e., angular velocity 60°), but barely detectable peroxidase activity. Also we have noticed that, in a given extract, peroxidase activity may often decline more rapidly than oxidase activity with time in storage. If extracts are treated with PVP soon after

Table IV. Effect of Substrate Levels on IAA Oxidase Activity of Birch Enzyme.

IAA levels are given; otherwise conditions are as given under "Materials and Methods."

IAA (u moles)	Total Rx. Time (min)	Vmax. (ugO ₂ /min)
0.075	2.0	0.85
0.15	4.5	1.22
0.30	9.0	1.35
0.60	17.0	1.30

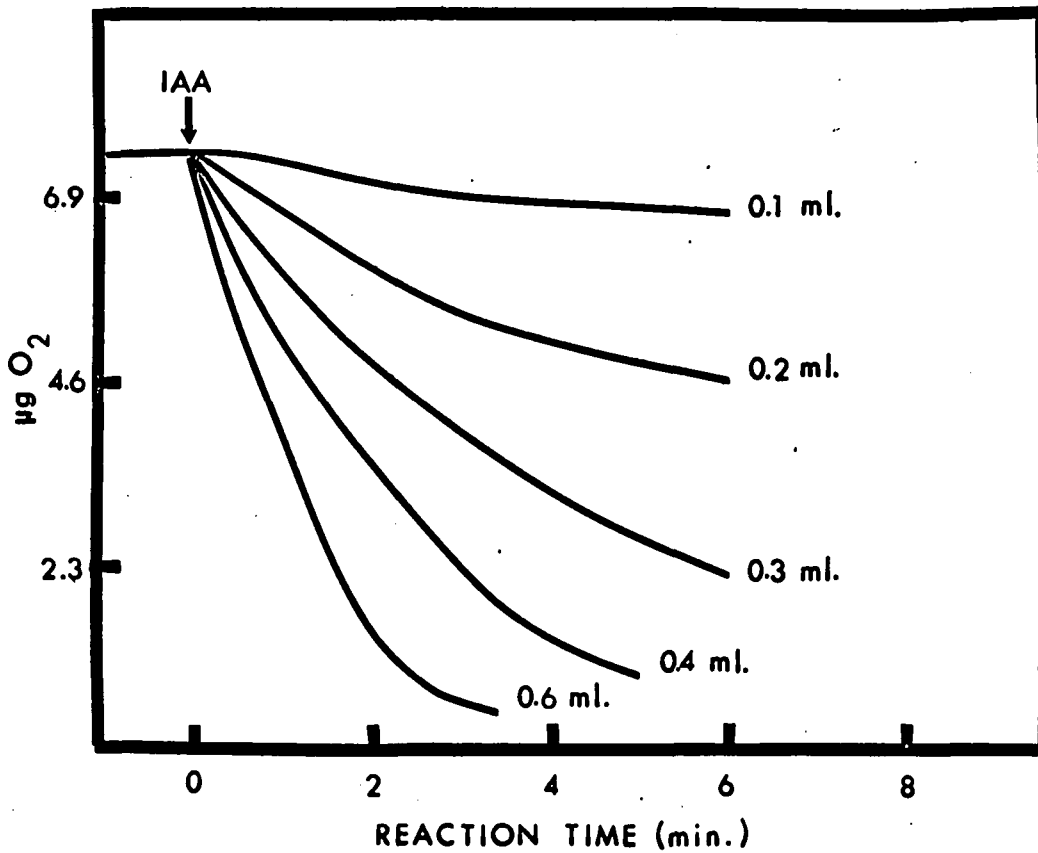


Fig. 14. Effect of enzyme concentration on initial velocity of IAA oxidase in birch leaf extracts.

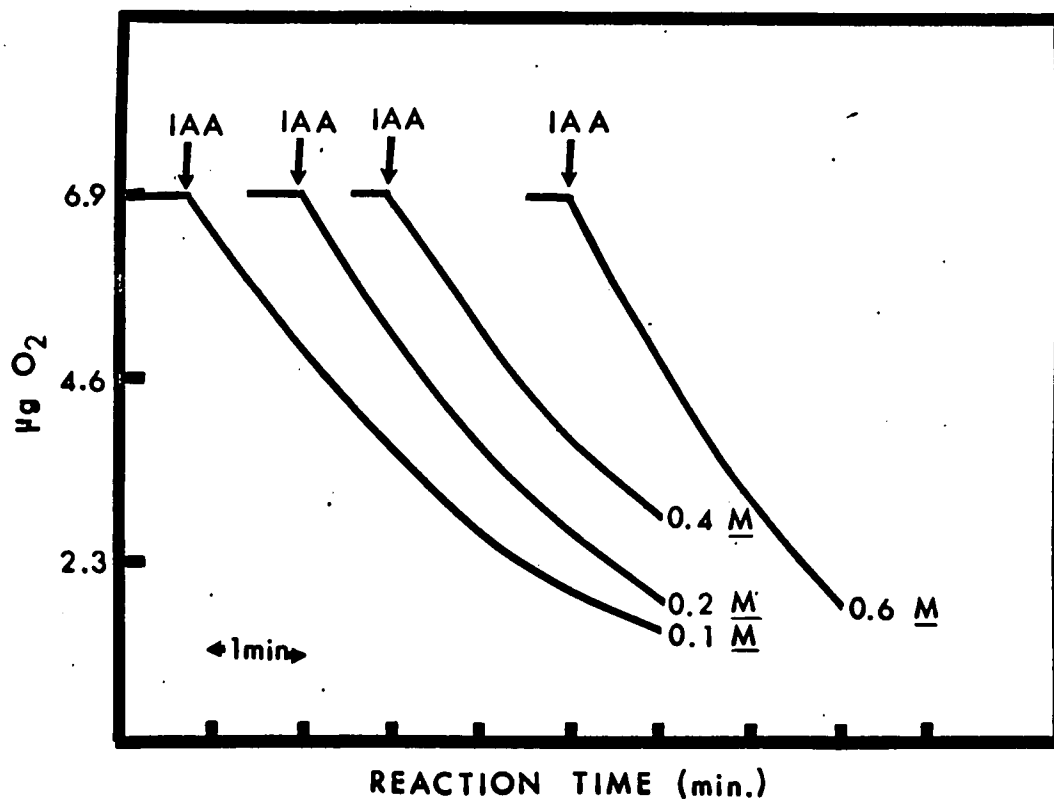


Fig. 15. Effect of acetate buffer strength on IAA oxidase activity in birch leaf extracts.

Table V. Comparison IAA Oxidase and Peroxidase in Crude Extracts after Two Months in Cold (4° C.) Storage.

Assays are given in "Methods and Materials."

Extract No.	IAA Oxidase (Angular velocity ^o)	Peroxidase (OD ₄₇₀ /19 min.)
G11	55	0.079
G12	48	0.058
G15	33	0.023

preparation and then stored in the cold, the shelf life of enzyme activity is much shorter.

Whole leaves could also be stored at freezer temperature (-12° C.) for many months (9 months in our experience), and retain levels of IAA oxidase activity up to 65° of maximum angular velocity.

Thermal Denaturation

In these tests, aliquots of PVP treated extract were subjected to various temperatures for 10 minutes in a water bath, cooled under tap water, reincubated at 35° C., and then assayed at this temperature for IAA oxidase activity. Peroxidase activity was measured at prevailing temperatures in the cuvet chamber of the spectrophotometer (Figure 16). Data from different birch leaf extracts varied somewhat, but some conclusions were possible.

Peroxidase activity of birch enzyme was generally maximum after the 55° treatment and declined steadily to zero activity at 85° . IAA oxidase activity was also at maximum after 55° treatment, but the maximum persisted or reappeared at higher temperatures before declining to near zero at 85° . In other words, IAA oxidase appeared to be a little more resistant to heat denaturation.

The increase in both types of activity between 35° and 55° suggested that thermolabile inhibitors were present in the curde extracts.

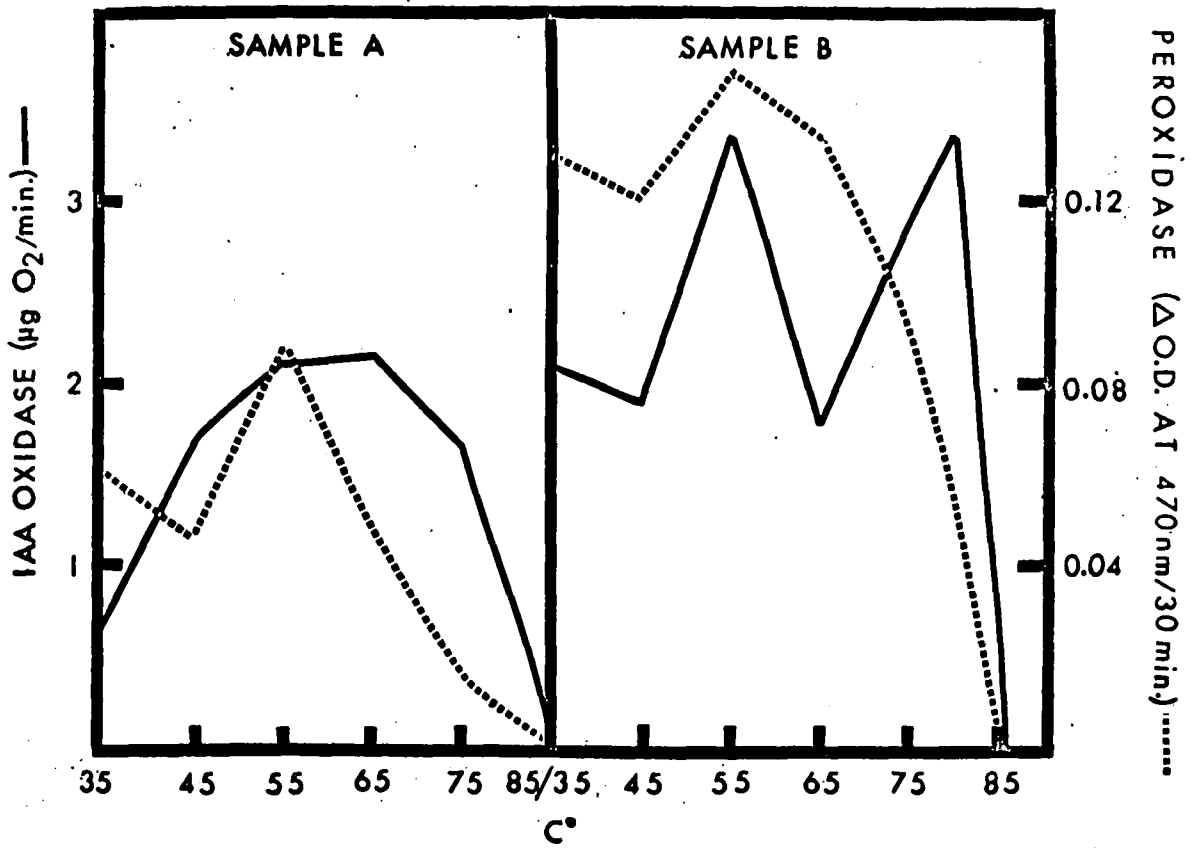


Fig. 16. Thermal inactivation of birch leaf enzymes.

Product Formation

Methyleneoxindole was tentatively identified as the final product from IAA oxidase activity of crude, birch enzyme (Figure 17). The characteristic double peaks for methyleneoxindole at 247 and 254 nm began to appear after two and a half hours. IAA itself shows minimum absorption around 242 nm and is maximum near 278 nm. As the IAA oxidase reaction proceeds, the peak and trough of unaltered IAA become inverted (the loss of absorption at 278 nm is not shown in Figure 17 due to extraneous material in the crude extract) as IAA changes to intermediates and finally to end product.

Purification and Fractionation

Ammonium Sulfate Precipitates

Early separations with ammonium sulfate (AS) were made in 10 per cent jumps (i.e., percentage of saturation) after the 30 per cent precipitate was removed. These tests showed that protein was evenly spread across the 7 precipitate groups and that IAA oxidase activity was spread across the middle and higher groups of salt concentration. Subsequently, larger AS fractions were taken, and the kinetic analyses for these are given in Figure 18. The data indicated that the AS precipitate coming down between 50 and 90 per cent saturation will include most of the IAA oxidase activity. Unfortunately, this range also included a large amount of the total protein so there was little gain in purification.

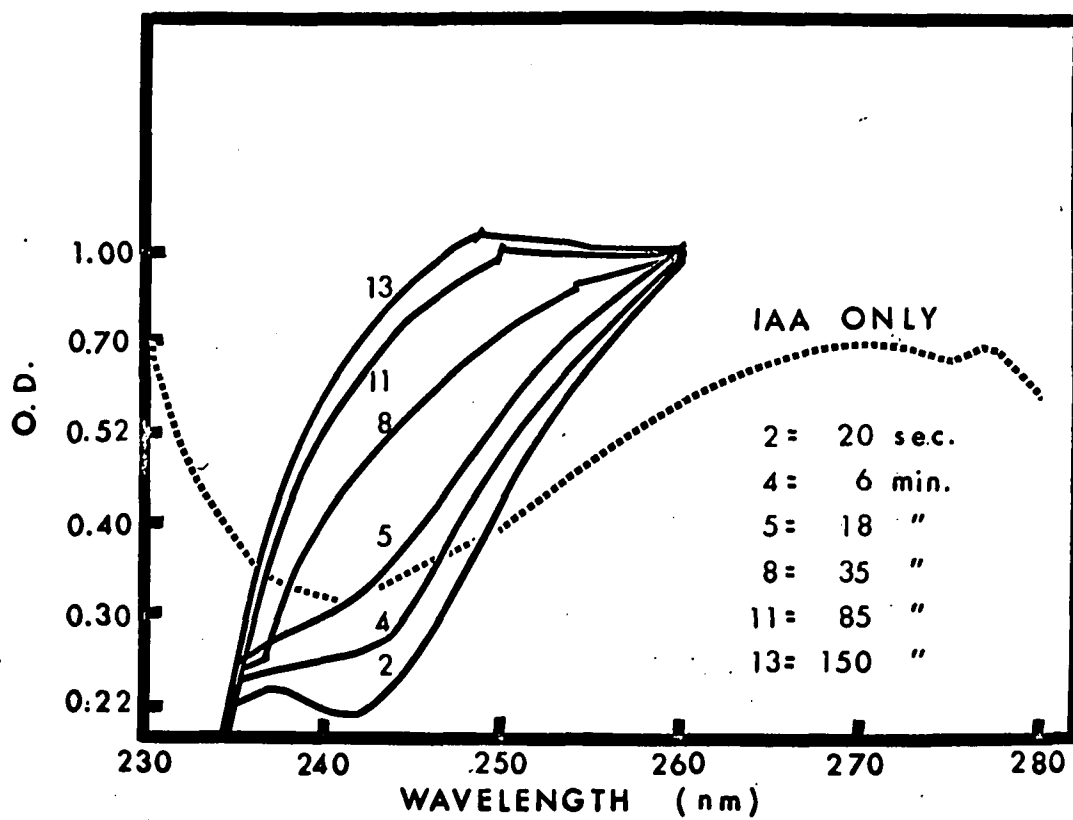


Fig. 17. Reaction spectra for birch enzyme showing appearance of product (3-Methyleneoxindole).

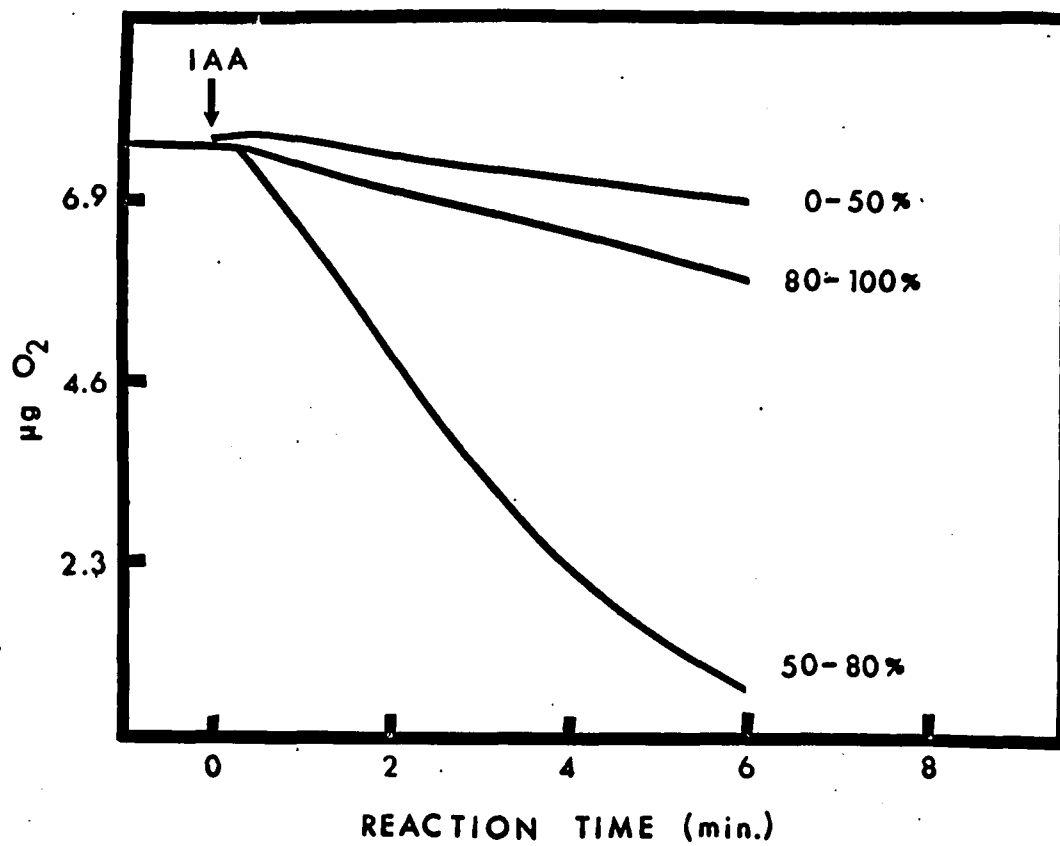


Fig. 18. IAA oxidase activity in ammonium sulfate fractions of birch leaf extracts.

Gel Filtration

Numerous attempts to purify the birch enzyme by gel filtration on Sephadex columns were largely unsuccessful. As discussed in an earlier section, the crude birch leaf extracts were very viscous. This property was undoubtedly responsible for our lack of success in obtaining useful fractionation of the total protein with porous gel. In most runs, protein was rather evenly spread across the entire elution diagram. Attempts to simply separate small molecules (e.g., salts) from large protein molecules were negative also, but did serve to verify that the high viscosity of crude extracts was preventing effective diffusion and separation (Figure 19).

Ultrafiltration

Effective partitioning of the total protein was finally achieved by using ultrafiltration through special membranes with different cut-off values for molecular size. A typical flow sheet and results obtained are given in Table VI.

Assuming that IAA oxidase has a molecular weight (MW) around 40,000, a membrane with a rated cut-off value of 100,000 was used first. This choice would also separate the desired enzyme from such contaminating undesirable enzymes as phenol oxidase (ca. 120,000 MW) and catalase (200,000 MW).

The initial filtration of crude extract yielded a clear, watery filtrate and effectively retained the highly viscous materials of the crude extract. Upon reconcentration this filtrate had a slight yellow color.

The manufacturer's data indicated that 5 times the

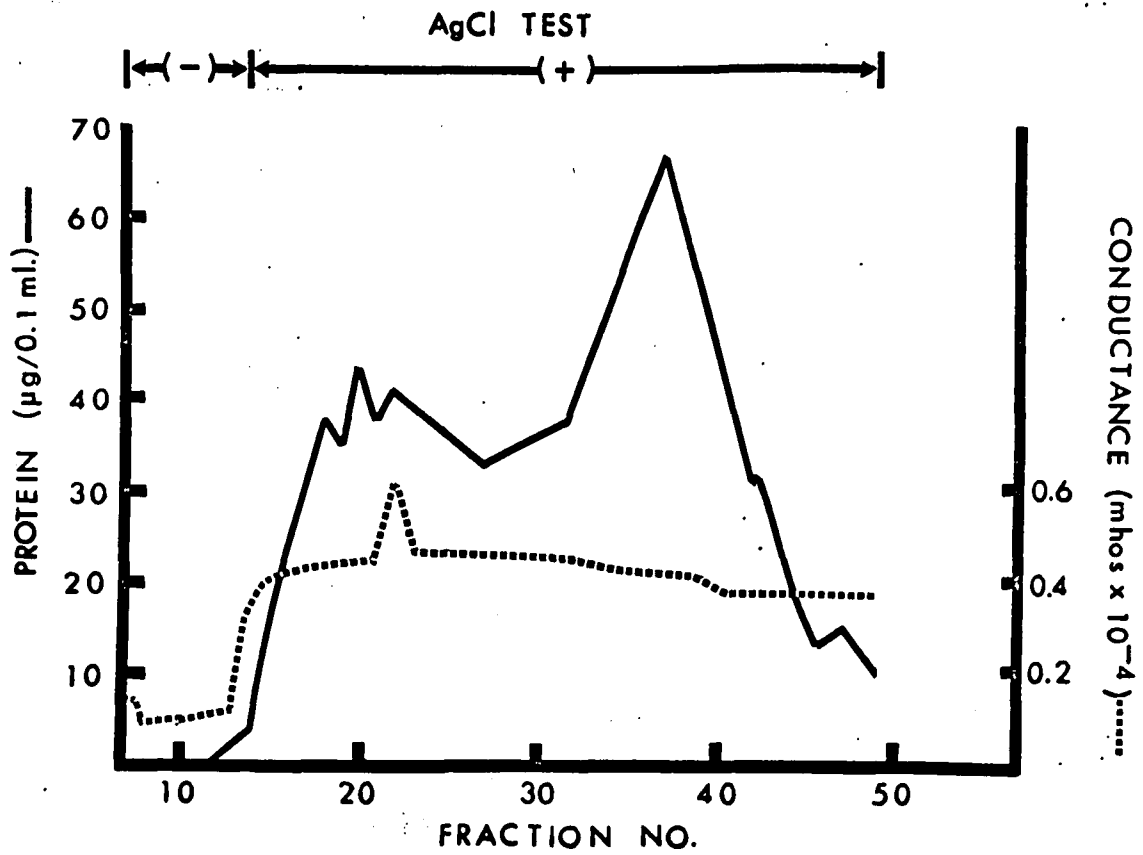


Fig. 19. Desalting of birch leaf extract on Sephadex G-25.

Table VI. Flow Sheet for Ultramembrane Filtration of Birch Leaf Extracts.

		<u>IAA oxidase</u> ($\mu\text{gO}_2/\text{min}$)	<u>Peroxidase</u> ($\text{OD}_{470}/19 \text{ min}$)
Crude extract (10 ml)		1.74	0.079
	<u>ml passed</u>		
16 psi N_2	10	0.94	
	20	0.42	
	30	0.31	
	40	0.31	
	50	0.37	
retentate →			
↓			
XM - 100 membrane			
	<u>ml collected</u>		
filtrate	10	0.52	
	10	0.27	
	10	0.16	
	10	0.12	
	10	0.06	
40 psi N_2			
	7 ml retentate →	0.57	0.032
↓			
UM-20E membrane			
filtrate (some loss of activity)			

sample volume should pass the membrane to insure complete passage of filterable molecules. Our results with several trials showed that some IAA oxidase activity is always retained by this membrane (see Table VI), but a fair portion is passed through. These first filtrates were combined and concentrated on a UM-20E membrane with cut-off values over the range 10,000 - 20,000 M.W. Filtrates concentrated on this membrane were less active than calculated. Apparently some activity was passed through. Another membrane (UM-10) with cut-off at 10,000 MW gave better retention of activity during concentration.

In order to examine the protein size distribution of these concentrates, several such preparations were chromatographed on polyacrylamide columns with known elution characteristics. An example of these results is given in Table VII and Figure 20. The partly purified sample from ultrafiltration had about half the oxidase activity of the crude extract and total oxidase activity recovered after columning was only one fifth of the crude extract. This loss was probably due in part to actual loss of enzyme (during filtration), and in part to loss of natural promotor (refer to p-coumaric data before and after dialysis) that was not entirely compensated for in the assay mixture.

The elution diagram has a protein peak just below 40,000 MW, and covers a range of sizes from 10,000 to 100,000 MW with the bulk of protein between 24,000 and 65,000 MW. Clearly, the ultrafiltration was quite effective in isolating

Table VII. Ultrafiltration Data for Sample Before Chromatography on Bio-Gel, P-100.

Fraction	IAA Oxidase ($\mu\text{gO}_2/\text{min}$)
Crude	1.27
Retentate (XM-100)	0.40
Retentate (UM-20E)	0.54

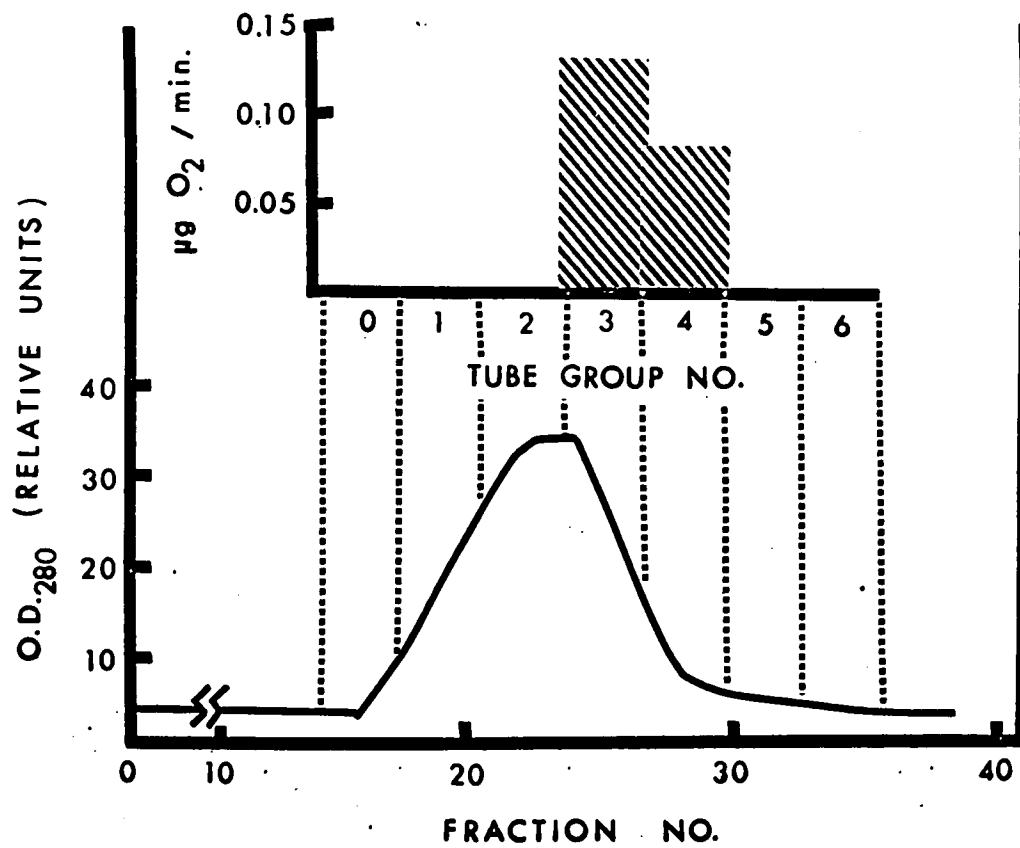


Fig. 20. Gel filtration of ultrafiltered extract from birch leaves (Bio-Gel, p-100).

protein around the desired size. These calculations, however, are crude, and a calibration of the column with commercially purified peroxidase would insure better determination of the true elution volume for 40,000 MW.

Since IAA oxidase in individual fractions was too dilute for meaningful assay, the fractions were arbitrarily grouped, concentrated to 3 ml, and then assayed (upper part Figure 20). Oxidase activity was confined to two groups of calculated MW less than 40,000. Some slight peroxidase activity was detected in these two groups also.

Gel Chromatography

Using purified enzyme prepared by ultrafiltration of crude extracts, several attempts were made to see if IAA oxidase activity and peroxidase activity could be separated on a column of SE-Sephadex as reported by Sequeira and Mineo (52).

Several of the early trials using preparations of moderate IAA oxidase activity gave highly reproducible protein elution diagrams. Every run yielded a diagram with three protein peaks at 1.3, 3.2, and 5.4 elution volumes. But the level of oxidase and peroxidase activity was very low in effluent fractions and difficult to measure. Therefore effluent fractions were grouped, using the protein elution diagram as a guide, and each of their total volumes was concentrated (by ultrafiltration) to the beginning sample volume of 3 ml. Assay of these concentrated effluent groups indicated that the main oxidase and peroxidase activity was located in group two.

This group formed a "valley" of protein between two peaks and had a mid-point elution volume of 2.3.

The best attempt at separating oxidase from peroxidase was made with a highly active extract. The filtrates from XM-100 membranes were concentrated on UM-10 membranes, and this purified enzyme contained about five times more oxidase activity than any prior preparation (Table VIII). This purified enzyme was then chromatographed on SE Sephadex, the effluent groups were reconcentrated to 3 ml (on UM-10 membrane) and measured for IAA oxidase and peroxidase activity. The results are summarized in Figure 21.

Protein eluted in three peaks as in all prior trials. IAA oxidase activity and peroxidase activity were only found in groups one, two, and three with the highest activity for both in group two. Additional groups (not shown in Figure 21) up to 6.9 elution volumes were assayed also, but no further enzyme activity was detected.

In order to verify these results with birch enzyme, a sample of commercially purified horseradish peroxidase (Nutritional Biochemicals Corporation) was chromatographed on the same column of SE-Sephadex with the same eluant. In this case, there was sufficient activity present to assay the individual effluent fractions. The results are summarized in Figure 22.

Essentially, the results obtained with birch enzyme were substantiated. The main peak of protein was found at 2.5 elution volumes, as were the only peaks for oxidase and

Table VIII. Membrane Ultrafilter Purification of Crude Birch Extract for Chromatography on SE-Sephadex Column.

Sample	IAA Oxidase ($\mu\text{gP}_2/\text{min}$)	Peroxidase ($\text{OD}_{470}/15 \text{ min}$)
Crude	6.45	0.183
Retentate (XM-100)	1.30	0.086
Filtrate (XM-100)		
1st 10 ml.	2.00	
2nd 10 ml.	1.10	
3rd 10 ml.	0.43	
Retentate (UM-10)	2.60	0.086

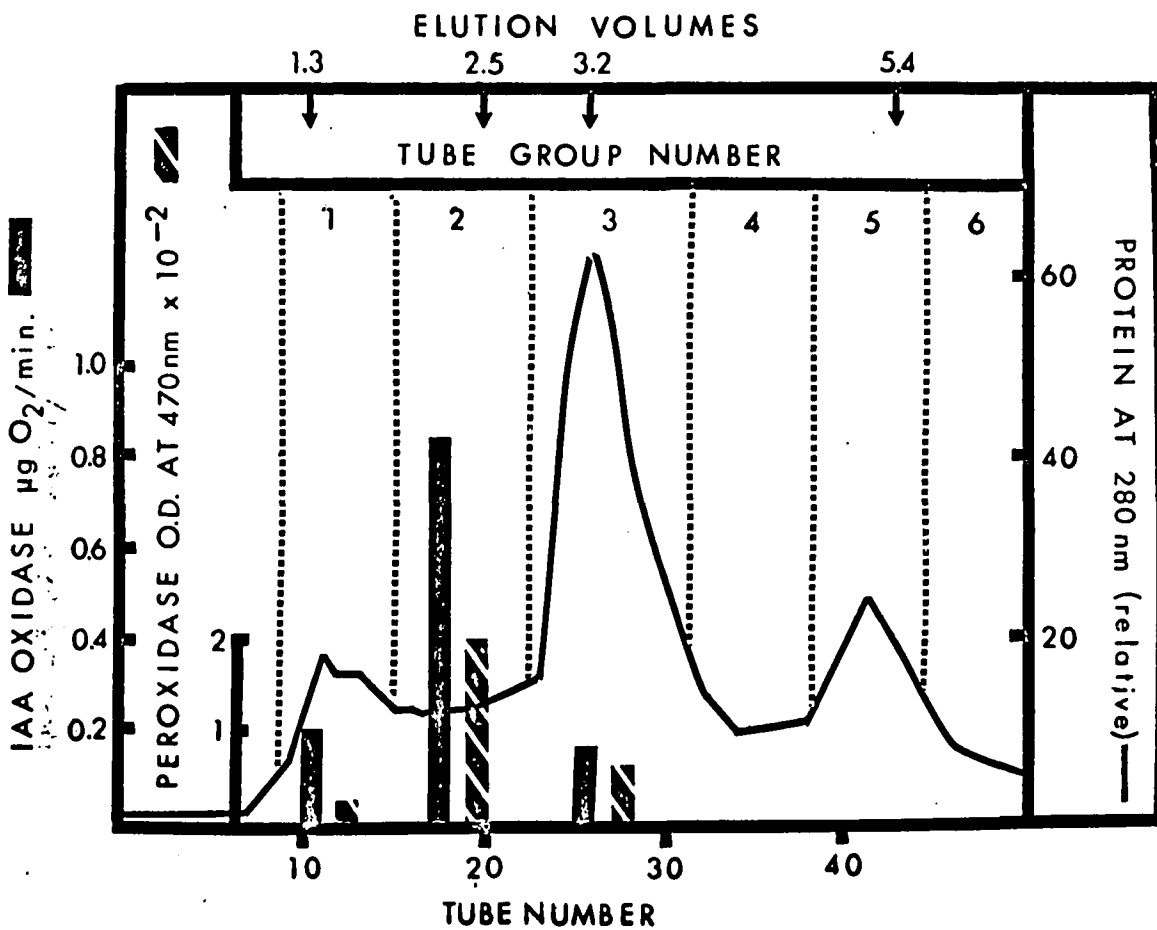


Fig. 21. Chromatography of birch leaf enzyme on SE-Sephadex, after ultrafiltration.

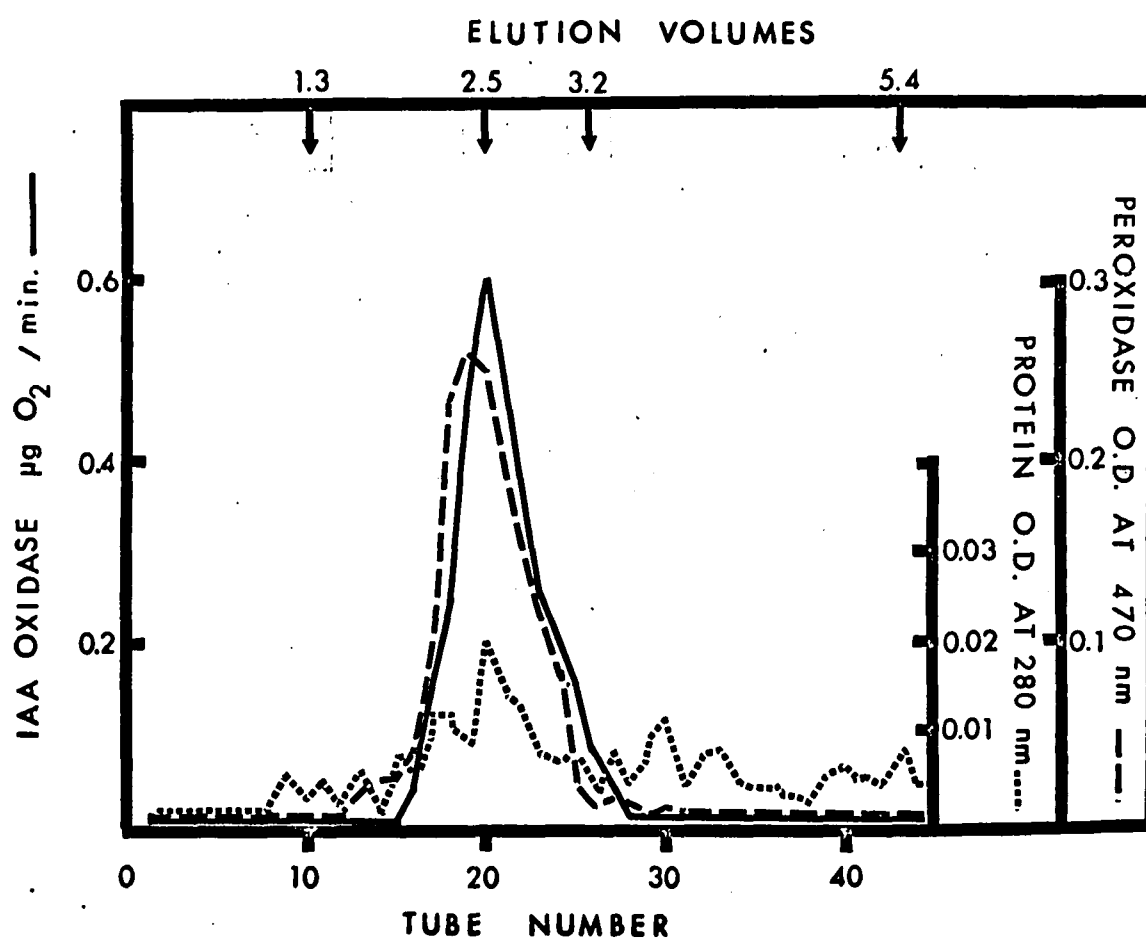


Fig. 22. Chromatography of commercial horseradish peroxidase (N.B.Co.) on SE-Sephadex.

peroxidase activity. No separate peak of IAA oxidase activity alone could be found at 5.4 elution volumes as reported by Sequeira and Mineo (52) for horseradish peroxidase obtained from the same company.

DISCUSSION

Extraction Problems

Three major problems were encountered in extracting IAA oxidase from yellow birch leaves. First was the problem of completely extracting all the enzyme present in a given quantity of tissue; second was the problem of highly viscous extracts that interfered with further purification; and third was the problem of numerous phenolic compounds that interfered with enzyme activity in a variety of ways.

Clearly, 0.05 M acetate buffer as extractant gave highly active preparations, and suggests that a large proportion of the total enzyme was obtained. Pineapple enzyme, claimed to be "...the most potent known source of indole-acetic acid oxidase" (21,22) could destroy 4.5 mg of IAA per gram of tissue in 10 minutes. Birch enzyme appears to be equally potent and, by calculation, could destroy from 3.2 to 5.0 mg IAA per gram of tissue in 10 minutes. However it is still not known if our extraction of oxidase activity was complete. This needs to be examined further. Extractants with higher ionic strength might help to recover all ionic species of oxidase from cellular debris (1). This would be even more critical in studies to examine the isoenzymes of IAA oxidase, because anionic isoenzymes are apparently lost quite easily during extraction (31).

The presence of high viscosity in aqueous extracts of fresh birch leaves (or their acetone powders) is probably due to carbohydrate polymers such as pectic substances (gal-

actans and arabans) and gums (containing rhamnose, galacturonic acid, etc.). In the work reported here, the viscosity problem was "side-stepped," but use of commercial enzymes such as pectinase and galactosidase might be helpful.

PVP was very effective in clearing aqueous extracts of phenolic compounds, but again it is not known if PVP was completely effective in this regard. Results with dialysis of crude extracts (discussed earlier) indicated that not all phenolics were removed. Chlorogenic acid is one phenolic that does not bind strongly to PVP (2), and could have been present in birch leaf extracts even after treatment with PVP. Chlorogenic acid has been reported as a strong inhibitor of IAA oxidase from several plants (24), so its presence or absence needs to be confirmed.

The use of thiols in their capacity as reducing agents provides another means for coping with phenols in plant extracts. Thiols give temporary protection by reducing quinones back to phenols, or thiols can give more permanent protection by combining with quinones to yield non-inhibitors. The auto-oxidation of thiols by contaminating traces of metal ions has to be considered when these reducing agents are used in extracting media.

Cofactor Requirements

Manganese and DCP

The results obtained in our work indicated clearly that neither Mn^{2+} nor DCP was essential to the IAA oxidase

activity of birch leaf enzyme. In fact, maximum velocity of the enzymatic reaction generally improved slightly when these two factors were omitted from the assay mixture. Presence of endogenous Mn^{2+} was ruled out when Mn^{2+} failed to promote the oxidase activity in exhaustively dialyzed or EDTA-treated extracts. In addition, the IAA oxidase function of commercial horseradish peroxidase showed no need for, or improvement with, added Mn^{2+} and/or DCP.

The literature on cofactor requirements of IAA oxidase is quite conflicting (discussed in an earlier section). In all reports (with which the author is familiar) some monophenol is either needed or improves IAA oxidase activity of plant extracts. Usually this has been DCP, or in the case of pineapple (21) and birch (present work) it is p-coumaric. But the results with Mn^{2+} generally show that it is definitely needed or not needed. Along with birch enzyme, neither fungus enzyme (45) nor citrus root enzyme (18) showed a need for Mn^{2+} . These conflicting results suggest two tentative explanations: (1) that the enzyme with IAA oxidase activity operates with different requirements in different plants; or (2) that in reality there is no need for these cofactors (especially Mn^{2+}). With regard to the latter, it seems possible that a reported "need" for Mn^{2+} in in vitro assays may be an artificial requirement due to extraneous compounds that are present.

Hydrogen Peroxide

Hydrogen peroxide was a strong stimulant to IAA

oxidase from birch leaves. In dialyzed extracts, H_2O_2 was absolutely required for oxidase activity. But catalase had no effect on IAA oxidase activity of HRPO in our work. Fox (10) also reported that catalase had no effect on HRPO. These results are interpreted to mean that a peroxide is not an intermediate in the enzymatic degradation of IAA. Ray (47) likewise found a stimulatory effect on Omphalia enzyme by H_2O_2 , but he felt that H_2O_2 served to bring the oxidative enzyme into an active state and was not needed as an intermediate reactant. This idea was deduced from his finding that catalase inhibited IAA oxidase activity of Omphalia when added to the reaction mixture initially, but catalase had very little effect on activity when added after the steady state condition was obtained. Ray (45) also found that very little IAA oxidase activity occurred in the absence of O_2 even when H_2O_2 was present, but peroxidase activity on pyrogallol was quite unaffected by the presence or absence of O_2 . Again, this evidence contradicts the idea that IAA is oxidized by some modified peroxidatic reaction.

What then is the function of H_2O_2 in the oxidative destruction of IAA by birch enzyme in vitro? The idea that H_2O_2 brings the enzyme into an active state is tenable, but has not been clearly demonstrated in the present work. However, based on the idea given earlier that there are no cofactor requirements for IAA oxidase, one can tentatively suggest that H_2O_2 compensates for an extraneous detrimental influence--namely chlorogenic acid. As stated, PVP does not efficiently remove chlorogenic acid from aqueous extracts.

Assuming that chlorogenic acid is coupled to some larger polymer in the birch extracts then dialysis would not remove it either. Hence the possibility exists that H_2O_2 may serve to oxidize small endogenous quantities of chlorogenic acid (or some natural counterpart) and thereby convert the acid into a non-inhibitory molecule. This idea is more tenable if one considers the fact that two additions of 0.1M H_2O_2 were made to the pineapple enzyme to remove strong phenolic inhibition before IAA oxidase activity could be demonstrated (21). Other classes of inhibitory substances may be involved also.

Inhibitors of much higher MW than phenolic compounds have been found in extracts from Japanese morning glory (55, 57). Protector -A^{1/} (Pr-A) had a MW around 200,000, protector -I (Pr-I) was about 8000 MW, and protector II (PR-II) was about 2000 MW. Pr-I induced a lag phase in IAA oxidase assay systems that could be removed by 0.01mM H_2O_2 or by pre-incubation with $MnCl_2$ (58). The sustained requirement of birch IAA oxidase for added H_2O_2 after dialysis and the increase in oxidase activity with heat treatments at 55°C suggest that similar high MW inhibitors are present in birch extracts too.

p-Coumaric Acid

The positive effect of p-coumaric acid on the IAA oxidase activity of birch enzyme has been clearly demonstrated in the present work. Since more p-coumaric is required to

^{1/} These workers refer to the IAA oxidase inhibitors as auxin "protectors."

achieve maximum activity with dialyzed enzyme, there must be some natural counterpart present in the crude extracts. This need for higher levels of p-coumaric with increased purification of the enzyme may account for the apparent loss of some oxidase activity in ultrafilter fractions and column chromatography fractions. Unfortunately, these purified enzyme fractions were routinely assayed with the lower p-coumaric levels used in crude extracts (0.30 to 0.45 μ moles), and not with the higher levels of p-coumaric (up to 1.45 μ moles) that were found to be necessary for maximum activity in later experiments with dialyzed enzyme.

The stimulatory effect of p-coumaric on IAA oxidase in birch leaves poses two questions: (1) by what means does p-coumaric promote oxidase activity; and (2) what are the variations in molecular structure that promote or inhibit enzyme activation?

The best answer to our first question presently seems to be that p-coumaric acid is an electron donor, as suggested by Fox et al. (9,10) for the same promotive effect of DCP on HRPO. Acting in this way, p-coumaric thereby substitutes for a postulated electron donor site on the enzyme which would be utilized when p-coumaric (or in other systems DCP) is absent. When this electron donor site on the enzyme is used, the free enzyme released after catalysis is partially inactive, but when p-coumaric (or DCP in other systems) is present, the regenerated free enzyme is fully active. Evidence that p-coumaric is, in fact, oxidized during the IAA oxidase

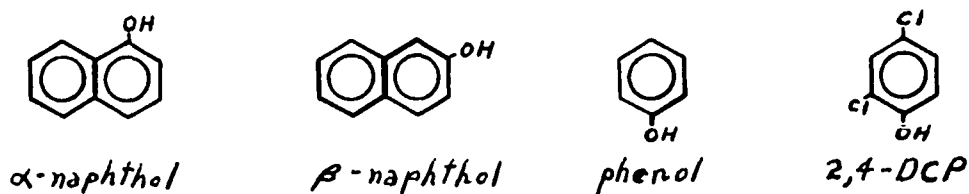
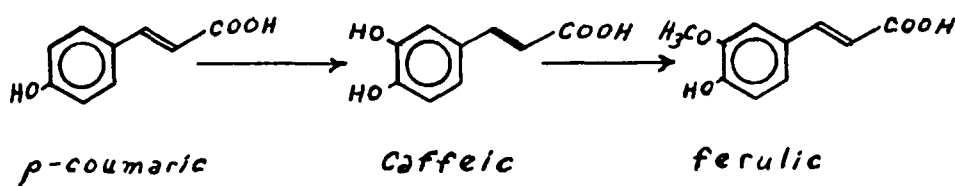
reaction has been demonstrated by Engelsma (7) for both HRPO and a partly purified enzyme from gherkin (Cucumis sativus) seedlings.

If the role of p-coumaric as electron donor is correct, then we have a regulating mechanism for the amount of IAA that is destroyed in vivo. For example, if p-coumaric is absent from the locus of IAA oxidase activity in the cell, then the amount of IAA destroyed is some function of the amount of enzyme that is present; since a fraction of active enzyme is rendered inactive during catalysis. But when p-coumaric is present, the quantity of IAA destroyed will be some function of the amount of p-coumaric present plus the amount of enzyme. Under these latter conditions more IAA would be destroyed than under the former because enzyme inactivation would not begin until all the p-coumaric was oxidized. Given this type of regulatory system, any factor that influences p-coumaric biosynthesis or p-coumaric transport to the locus of oxidase activity would indirectly affect IAA levels.

These considerations, however, must remain tentative until a clear demonstration has been made that birch enzyme can oxidize IAA without p-coumaric. In our partly purified preparations, the electron donor site on birch enzyme may be blocked by some small molecule or by allosteric hindrance. Possibly this "block" of the electron donor site will be removed with highly purified enzyme as in the case of HRPO. Or more likely, the electron donor site on birch enzyme has been oxidized by quinones during extraction and therefore p-coumaric

becomes an absolute requirement. The use of sulfhydryl reagents and EDTA during extraction may help to clarify this question.

With regard to question two (posed above), it seems clear that *p*-coumaric reacts somewhere on the enzyme surface; because other hydroxylated derivatives of cinnamic acid do not work as well and may be inhibitory. (See structural formulas below).



In pineapple enzyme (21), caffeic or its depside chlorogenic, are inhibitory simply by addition of one hydroxyl group. If one of these hydroxyls is made obscure by methylation, as with ferulic, then an inhibitor becomes a promoter to a limited extent. Ortho-coumaric, though a monophenol, was inactive with

pineapple enzyme. Phenol itself was only slightly promotive so additional structure is apparently required. In the case of DCP, this additional structure is simply two chloride atoms. With α - and β -naphthol the additional structure is a benzene ring. However, the naphthols inhibited pea enzyme (17), but were promoters to pineapple enzyme.

Though DCP has been an IAA oxidase promoter for many plant extracts its natural occurrence is doubtful. Para-coumaric may be more of a universal IAA oxidase promoter in nature than is generally appreciated. In the natural state, p-coumaric has been isolated as a depside with quinic (22), and as the triglucoside of several flavanols (11). The presence and form of p-coumaric in birch leaves has yet to be determined.

Locus of IAA Oxidase Activity

Our column chromatography results with partly purified birch enzyme and with commercial HRPO, clearly support the idea that IAA oxidase activity and peroxidase activity are both catalytic functions of the same enzyme. The thermal denaturation of oxidase and peroxidase activity in birch have some differences and some similarities. But this would not be unexpected if there are two catalytic centers on the same molecule. The idea proposed by Siegel and Galston (54) that apoenzyme alone controls IAA oxidase activity, while both apoenzyme and heme in combined form are necessary for peroxidase activity, helps to explain why heat inactivation may follow a different course for each catalytic activity. Also it is important to

note that both types of activity in birch are optimal at the same low pH, and that IAA oxidase activity of HRPO is maximal at about the same pH as IAA oxidase activity in birch. Recently, Turin^{2/} has found that both types of activity (obtained from tobacco callus) remained together after Sephadex G-100 gel filtration and DEAE Sephadex gel chromatography. Similar results have been reported by Stutz (59) and Ray (45) also. In summary then, the weight of evidence does not support the argument of Sequeira and Mineo (51) that the two types of catalytic activity are due to two different enzymes.

Using the same system as the above two authors, both types of enzyme activity were maximal at about 2.4 elution volumes; whereas they found combined activities at 1.3 elution volumes, mainly peroxidase at 3.2 elution volumes, and mainly IAA oxidase at 5.4 elution volumes. It is interesting to note that these three elution volumes correspond to the three protein peaks eluted from SE-Sephadex with birch enzyme. It is quite probable that Sequeira and Mineo's results were artifacts due to: (1) problems with contaminating phenols; (2) binding of enzyme to higher M.W. components in the extracts^{3/} in such a way as to prohibit efficient separations; and (3) use of the Salkowski method (known to give erroneous results at times) to assay activity. The fact that enzyme activities of HRPO (N.B.Co.) eluted in the same pattern as enzyme activities of birch enzyme lends further support to the validity of our results.

^{2/} (Mrs.) Barbara A. Turin, Biologist, Owens-Illinois Technical Center, Toledo, Ohio (personal correspondence).

^{3/} This problem was encountered by Turin with tobacco callus extracts, and is also suggested by the fact that Sequeira and Mineo obtained further segregation of their combined activity peak at 1.3 elution volumes upon rechromatography.

The best approach now would probably be to make a detailed examination of the peroxidase isoenzymes for the locus of major IAA oxidase activity. Macnicol's (36) work indicates that some isoenzymes of peroxidase may have far greater IAA oxidase activity than others. Both cationic and anionic HRPO isoenzymes have been effectively separated on columns of CM-⁴/ cellulose and DEAE-⁴/ cellulose (53), but electrophoresis may permit more sensitivity in working with low concentrations of enzyme. Peroxidase activity is readily detected on electrophoretic bands due to the colored product, but, to the author's knowledge, IAA oxidase detection on electrophoretic bands has not been accomplished. Our work with DMACA (see Appendix), to measure the appearance of IAA oxidase reaction products, suggested that this reagent may be used effectively to detect IAA oxidase of electrophoretic bands.

⁴/ CM = carboxymethyl, DEAE = diethylaminoethyl.

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APPENDIX

Standard curves for assay methods given under Methods and Materials are shown in Figures 23, 24, and 25. Figure 26 shows the results of assaying birch IAA oxidase activity with the DMACA reagent. This reagent forms a wine red compound when complexed with the reaction products of IAA oxidase activity. In our limited work, the assay appears to be sensitive enough for wider use, but the results cannot be quantified unless calibrated with some other technique for assaying activity. Hence the usefulness of DMACA reagent will be mainly for qualitative evaluation.

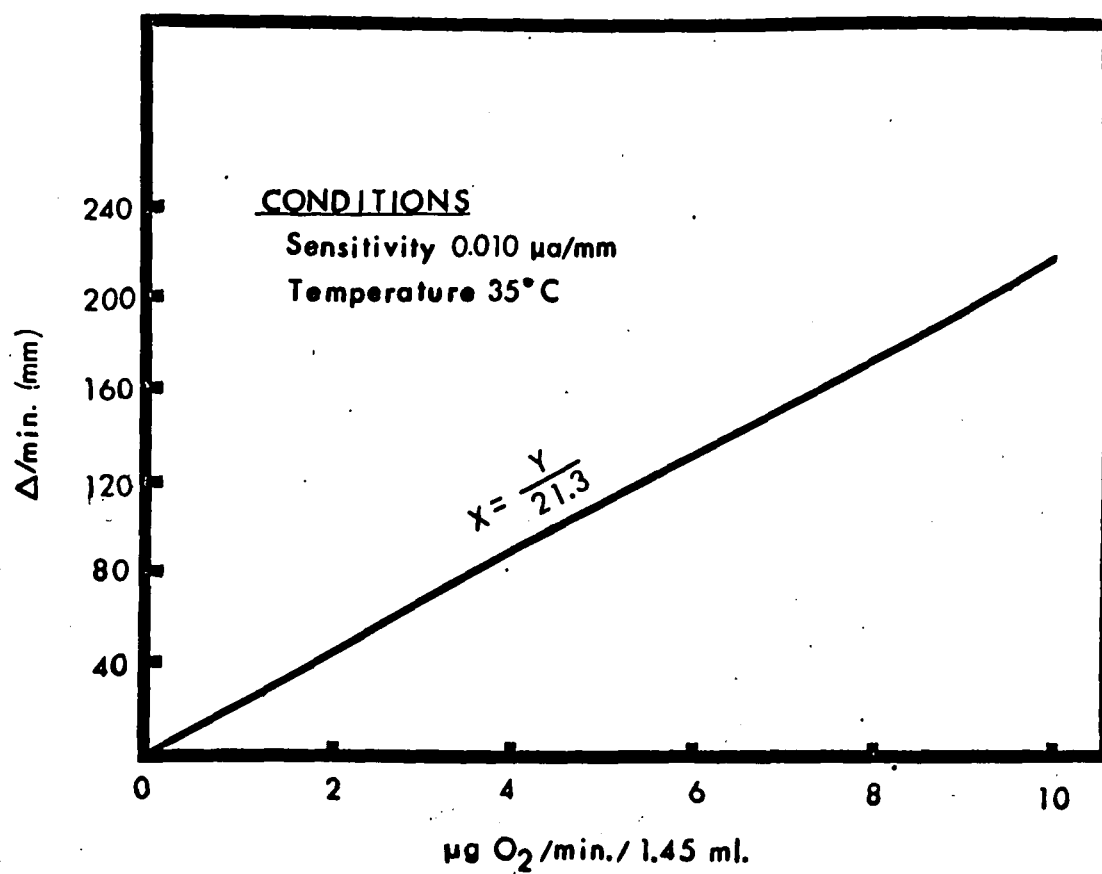


Fig. 23. Oxygen cathode standard curve.

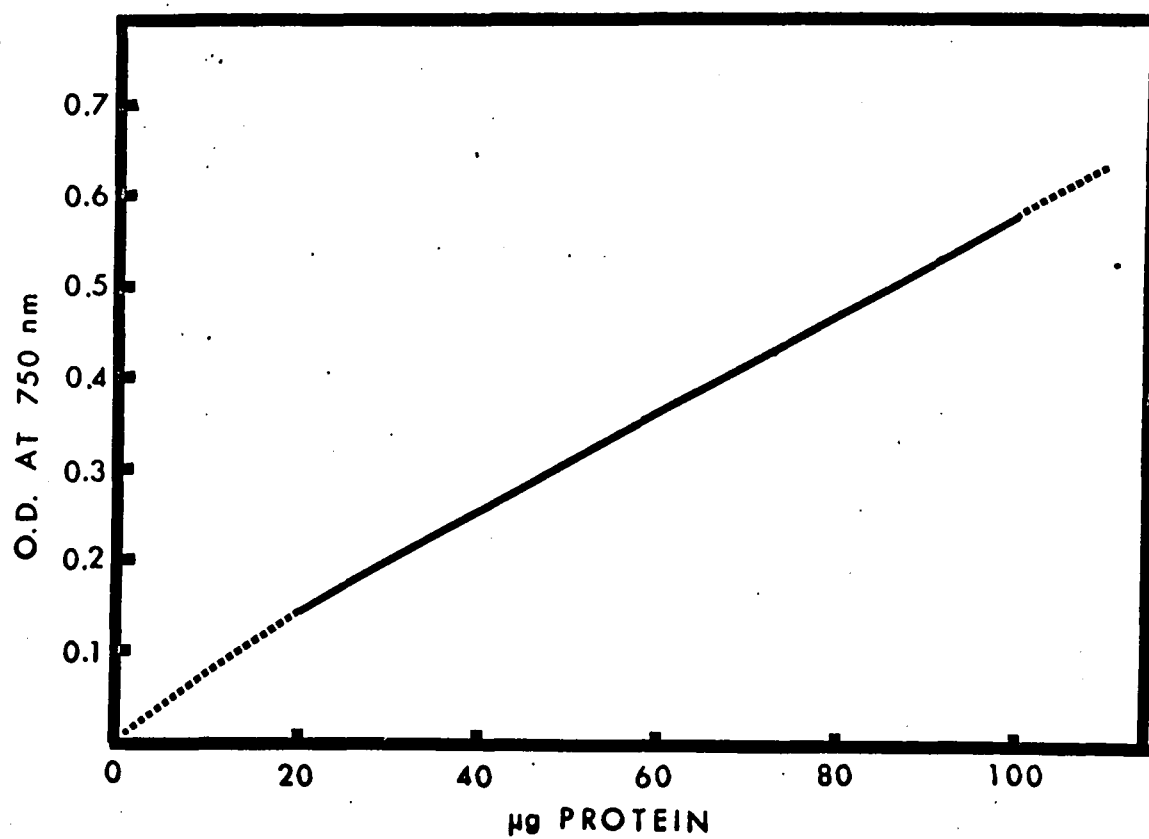


Fig. 24. Folin-Lowry protein standard curve.

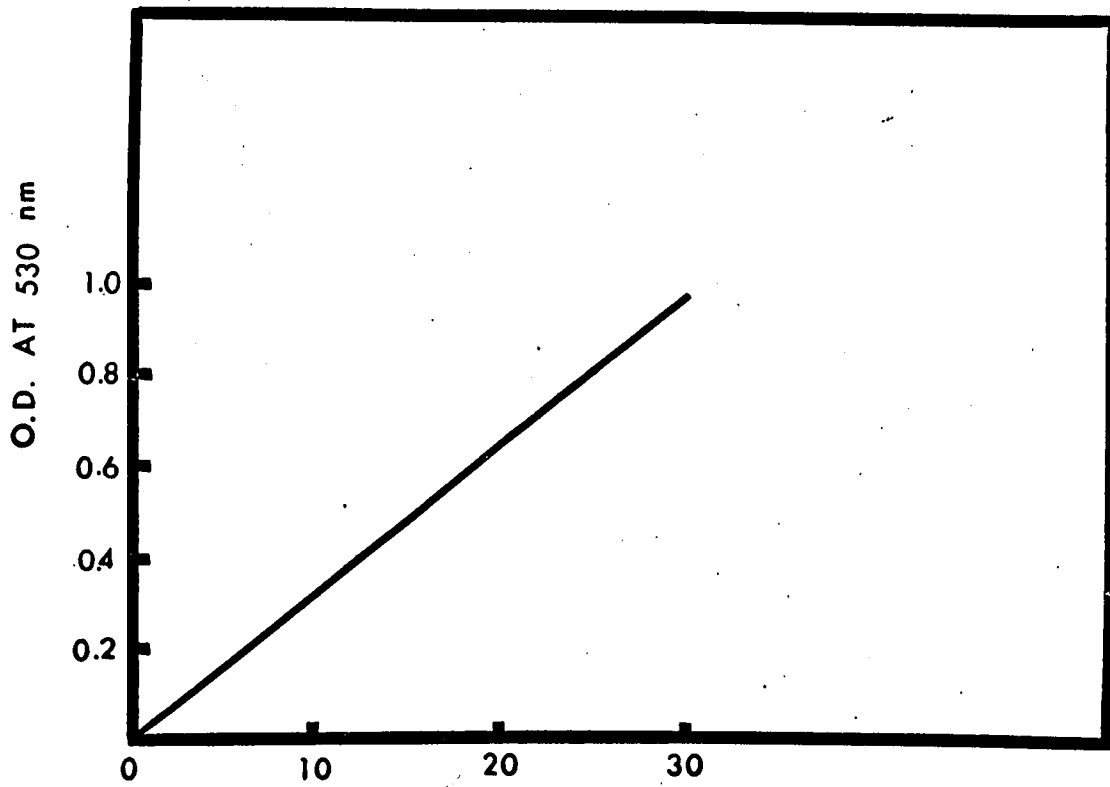


Fig. 25. Salkowski standard curve.

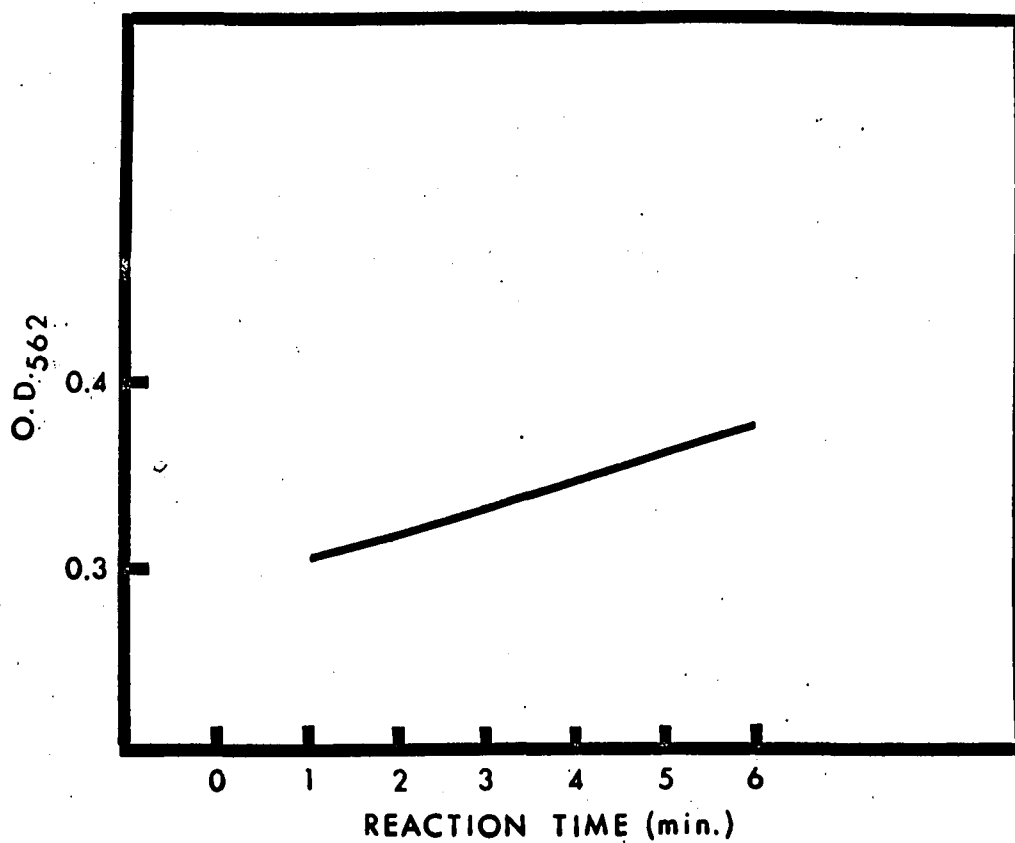


Fig. 26. Assay of IAA oxidase activity in birch leaf extracts with dimethyl-amino cinnamaldehyde (DMACA).