THE METABOLIC PATHWAY OF INDOLEACETIC ACID INACTIVATION IN HIGHER PLANTS WITH ESPECIAL REFERENCE TO TRITICUM COMPACTUM HOST.

A Thesis
Submitted to the Faculty of
Graduate Studies and Research,
University of Manitoba,
in Partial Fulfillment
of the Requirements
for the Degree of
Doctor of Philosophy

by
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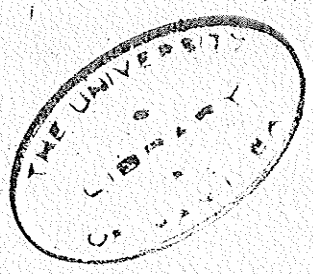
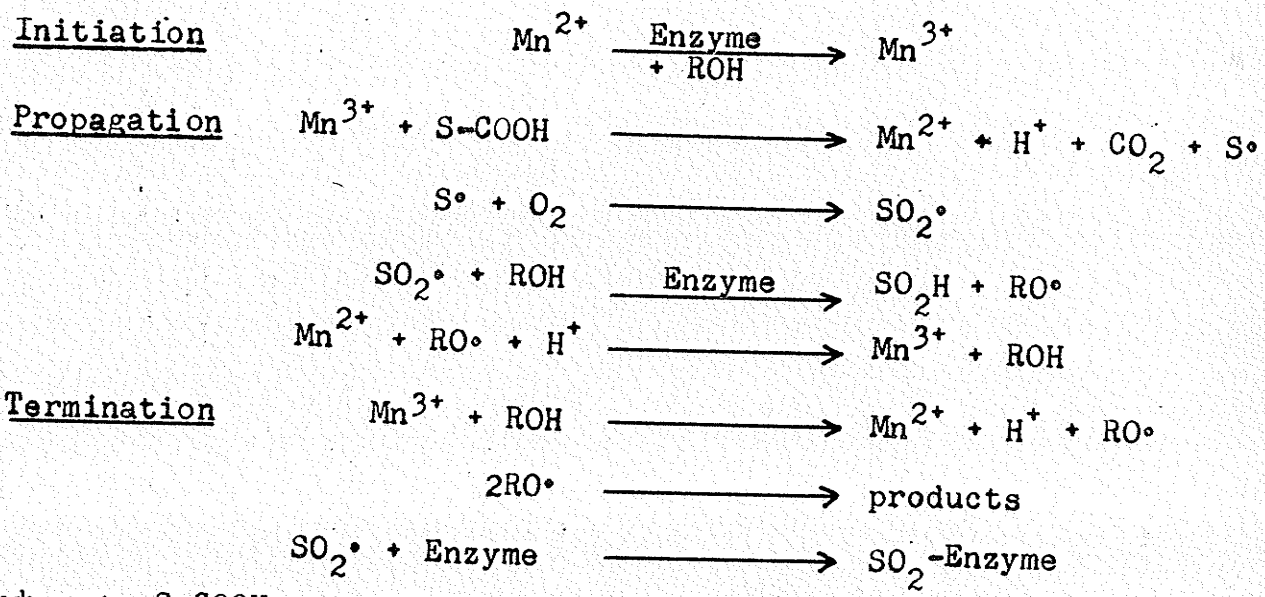
Winnipeg, Manitoba
July, 1956



ABSTRACT

The decarboxylation and oxidation of indoleacetic acid is catalysed by either peroxidase or catalase in the presence of manganese and a monohydroxyphenol or resorcinol. The kinetics of the system and the non-enzymic reactions of indoleacetic acid with manganic ions indicate the following chain reaction sequence for enzymic indoleacetic acid degradation:



where: S-COOH = indoleacetic acid (S=skatole nucleus), ROH=phenolic cofactor, Enzyme=peroxidase or catalase.

In accordance with the autoxidative nature of the reaction system, inhibitors act either as chain-stopping agents and cause an extension of the induction period e.g. catechol, or as chain-transferring agents which cause retardation e.g. riboflavin or hydroquinone.

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ACKNOWLEDGEMENTS

The author is greatly indebted to Dr. E. R. Waygood for the many ways in which he has contributed to the present studies. Thanks are also due for the assistance of Misses Ann Oaks, Sally Dangerfield, Christina Gow, Sigrid Lichti, Frances Brown, Mrs. Johanna Kroeker and Mr. D. Bissett. The sample of scopoletin used in these studies was generously donated by Dr. W. A. Andreae. The investigation was supported by grants by the Charles F. Kettering Foundation, Yellow Springs, Ohio, U.S.A., the National Research Council of Canada, and the Canada Department of Agriculture, Science Service.

I INTRODUCTION

Indoleacetic acid is widely distributed in higher plants and its concentration within a cell appears to be a determining factor in all early stages of cellular differentiation, organ development and growth processes (see review, Audus, 1953). Thus, by virtue of its natural occurrence and wide variety of effects on growth, indoleacetic acid is considered to be the "master" non-specific hormone of plants (Thimann, 1954). Although evidence has been accumulating for some twenty years to support this contention, the mechanism of action of indoleacetic acid is not yet understood.

In general terms, plant growth may be envisioned as under the control of a "growth centre" or "reactive entity" within the cell composed of a protein together with either indoleacetic acid itself or a derivative (Muir and Hansch, 1955). Whether indoleacetic acid remains unchanged by acting as a cofactor-catalyst in the growth process or is utilized in an essential reaction is not known (Gordon,1949). Closer examination of this central problem has been hampered by a lack of knowledge of the metabolism of indoleacetic acid, particularly of the catabolic processes which are believed to be responsible in large part for controlling the concentration of the hormone at the growth centre site (Larsen, 1951).

There has been little or no agreement on the requirements for and the mechanism and products of indoleacetic acid inactivation. This has naturally contributed a large element of uncertainty to explanations of the way in which environmental conditions influence the concentration of indoleacetic acid and thereby growth (Larsen, 1951). Furthermore, no decision can yet be made on the possibility that an intermediate formed from indoleacetic acid maybe the active growth-regulating entity.

In the present investigations an attempt has been made to reconcile the conflicting data available on the enzymes and cofactors required to catalyse complete indoleacetic acid decarboxylation and oxidation in vitro. The kinetics of enzyme, substrate and cofactor requirements and the mechanisms of retardation and inhibition of the systems have been studied in detail. From these results a reaction sequence is proposed which for the first time offers a detailed description of the pathway of indoleacetic acid breakdown and hence of some of the reactive potentialities of the indoleacetic acid molecule. These findings should provide a foundation for further biochemical investigation into the nature of the growth process.

II LITERATURE REVIEW

The growth of plant organs is conditioned by a wide variety of external stimuli including temperature, light, nutrition, etc., the effects of which represent the integrated influences of many metabolic processes. However, in recent years evidence has accumulated that many growth responses are elicited through direct or indirect environmental effects on specific growth hormones. Thus in order to characterize the way in which normal growth patterns are established, many studies have been made on the nature, distribution and effects of naturally occurring growth-regulating substances.

The first clear demonstration of the existence of plant hormones resulted from the now classical experiments of Went (1928, 1935) and associates on the bending of etiolated oat coleoptiles. Bending occurred towards the source of a unilateral white or blue light or away from the eccentric application of extracts from the coleoptiles themselves. After ingenious experimentation, it was concluded that bending was due to the unequal distribution within the coleoptile of a growth-promoting substance or "auxin" (Went, 1935, Wildman and Bonner, 1948).

Subsequently, Kogl et. al. (1934) isolated from biological sources three chemically pure substances which in very low concentrations also caused the bending of oat coleoptiles.

Two of these are now thought to be artifacts (Zimmerman, 1947), but the third, "heteroauxin" or indole-3-acetic acid (IAA), was eventually proven (Wildman and Bonner, 1948) to be the natural hormone in oat coleoptiles and to account for about 80% of its phototropic response. IAA has since been found to occur in all plant organs and species investigated with the exception of the apple (Luckwill and Powell, 1956) and tobacco leaves (Vlitos et. al., 1956) and but for these reports is now considered to be the principal native auxin of higher plants (Gordon and Nieva, 1949, Bonner and Bandurski, 1952).

When fed to a plant, IAA has a multiplicity of effects on growth phenomena ranging from stimulation to inhibition of flowering, fruiting, rooting and budding (see review, Audus, 1953). IAA also affects the linear growth of roots, stems and leaves and appears to exert a control over geotropism and phototropism. Whether IAA stimulates or inhibits growth depends in part on its concentration and in part on the particular plant organ and species under study (Went, 1935, Muir and Hansch, 1955). Nevertheless, irrespective of the plant material used, external stimuli and normal metabolic events could alter the concentration of IAA in a plant organ by any of three processes i.e. by influencing translocation, synthesis or destruction of the hormone.

It has been established (Wildman et.al., 1947, Gordon and Nieva, 1949, Galston, 1949a) that the enzymic conversion of

tryptophane to IAA is the chief pathway of IAA synthesis in plants and takes place mainly in apical meristems of aerial organs e.g. buds and flowers (Wildman et. al., 1947). Synthesis of IAA is probably continuous (see review, Gordon, 1949) provided the source of tryptophane is not (a) exhausted, which is unlikely except in cases of starvation or (b) interrupted, which may occur for example as one effect of zinc deficiency (Tsui, 1948).

The mechanism by which active translocation of IAA occurs is not well understood but there is generally a pronounced downward movement from the site of synthesis (Galston, 1950b) which can proceed against a concentration gradient (Went and White, 1939). Lateral or upward movement takes place if IAA enters the transpiration stream, but when located within living aerial tissues, polar basipetal movement resumes (Skoog, 1938). The rate of translocation within a given tissue appears to be relatively constant (Wildman et. al., 1947, Galston, 1950b) and is probably influenced by environmental conditions only when these are extreme e.g. local heating or chilling.

With our present knowledge it seems probable that the chief factor governing variation in IAA concentration <u>in vivo</u> under normal conditions is the process of IAA inactivation (Van Overbeek, 1938, Galston and Dalberg, 1954). This view has only been questioned once (Briggs et. al., 1955b) and then as a result of negative evidence and the unsatisfactory state of

knowledge of the mechanism of IAA breakdown.

Many attempts have been made to extract from plant sources enzyme systems which are capable of destroying IAA. The system which has received most extensive study has been obtained from etiolated pea epicotyls by grinding and centrifuging (Tang and Bonner, 1947, Hillman and Galston, 1956) followed in most cases by partial purification with acetone (Tang and Bonner, 1948, Galston and Baker, 1951, Goldacre, 1951, Andreae and Andreae, 1953) and/or ammonium sulphate (Wagenknecht and Burris, 1950, Galston et. al., 1953, Manning and Galston, 1955). Extracts active in destroying IAA have been prepared from other plant sources notably: green pea epicotyls (Galston and Baker, 1951), bean roots (Wagenknecht and Burris, 1950, Kenten, 1955), pineapple tissue (Gortner and Kent, 1953), horseradish roots (Goldacre, 1951, Galston et. al., 1953, Goldacre et.al., 1953, Kenten, 1955), the fern Osmunda cinnamomea L. (Briggs et. al., 1955a, Briggs et. al., 1955b), wheat leaves (Waygood et. al., 1956) and the fungus Omphalia flavida (Segueira and Steeves, 1954, Ray and Thimann, 1955).

The literature on IAA inactivation has been reviewed by Larsen up to 1951 at which time there was little agreement on the conditions required for complete IAA destruction. It was established that for partial IAA destruction under the catalysis of undialysed extracts from etiolated pea epicotyls and bean roots,

oxygen was required and consumed and an equal amount of carbon dioxide was released i.e. the RQ of the reaction was unity (Larsen, 1940, Tang and Bonner, 1947, Tang and Bonner, 1948, Wagenknecht and Burris, 1950). However, the stoichiometric relationship between the gas exchanged and IAA destroyed had received no attention and none of the systems were capable of completely destroying more than micromolar concentrations of IAA. There were indications therefore that the systems as described were incomplete i.e. that cofactors and/or enzymes were present in suboptimum amount. Hence conflicting reports of the properties of these inefficient systems must be regarded with caution until they can be tested on systems in which IAA is completely destroyed.

Enzyme systems have recently been described by Kenten (1955) and Waygood et. al. (1956) which completely destroy as much as 2×10^{-3} M IAA with the concomitant uptake and release of one mole each of oxygen and carbon dioxide per mole of IAA. Both systems contained peroxidase:- horseradish (Kenten, 1955) and wheat leaf (Waygood et. al., 1956), and were stimulated by the addition of certain organic cofactors e.g. resorcinol or monohydric phenols. The systems differed in that manganese was required as well as an organic cofactor for full activity in the wheat leaf system whereas manganese had only a small stimulatory effect on the horseradish peroxidase system. The literature on IAA inactivation will be reviewed with regard to its support or

conflict with the findings of these authors.

Several lines of evidence support the contention that peroxidase participates in IAA oxidation. The enzyme employed by Kenten (1955) was a highly active crystalline peroxidase prepared from horseradish by the method of Kenten and Mann (1954) and further purified by acid-ammonium sulphate precipitation and dialysis to remove flavins and other soluble impurities. Extracts from other plant sources which destroy IAA have almost all been shown to contain peroxidase (Tang and Bonner, 1947, Wagenknecht and Burris, 1950, Galston et. al., 1953, Gortner and Kent, 1953, Ray and Thimann, 1955, Briggs et. al., 1955a) and to be inhibited by agents e.g. cyanide, which form irreversible complexes with porphyrins (Tang and Bonner, 1947, Andreae and Andreae, 1953). Briggs et. al. (1955a) found a correlation between the peroxidase (and catalase) content and the IAA "oxidase" activity of fern leaf extracts and Goldacre (1953) reported similar results with extracts from peas. Finally, peroxidase is adaptively formed in pea seedlings fed IAA thus Galston and Dalberg (1954) suggested that plants may protect themselves from deleterious effects of excess IAA by developing part of the enzymic system which destroys the hormone.

Polyphenol oxidase may also catalyse IAA inactivation since some IAA "oxidase" systems are inhibited by known copper-chelating agents e.g. diethylthiocarbamate (Wagenknecht

and Burris, 1950, Briggs et. al., 1955b). In addition, a tyrosinase from mushrooms has been reported (Briggs and Ray, 1956) to be capable of partially inactivating IAA. However, this reaction probably has little general physiological significance since polyphenol oxidase has a limited distribution in higher plants (Onslow, 1931).

The participation of a peroxidatic reaction in the reaction sequence for IAA destruction is strongly implied by the many findings that in vitro IAA oxidation is stimulated by the addition of certain known hydrogen donors in peroxidatic reactions. These include monohydroxyphenols, notably dichlorophenol (Kenten, 1955, Hillman and Galston, 1956, Goldacre et. al., 1953, Lockhart, 1955, Waygood et. al., 1956, Hillman and Galston, 1956), resorcinol (Kenten, 1955, Waygood et.al., 1956), and two substances which are suspected hydrogen donors:- maleic hydrazide (Andreae and Andreae, 1953, Kenten, 1955) and methyl umbelliferone (Andreae and Andreae 1953). These substances are probably involved in a peroxidation with peroxide derived from the reactions of IAA itself. Indirect evidence has been found that IAA is peroxigenic in the presence of peroxidase extracts from pea epicotyls (Andreae and Andreae, 1953, Andreae, 1955b) and also when fed to intact pea sections (Siegel and Galston, 1955). Furthermore, there seems to be little doubt that a peroxide is utilized during IAA degradation since IAA inactivation is promoted

by the addition of hydrogen peroxide directly (Goldacre, 1951, Galston et. al., 1953,Briggs et. al., 1955a, Lockhart, 1955) or indirectly via a peroxigenic oxidase system (Waygood et. al, 1956). It must be made clear however, that the systems described by Waygood et. al., (1956) and Kenten (1955) did not require added peroxide to achieve complete IAA breakdown. Hence IAA inactivation appears to be a cyclic reaction involving at least peroxidase and a phenolic cofactor and in which peroxide is both produced and utilized.

It has been suggested by Galston and coworkers that a flavoprotein enzyme supplements the action of peroxidase in IAA breakdown. Light promoted IAA breakdown by crude extracts from pea epicotyls (Galston and Baker, 1949, Galston, 1950a, Galston and Baker, 1951, Galston et. al., 1953) and the action spectrum of the photosensitized inactivation resembled the absorption spectrum of riboflavin (Galston and Baker, 1949, Galston, 1950a). Since high concentrations of riboflavin destroyed IAA non-enzymically in light (Galston, 1949b), Galston suggested that a flavoprotein was required to catalyse peroxide formation from IAA. This possibility was attractive since the spectrum of riboflavin also parallels the action spectrum of phototropic responses that are known to be influenced by IAA (Galston, 1949b, Galston and Baker, 1949, Galston, 1950b). However, riboflavin itself was not shown to stimulate enzymic

IAA oxidation and the systems used by Galston were relatively inefficient in oxidizing IAA. Furthermore, riboflavin was evidently not required for complete IAA breakdown by peroxidase systems (Kenten, 1955, Maclachlan and Waygood, 1956) nor did light increase the rate or amount of breakdown in the wheat leaf system (Waygood et. al., 1956). It must be concluded as implied by Galston himself in a recent paper (Hillman and Galston, 1956), that the existence of a flavoprotein component of IAA "oxidase" is questionable.

Catalase is known to act peroxidatically (Chance, 1951, Keilin and Hartree, 1955, Laser, 1955) and under certain conditions might be expected to replace peroxidase as enzyme catalyst in IAA breakdown. However, reports of its effect on the course of IAA oxidation have varied widely according to the source of the system used to catalyse the reaction. Thus catalase completely inhibited IAA oxidation catalysed by pea epicotyl extracts in the dark (Goldacre, 1951, Andreae and Andreae, 1953), extended the induction period with wheat leaf extracts (Waygood et. al., 1956) and enhanced the rate of IAA oxidation catalysed by horseradish peroxidase (Kenten, 1955). Inhibition by catalase has been interpreted (Goldacre, 1951, Andreae and Andreae, 1953, Waygood et. al., 1956) as indicating that an intermediate peroxide required for IAA oxidation was catalatically destroyed by catalase. This explanation seems

unlikely since at the low concentrations of peroxide which could arise in the extracts, catalase would not act catalatically but peroxidatically. It is possible that the inhibitory effects of catalase were due to thermostable dialysable inhibitors in the catalase preparations and not to catalase itself. Natural inhibitors have been reported to contaminate almost all plant extracts investigated (Tang and Bonner, 1947, Wagenknecht and Burris, 1950, Gortner and Kent, 1953, Briggs et. al., 1955 a, Waygood et. al., 1956) but the nature of these inhibitors and their possible relationship with catalase inhibition have yet to be demonstrated.

Although most investigators are agreed that peroxidase and a phenolic cofactor are required for IAA oxidation the requirement for manganese is less well established. Wagenknecht and Burris (1950) first demonstrated that IAA "oxidase" systems which had been rendered inactive by dialysis were partially to completely reactivated by the addition of manganous ions. Magnesium, cupric, zinc and ferric ions would not substitute. Most other investigators (Tang and Bonner, 1947, Goldacre, 1951, Gortner and Kent, 1953, Kenten, 1955, Ray and Thimann, 1955, Lockhart, 1955) found no obvious manganese requirement, but this could have been due to the presence of sufficient manganese endogenously since dialysis was never used to clarify the systems. Galston and Baker (1951) identified the manganous ion as a photolabile inhibitor present in crude

pea epicotyl preparations but later (Goldacre et. al., 1953) found that 10^{-6}M to 10^{-4}M manganese enhanced the activity of well-dialysed brei. It was therefore not surprising that Waygood et. al. (1956) found a marked manganese requirement for IAA inactivation by dialysed peroxidase extracts from wheat leaves.

With respect to the mechanism of manganese activation, Waygood et. al. (1956) were first to suggest that manganese underwent oxidation and reduction during IAA oxidation and that manganic ions (trivalent manganese) initiated the degradation of IAA. On the basis of similar results with a system from peas, Hillman and Galston (1956) also postulated the participation of manganic ions. In support of this hypothesis it was pointed out (Waygood et. al., 1956, Hillman and Galston, 1956) that the requirements for complete IAA oxidation were identical with the requirements described by Kenten and Mann (1950, 1952) for the enzymic oxidation of manganese.

The oxidation of manganese by the "Kenten-Mann" system (1950, 1952) is accomplished as follows: -
(a) utilization of hydrogen peroxide by peroxidase for the oxidation of those phenolic substances (ROH) e.g. monohydroxyphenols or resorcinol, which do not readily form stable oxidation products; (b) oxidation of manganous to manganic ions by the semiquinol (RO.) of the phenolic cofactor and resulting semi-

quinol reduction; (c) chelation of manganic ions in stable complex by such agents as pyrophosphate or citrate, i.e.:

(a) $H_2O_2 + 2ROH \xrightarrow{\text{peroxidase}} 2H_2O + 2\,RO^{\bullet}$

(b) $RO^{\bullet} + Mn^{2+} + H^{+} \longrightarrow ROH + Mn^{3+}$

(c) $Mn^{3+} + \text{citrate} \longrightarrow \text{manganicitrate}$

Waygood et. al. (1956) suggested that peroxide produced during the degradation of IAA was utilized by peroxidase for the oxidation of phenolic cofactors (reaction a) which then oxidized manganese (reaction b). In the absence of pyrophosphate or citrate manganic ions would oxidize IAA by virtue of their extremely high redox potential (Maclachlan and Waygood, 1956). The fact that pyrophosphate and citrate completely prevents IAA breakdown (Waygood et. al., 1956) lends support to this hypothesis.

The theory of catalysis of IAA breakdown by the Kenten-Mann system not only accounts for the role of peroxidase, manganese and phenolic cofactors but also assimilates reports that peroxide is produced and utilized during IAA oxidation. Participation of the Kenten-Mann system implies that IAA oxidation is actually a peroxidase-controlled autoxidation and as such a unique process which has not been documented elsewhere. However, note should be made of suggestions that an autoxidation may also be involved in tyrosine (La Du and Zannoni, 1956) and tryptophane (Knox and Mehler, 1950, Dalgliesh, 1951) metabolism.

Such a process would be expected to obey the classical laws of autoxidation (Waters, 1947, Waters and Wickham-Jones, 1951) and therefore to be subject to verification by appropriate kinetic investigation.

It appears that the only enzyme required for IAA autoxidation is peroxidase which functions indirectly by providing a source of manganic ions. Therefore the actual reactions undergone by IAA should occur spontaneously in the presence of oxidized manganese alone. This prediction has been confirmed by Maclachlan and Waygood (1956) who isolated the reaction between IAA and manganic ions using the organic chelating agent ethylenediamimetetraacetic acid (versene) to bind manganic ions in complex at a redox potential of ca. 0.80 v. At pH 6.0 and in an oxygen atmosphere the reactions of IAA in the presence of manganiversene were identical with those in the enzymic system in the following respects: (a) the release of one equivalent of carbon dioxide slightly preceeded the consumption of one mole of oxygen per mole of IAA; (b) the yellow **and** product possessed characteristic solubility relations, crystal structure and chromatographic Rf value. These findings at once confirmed the participation of manganic ions in the enzymic breakdown of IAA and obviated the need for postulating a more complex system of catalysts and cofactors in the enzymic system other than those required for the formation of manganic ions.

On analogy with the well documented reactions of the cobaltic ion (Bawn and White, 1951a, 1951b, 1951c) the following reaction sequence was proposed (Maclachlan and Waygood 1956) for the spontaneous manganic ion - initiated degradation of IAA (IAA = S-COOH, where S represents the skatole nucleus) i.e.:

$$S\text{-}COOH + Mn^{3+} \longrightarrow S\text{-}COO\cdot + H^{+} + Mn^{2+}$$

$$S\text{-}COO\cdot \longrightarrow CO_2 + S\cdot$$

$$S\cdot + O_2 \longrightarrow SO_2\cdot$$

The final yellow product of the reaction was thought to be formed from the oxidized skatole radical ($SO_2\cdot$) or skatole peroxide by the addition of an hydrogen atom derived from other constituents of the reaction system e.g.: versene. The structure of the product and proof of its empirical formula ($SO_2H = C_9H_9O_2N$) were not determined.

It should be noted that under certain conditions little or none of the yellow product was obtained in the non-enzymic system. Thus anaerobically, to some extent in air, and even in an oxygen atmosphere below pH 5.0, a purple-black precipitate was formed indicating that polymerization had taken place. The skatole radical (S·) was considered to be a highly reactive intermediate and its condensation or reaction with various components of the medium would result in more than one

reaction product. This may explain the difficulties that investigators have had in identifying the end product of the enzymically-controlled reaction (Manning and Galston, 1955).

In summary then, the following constituents may be considered as established requirements for the enzymic decarboxylation and oxidation of IAA: (a) manganese, (b) an organic cofactor which is capable of acting as a redox catalyst for the oxidation of manganese by the Kenten-Mann system, (c) an enzyme catalyst for a peroxidatic reaction. The reactions of the non-enzymic system whereby IAA is oxidatively decarboxylated probably also take place in the enzymic system. On this basis it is concluded that the enzymic degradation of IAA is initiated by manganic ions and thereafter IAA decarboxylation and oxidation proceeds spontaneously. Operation of the Kenten-Mann system explains the roles of peroxidase and the phenolic cofactors. If these conclusions are correct the enzymic system must be considered to produce an intermediate during IAA degradation which contributes directly or indirectly to further IAA breakdown. A kinetic examination of the enzymic system should therefore reveal characteristics of an autocatalytic system.

III METHODS AND MATERIALS

(A) Enzyme Preparation

Leaves of wheat, Triticum compactum Host. var. Little Club were harvested after growing from 12-15 days under normal diurnal conditions in greenhouse soil. The leaves were ground with sand in a cold mortar and the brei was squeezed through nylon or cheesecloth. The volume of extract obtained in milliliters was generally close to half of the fresh weight of leaves in grams. The press juice was centrifuged at 20,000 xg at 2°C for 30 minutes and dialysed against distilled water in a rocking dialyser at 4°C. Unless otherwise indicated the brei was dialysed against 20 volumes of water for each of three consecutive 24 hour periods. The final dialysate was centrifuged as before and the supernatant stored at -15°C. Intermittent freezing and thawing during usage rendered visibly clear preparations containing little or no green material and approximately 0.4 mgms. protein nitrogen per milliliter.

Solutions of commercially purified horseradish peroxidase and crude beef liver catalase were purchased from Nutritional Biochemicals Corp. and dialysed in the same way as wheat leaf extracts to result in final concentrations of 0.10% and 0.55% W/V respectively.

(B) Manometric Methods:

The decarboxylation and oxidation of IAA was followed by measuring gas exchange by the direct method of Warburg in standard respirometers. Flasks of volume ca. 20 c.c. contained the components in a liquid volume of 3.0 ml. with 0.2 ml. 2N KOH in the central well. The concentrations of the components of a "standard" system for IAA oxidation were as follows: Enzyme, 0.5 ml; orthophosphate buffer pH 6.0, 150 μM; manganous chloride, 3·0 μM; resorcinol, 1.5 μM. Reaction was usually begun by the addition of the sodium or ammonium salt of IAA at pH 6.0 (6.67 μM = 158 μl gas exchange at molar equivalence). All experiments except those so indicated were performed at 29.5°C.

(C) Colorimetric Methods:

The concentration of IAA was determined colorimetrically by use of the Salkowski reagent ($FeCl_3$ - perchloric acid) in a procedure described by Gordon and Weber (1951). To estimate the amount of IAA destroyed in a reaction, one or two tenths milliliters of the contents of a Warburg flask were added to enough water to make up a volume of 2.0 ml. The Salkowski reagent (3.0 ml.) was added and the protein precipitate centrifuged off. After 30 minutes the solution density was read in a Coleman Universal Spectrophotometer at 520 mμ and the concentration of IAA obtained by reference to a standard concentration/density curve.

IV EXPERIMENTAL RESULTS

(A) General Features of IAA Degradation:

1. Enzyme and Cofactor Requirements:

When IAA was added to a dialysed wheat leaf preparation in orthophosphate buffer at pH 6.0 no destruction of the acid took place. If, however manganous ions were present, partial destruction occurred with the uptake and release of an equivalent volume of oxygen and carbon dioxide. If in addition to manganous ions, a monohydroxy phenol or resorcinol were present, IAA was completely destroyed with the concomitant uptake and release of one mole each of oxygen and carbon dioxide per mole of IAA. The oxidation was thermolabile, no carbon dioxide was released in a nitrogen atmosphere and in neither of these cases was IAA converted to a non-Salkowski reacting substance. The gaseous exchange of the complete system in the absence of one or more of the components is shown in Figure 1.

A characteristic feature of the progress curves for gas exchange of systems catalysed by 16-24 hr. dialysed preparations from summer-grown wheat leaves was a lag or induction phase lasting for 10 to 20 min. Extracts from winter grown leaves gave a lag phase of only 2 to 8 min. due apparently to a lower concentration of dialysable natural inhibitors of IAA oxidation. In spite of extended dialysis the lag phase was never completely overcome (Fig. 1). In this respect then, IAA oxidation

Figure 1. Cofactors for enzymic decarboxylation and oxidation of indoleacetic acid.

Standard system: 0.5 ml. wheat leaf extract (0.20 mgms. protein N); 5×10^{-4}M resorcinol; 10^{-3}M $MnCl_2$; 5×10^{-2}M phosphate, pH 6.0; 2.22×10^{-3}M IAA = 158 µL. O_2 at molar equivalence; final vol. 3.0 ml.; temp. 29.5°C.

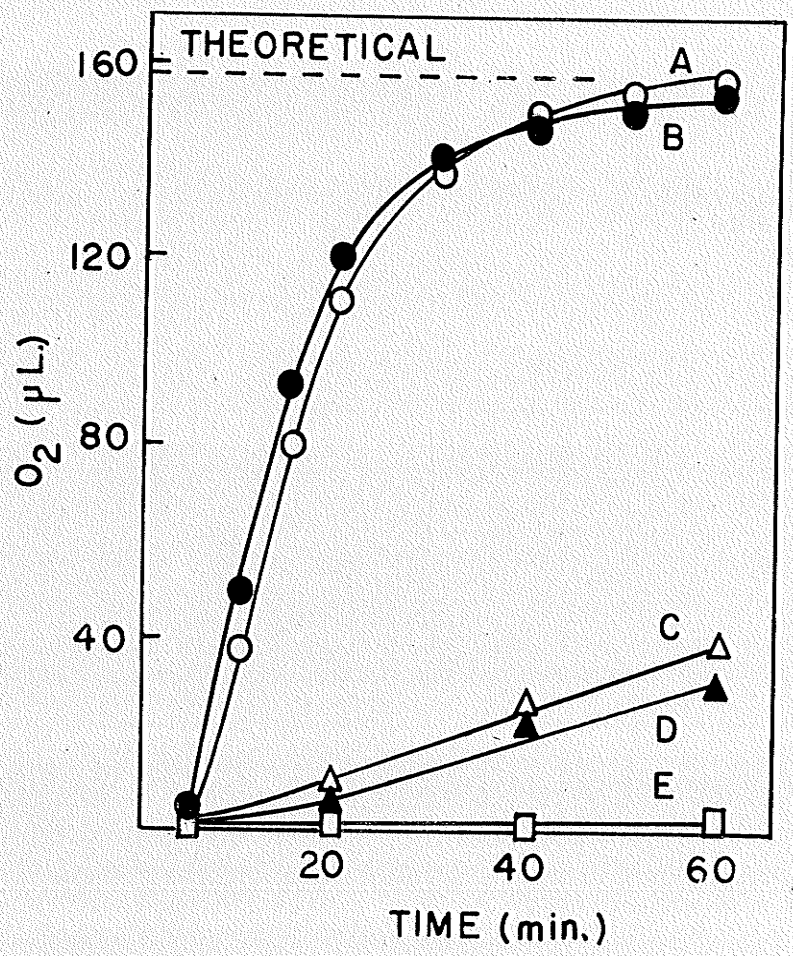
A. O_2 uptake of complete system.

B. CO_2 release of complete system.

C. O_2 uptake of system minus resorcinol.

D. O_2 uptake of system minus manganese.

E. O_2 uptake of system minus manganese and resorcinol.


THEORETICAL
160
120
80
40
O_2 (μL.)
A
B
C
D
E
20
40
60
TIME (min.)

resembles all autoxidation systems and the induction period may be considered as the time necessary for the system to build up a threshold concentration of the intermediate which initiates reaction i.e. manganic ions.

The decarboxylation and oxidation of IAA in the presence of manganese and an organic cofactor was catalysed by wheat leaf extracts (Fig. 1) and also by purified horseradish peroxidase and well dialysed beef liver catalase. The initial velocities i.e. the maximum velocity in any 10 min., and the absolute amounts of oxygen consumed by IAA after the attainment of equilibrium in the presence of these three enzyme extracts and various organic cofactors are compared in Table 1. Salkowski measurements on each system confirmed that the amount of IAA destroyed corresponded to the ratio oxygen consumed to theoretical consumption for molar equivalence. The product of the reactions in each case was a soluble yellow substance identical in crystal structure and solubility relations to the non-enzymic product (Maclachlan and Waygood, 1956). Although not shown in Table 1 there was invariably a lag phase with all cofactors and enzymes. Carbon dioxide was liberated always prior to the onset of oxygen consumption as illustrated in Figure 1, indicating that in each case decarboxylation of IAA precedes aerobic oxidation.

The oxidation of IAA in the presence of manganese

Table I Effect of Organic Cofactors on Indoleacetic Acid Oxidation by Catalase and Peroxidase Extracts[γ]

System	Maximum Velocity (μL. O_2/5 min.)				
Organic Cofactor:	Dichloro-phenol	Maleic Hydrazide	Natural Factor	Resorcinol	None
Beef Liver catalase	39.0	49.5	0	18.0	0
Wheat Leaf extract	44.5	25.0	17.5	31.5	6.0
Horseradish peroxidase	67.5	76.5	43.5	68.5	14.0
	Oxygen Consumed at Equilibrium (μL. O_2/3 hrs.)				
Beef liver catalase	157	150	0	86	0
Wheat leaf extract	153	146	128	162	36
Horseradish peroxidase	136	153	126	149	92

[γ]Systems contain peroxidase (0.2 mgms), catalase (5.5 mgms) or wheat leaf extract (0.2 mgms protein), manganese (3μ gms), orthophosphate pH 6.0 (150 μ gms), IAA (6.67 μ gms = 158 μl) in a final volume of 3.0 ml. Cofactor concentrations as follows: dichlorophenol, 1.5 μ gms; maleic hydrazide, 30μ gms; natural factor, 0.15 ml.; resorcinal, 1.5 μ gms. Temperature 29.5°C.

and wheat leaf or peroxidase extracts was stimulated by a soluble fraction (natural factor) from wheat leaves (Table 1) which was effective in replacing the artificial organic cofactor, e.g.: resorcinol, in the system. The factor was prepared from the dialysing medium of the wheat leaf extracts as follows: The medium from the first 24 hours dialysis was concentrated in vacuo at 45°C, acidified to pH 2.0 with phosphoric acid and extracted with three volumes of ether. The yellow residue from the ether layer after evaporation was taken up in water, adjusted to pH 6.0 with NaOH and made to 1/10 of the volume of the original enzyme extract. When 0.15 ml. of this preparation was used in conjunction with 0.5 ml. of enzyme extract in systems for IAA oxidation, an approximately threefold concentration and purification of the active principle was effected. When 0.5 ml. of the natural factor extract was used, the induction phase which preceded IAA oxidation was extended from ca. 10 min. to ca. 80 min. thus indicating the presence of natural inhibitors as well as activators of the oxidation.

The relative rates of IAA oxidation catalysed by peroxidase or catalase (Table 1) varied in the presence of different cofactors indicating reaction or complex formation between enzyme and cofactor. The catalase system differed from the peroxidase and wheat leaf systems in that it was completely inactive towards IAA in the absence of an organic cofactor or

in the presence of the natural factor. This suggests that peroxidase but not catalase possesses an affinity for naturally occurring cofactors (see also p. 39 and Table III). Similarly Kenten (1955) and Goldacre and Galston (1953) have concluded that natural cofactors exist in pea epicotyls and bean roots.

Resorcinol at the same concentration as dichlorophenol (5×10^{-4}M) was equally effective in promoting IAA oxidation in the horseradish peroxidase system, but less effective in the wheat leaf system (Table 1). In the catalase-resorcinol system a 30 min. induction period occurred, the rate was lower and less IAA was oxidized at equilibrium (Table 1). Further experiments revealed slight, but reproducible, differences between catalase and other systems in the optima of pH and resorcinol concentrations. These are shown later (Table III) but because of such quantitative differences between the catalase and peroxidase systems it is suggested that the activity of the wheat leaf extract, which contains both enzymes, is due to peroxidase chiefly. It may be noted that resorcinol, under optimum conditions in the catalase system, promoted the most rapid rate of IAA oxidation (63 μL./5 min.) found in the entire catalase investigation. Further studies of the kinetics of the catalase and peroxidase reactions would undoubtedly reveal other quantitative differences, nevertheless with respect to cofactor requirements the two systems are evidently identical.

2. Non-Enzymic Manganic Ion Requirements

A method was previously described (Maclachlan and Waygood, 1956) for preparing a manganiversene complex which was reasonably stable at pH 6.0 yet possessed a sufficiently high redox potential (greater than 0.7 v) to react with IAA. Manganoversene is oxidized to manganiversene by either excess solid lead dioxide or excess manganese dioxide and in most experiments with IAA the oxidant was allowed to remain suspended in solution. This reaction system had the advantage of allowing manganic ions to be continuously generated but the disadvantage of producing the ions at an unknown concentration. Thus, it was of interest to prepare manganiversene and remove the oxidant by centrifugation since by incubating IAA with known amounts of manganiversene the stoichiometry of its reaction with manganic ions could be determined.

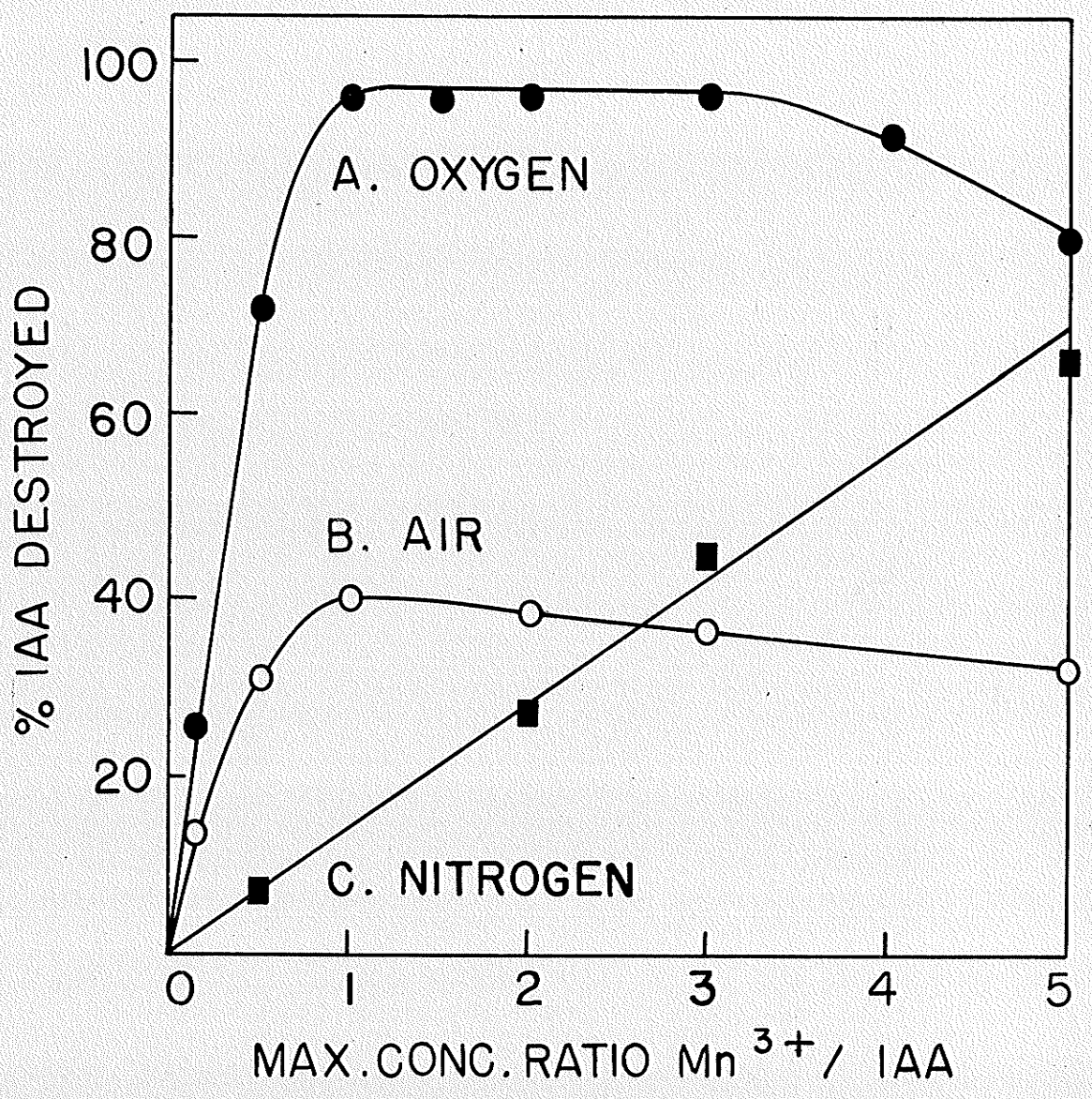
Manganiversene was freshly prepared, freed of oxidant and varying amounts were added to the main compartment of standard respirometers containing orthophosphate buffer pH 6.0 and, in sidearms, IAA. These were brought to temperature equilibrium (29.5°C) and a period of 20 min. was allowed to elapse between the preparation of manganiversene and the addition of IAA. The experiment was performed in air and in oxygen and nitrogen atmospheres with up to a maximum total manganese/IAA concentration ratio of 5/1. After gas exchange had reached

equilibrium the flask contents were analysed for IAA by the Salkowski test. The amount of IAA destroyed is plotted as a function of the total manganese/IAA concentration ratio in Figure 2.

It should be realized that the units of the abscissa of Figure 2 represent the maximum possible manganic ion concentration having been calculated from the total manganese ($Mn^{2+} + Mn^{3+}$). Manganiversene is least stable at pH 6.0 (Maclachlan and Waygood, 1956) and in 20 min. partially decomposes. Manganoversene is colorless but manganiversene is red (λ_{max} = 500 mμ.), thus the rate of decolorization of the latter permits a convenient measurement of the rate of spontaneous degradation of manganic ions. The optical density of several manganiversene solutions was found to decrease independently of the original concentration by 21-24% per 20 min. at pH 6.0 and room temperature. However, the actual manganic ion concentration was probably even less than ca. 75-80% of that indicated in Figure 2 since manganese oxidation did not appear to go to completion using lead dioxide as oxidant. When manganiversene was titrated 20 minutes after preparation with ferrous sulphate, generally close to 50% and never more than 61% of the total manganese was found in the manganic form.

Figure 2. Indoleacetic acid destruction in the presence of increasing concentrations of manganiversene in air, oxygen and nitrogen atmospheres.

Systems: manganiversene (see text for preparation); 5 x 10^{-2}M phosphate, pH 6.0; 2.22 x 10^{-3}M IAA; Final vol. 3.0 ml.; temp. 29.5°C. Oxygen, air and nitrogen atmospheres as indicated. % IAA destroyed determined by Salkowski test.


100
80
60
40
20
% IAA DESTROYED
A. OXYGEN
B. AIR
C. NITROGEN
0
1
2
3
4
5
MAX. CONC. RATIO Mn 3+ / IAA

On this basis, complete IAA degradation occurred in the presence of less than equimolar amounts of manganic ions (Fig. 2:A). In one instance, 72.5% IAA destruction occurred in the presence of 0.5 moles Mn^{2+} + Mn^{3+} per mole IAA which indicates that only about 0.35 moles Mn^{3+} were required per mole IAA destroyed. Thus there can be no doubt that IAA does not necessarily react stoichiometrically with manganic ions but once initiated, IAA degradation may to some extent proceed catalytically. From the above calculations as much as 65% of the IAA destruction may occur via autocatalytic pathways.

Catalytic IAA breakdown in the presence of limiting amounts of manganiversene took place only with a high oxygen tension (Fig. 2:A). This implies that an oxidized intermediate of IAA breakdown possesses a high enough redox potential to react with IAA and propagate an autoxidation sequence. In terms of the free radical mechanism previously proposed (Maclachlan and Waygood, 1956), the overall propagation sequence, as a consequence of initiation by manganic ions, may be represented as follows, where S-COOH = IAA; $SO_2^{\bullet}$ = oxidized skatole radical = skatole peroxide; SO_2H = final product; $S^{\bullet}$ = skatole radical.

$$SO_2\cdot + S\text{-}COOH \longrightarrow SO_2H + S\cdot + CO_2$$

$$S\cdot + O_2 \longrightarrow SO_2\cdot$$

It should be understood that these equations represent the overall process of autoxidation and do not attempt to explain the mechanism which may involve other intermediate reactions e.g.: oxidation of manganous ions by $SO_2\cdot$ and resulting IAA oxidation by manganic ions so generated.

At any given manganiversene concentration less IAA was destroyed in air (Fig. 2:B) than in oxygen (Fig. 2:A). This was interpreted (Maclachlan and Waygood, 1956) as an indication that the decarboxylation product of IAA i.e. the skatole radical, S·, tended to react with manganic ions rather than with oxygen. In all systems manganiversene was completely decolorized in less than 10 minutes, thus S· and $SO_2\cdot$ are probably capable of reacting with versene with the result that manganiversene is decomposed in a chain reaction. In nitrogen, at high manganiversene concentrations in air and even in oxygen below pH 5.0, a a black precipitate formed which was found to contain some IAA even after vigorous washing (Maclachlan and Waygood, 1956). This indicated that condensation of the decarboxylation products with themselves or IAA could also take place. The one system component with which these free radicals did not appear to react was water. This may be stated with confidence since the amount of oxygen consumed by the systems was never found to be less than the carbon dioxide released. This result would not be expected if water were decomposed by radicals with the formation of

hydroxyl radicals and hydrogen peroxide. It should be noted that reactions of the intermediate radicals, particularly $SO_2\cdot$, are important in interpreting the results of the enzymic system since it was concluded (see Literature Review) that a product of IAA breakdown was utilized to propagate further breakdown i.e.: the enzymic system was cyclic.

(B) Kinetics of Enzymic Indoleacetic Acid Degradation

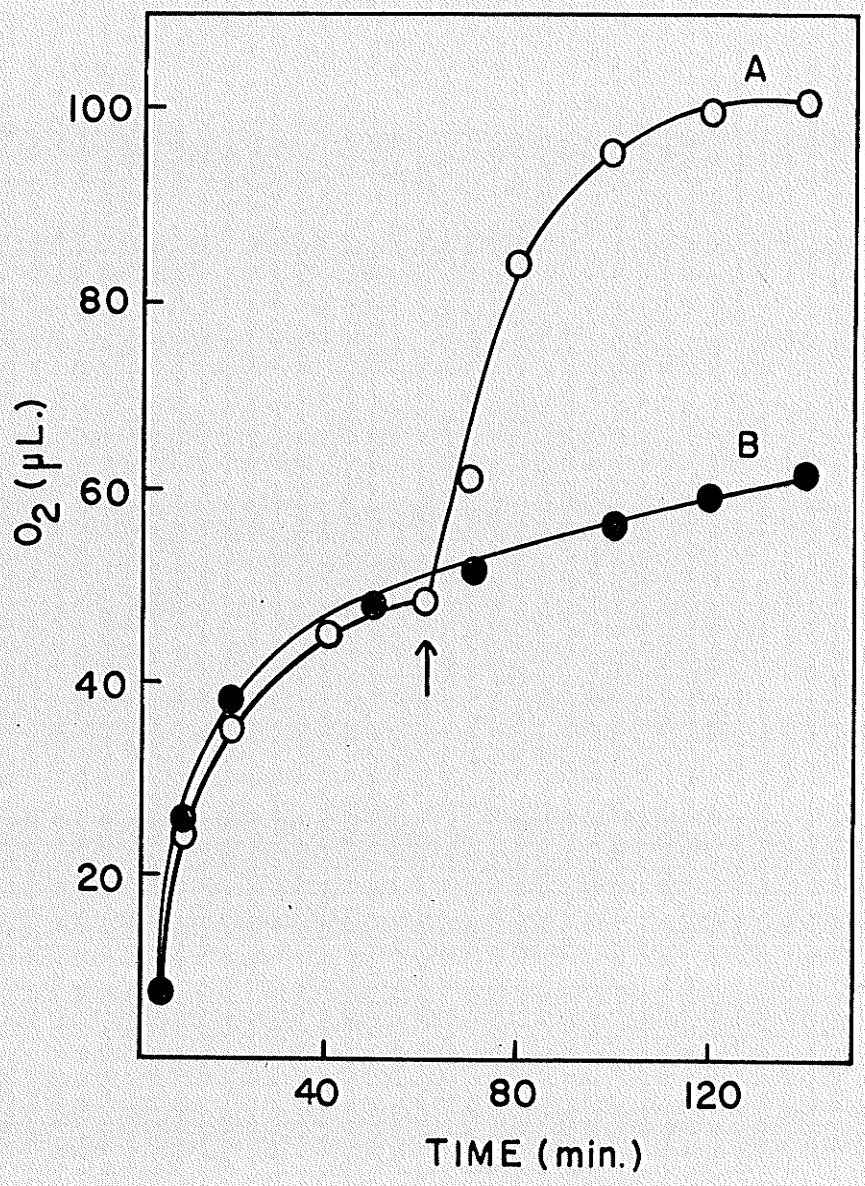
1. Enzyme Concentration:

In the presence of low concentrations of wheat leaf enzyme the progress curves for IAA oxidation attained an equilibrium lower than the theoretical oxygen equivalence (Fig. 3). At these low equilibria, peroxidatic activity of the systems could not be detected by the benzidine test and rapid oxidation resumed only if more enzyme were added (Fig. 3:A). The addition of IAA (Fig. 3:B) or any constituent of the system other than enzyme had no effect on the rate of oxidation. Evidently the peroxidatic activity of the extracts was destroyed or strongly inhibited during IAA oxidation. Similar effects were described by Kenten (1955) with the horseradish peroxidase-catalysed oxidation of IAA.

The peroxidatic activity of catalase appeared to be inactivated during IAA oxidation in the same way as wheat leaf extract and horseradish peroxidase. With low catalase concentrations (0.05%) the rate of oxygen uptake declined prematurely and resumed only if more catalase (or peroxidase) were added. When hydrogen peroxide was introduced to the flasks at this stage oxygen was evolved vigorously. Slightly more oxygen was evolved (114%) from a system containing all the components with the exception of IAA, and less was evolved (90%) from a solution of catalase only at the same concentration.

Figure 3. Enzyme inactivation during indoleacetic acid oxidation.

Systems standard except enzyme concentration = 0.05 ml. At arrow (60 min.), A: 0.05 ml. enzyme added, B: 6.67 μM. IAA added.


A
B
100
80
60
40
20
O_2 (μL.)
40
80
120
TIME (min.)

It was concluded that during the oxidation of IAA the catalatic activity of catalase was retained whereas its peroxidatic activity was inhibited. Inactivation of both catalase and peroxidase is probably reversible and due to strong binding of a reaction intermediate at peroxidatic sites.

In the presence of optimum (see later sections) concentrations of resorcinol and manganese the effects of enzyme inactivation are shown by wheat leaf systems containing up to 0.25 ml. of enzyme (Fig. 4:C-E) in which theoretical oxygen equivalence was not attained. The change in slope at about 30 minutes indicates the point where the enzyme concentration became limiting. Theoretical amounts of oxygen were consumed when 0.5 ml. of enzyme was used (Fig. 4:B), but higher concentrations of enzyme (Fig. 4:A) decreased the amount of oxygen uptake in this particular experiment. Analogous results were obtained using DCP in place of resorcinol at the same concentration (5×10^{-4}M).

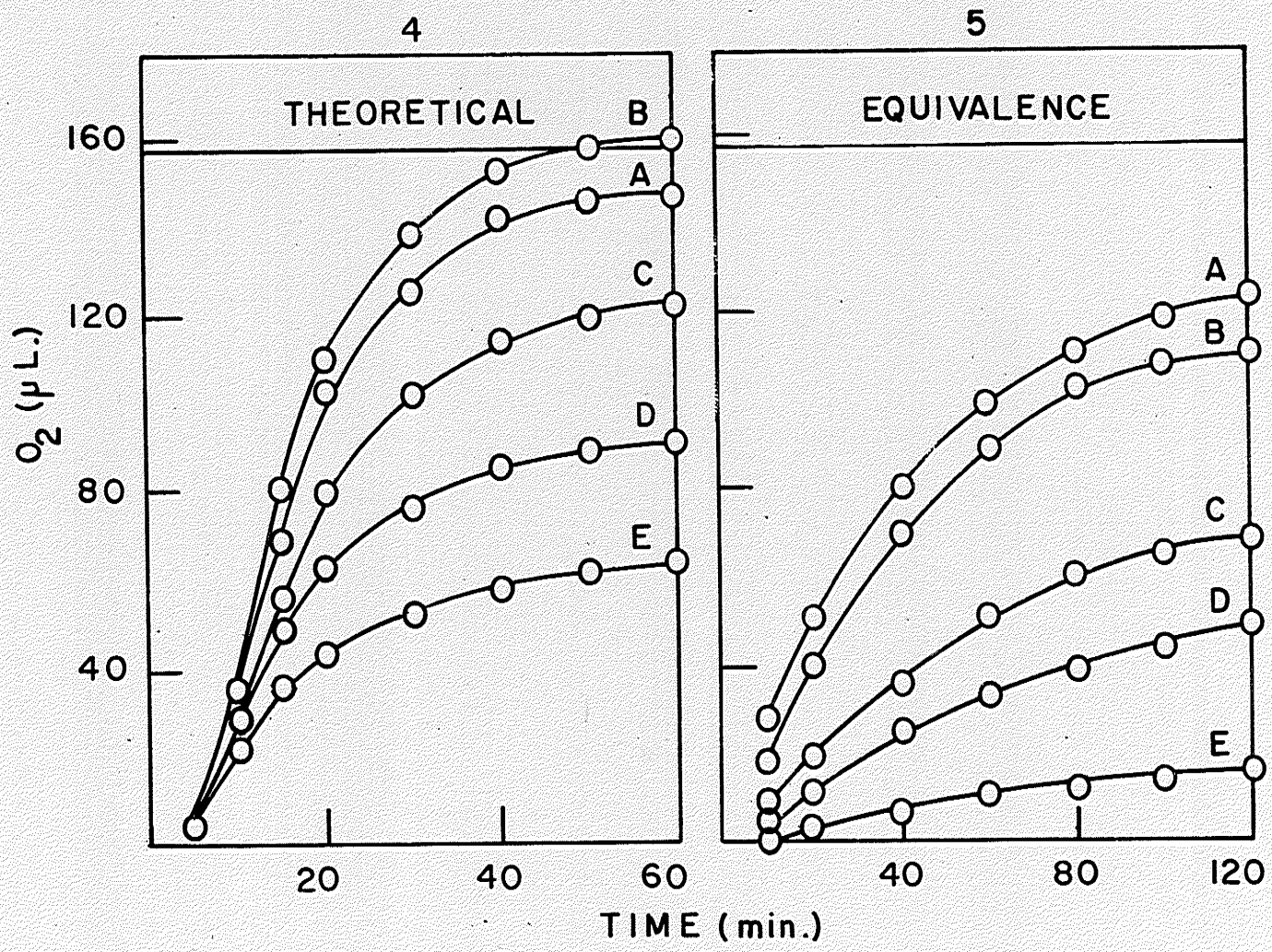
The effect of varying the enzyme concentration in the presence of <u>suboptimum</u> amounts of resorcinol (5×10^{-5}M) are shown for comparison in Figure 5. As expected, the initial velocities of IAA oxidation were lower at each enzyme concentration than in the corresponding systems containing optimum amounts of resorcinol (Fig. 4). But in addition, lower final equilibria were attained. Thus, for example, with 0.25 ml.

Figure 4. Effect of enzyme concentration on indoleacetic acid oxidation with optimum cofactor.

Systems standard with enzyme conc. in ml.: A, 1.0; B, 0.50; C, 0.25; D, 0.10; E, 0.05. Resorcinol conc. : 5×10^{-4} M.

Figure 5. Effect of enzyme concentration with suboptimum cofactor.

Systems same as Fig. 4 except resorcinol = 5×10^{-5} M.


4
5
THEORETICAL
EQUIVALENCE
B
A
C
D
E
160
120
80
40
O_2 (μL.)
20
40
60
40
80
120
TIME (min.)

of enzyme about 120 µl. oxygen were consumed at equilibrium (Fig. 4:C) as compared to about 70 µl. (Fig. 5:C) at the lower cofactor concentration. In similar experiments using the natural factor (concentration unknown, but suboptimum), 0.25 ml. of enzyme catalysed the consumption of only 40 µl oxygen at equilibrium. With either the natural factor or limiting resorcinol it was again found that the enzyme was the only component of the systems which, when added at equilibrium, brought about a significant increase in the rate of IAA oxidation. Therefore from a comparison of Figures 4 and 5 it is evident that more enzyme was inactivated at low cofactor concentrations than at the optimum.

The intermediate causing inactivation appeared to be an oxidized substance since in any one experiment the oxygen consumed at equilibrium was related to the amount of enzyme inactivated e.g. Fig. 4:C-E. But since more enzyme was inactivated at low concentrations of cofactor it must be concluded that optimum amounts of cofactor had a 'protective' effect on the enzyme. Dependence of enzyme "inactivation" on the enzyme/cofactor concentration ratio is satisfactorily explained if it is assumed that the substance which causes inactivation may react <u>either</u> with the enzyme <u>or</u> with the cofactor. It is suggested that the oxidized skatole radical (skatole peroxide) is the most probable inactivating agent and that in the absence

of sufficient hydrogen donor (cofactor) a relatively stable and inactive enzyme-peroxide-skatole complex is formed.

The enzyme concentration/activity curves for resorcinol, DCP and the natural factor (Fig. 6) all deviate from linearity at high enzyme concentrations. Similar results occur with catalase and have been reported in other IAA "oxidase" systems (Goldacre, 1951, Gortner and Kent, 1953, Goldacre et.al., 1953). In all of these systems this effect is understandable if it is assumed that the enzyme-inactivating intermediate was also an oxidant of a natural or artifical cofactor and as such promoted the oxidation of manganese and IAA. By increasing the concentration ratio of enzyme/cofactor more of the intermediate would be diverted from chain oxidation of IAA to inactivation of the enzyme. Thus, due to competition, a linear increase in activity with enzyme concentration would not be expected except at low enzyme/cofactor concentration ratios i.e.: where the enzyme was saturated with hydrogen donor.

2. Substrate Concentration

A typical set of progress curves for the oxidation of variable amounts of IAA (0.56 to 3.33 x 10^{-3}M) is shown in Figure 7. There are three distinctive features of the curves. Firstly, the lag phase which preceded IAA oxidation was due to incomplete removal of endogenous inhibitors since the wheat leaf enzyme used in this particular experiment had

Figure 6. Effect of enzyme concentration on the oxidation rate with various cofactors.

Systems standard with: A, 5×10^{-4}M dichlorophenol; B, 5×10^{-4}M resorcinol; C, 0.15 ml. natural factor; D, 5×10^{-5}M resorcinol.

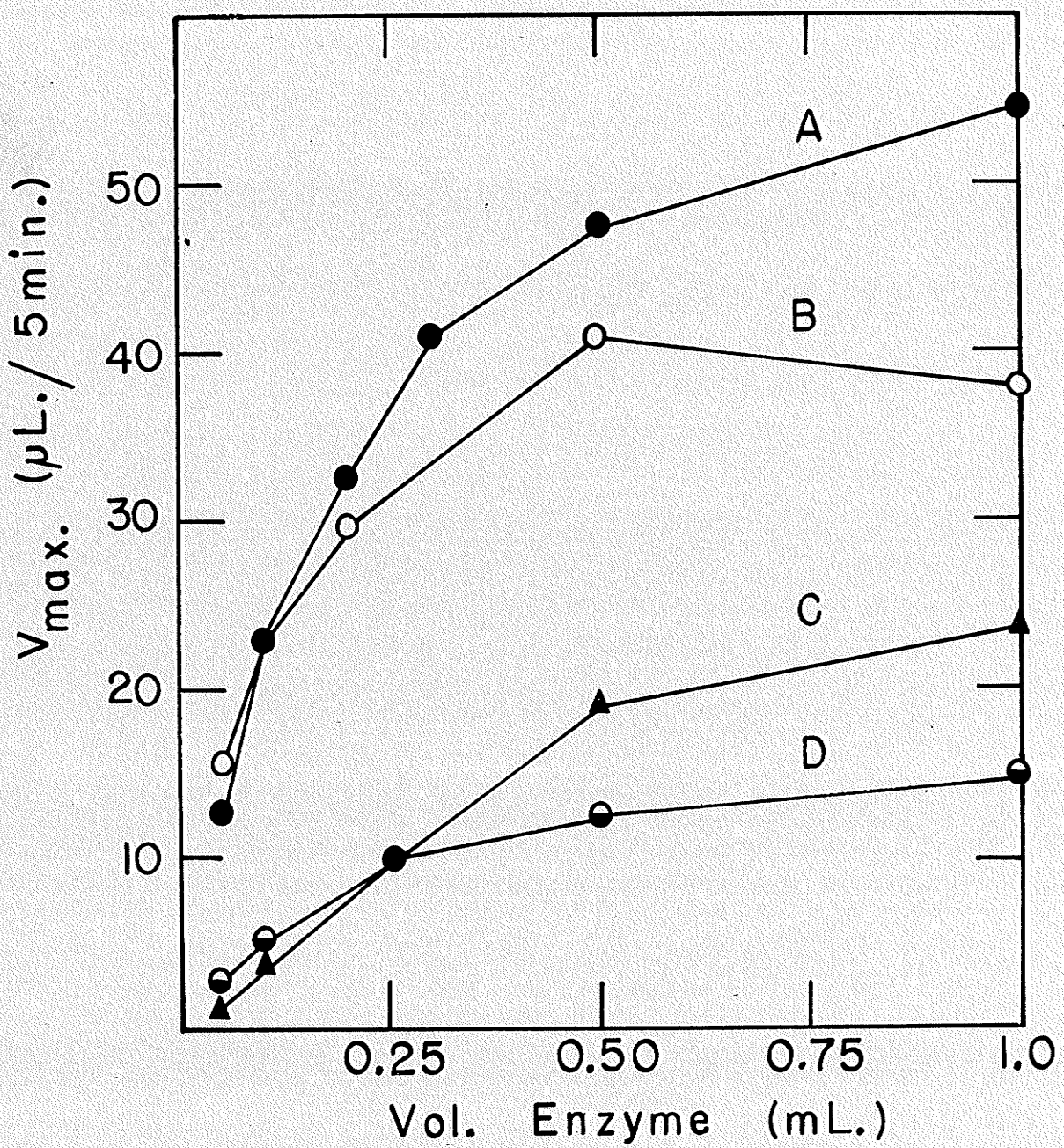
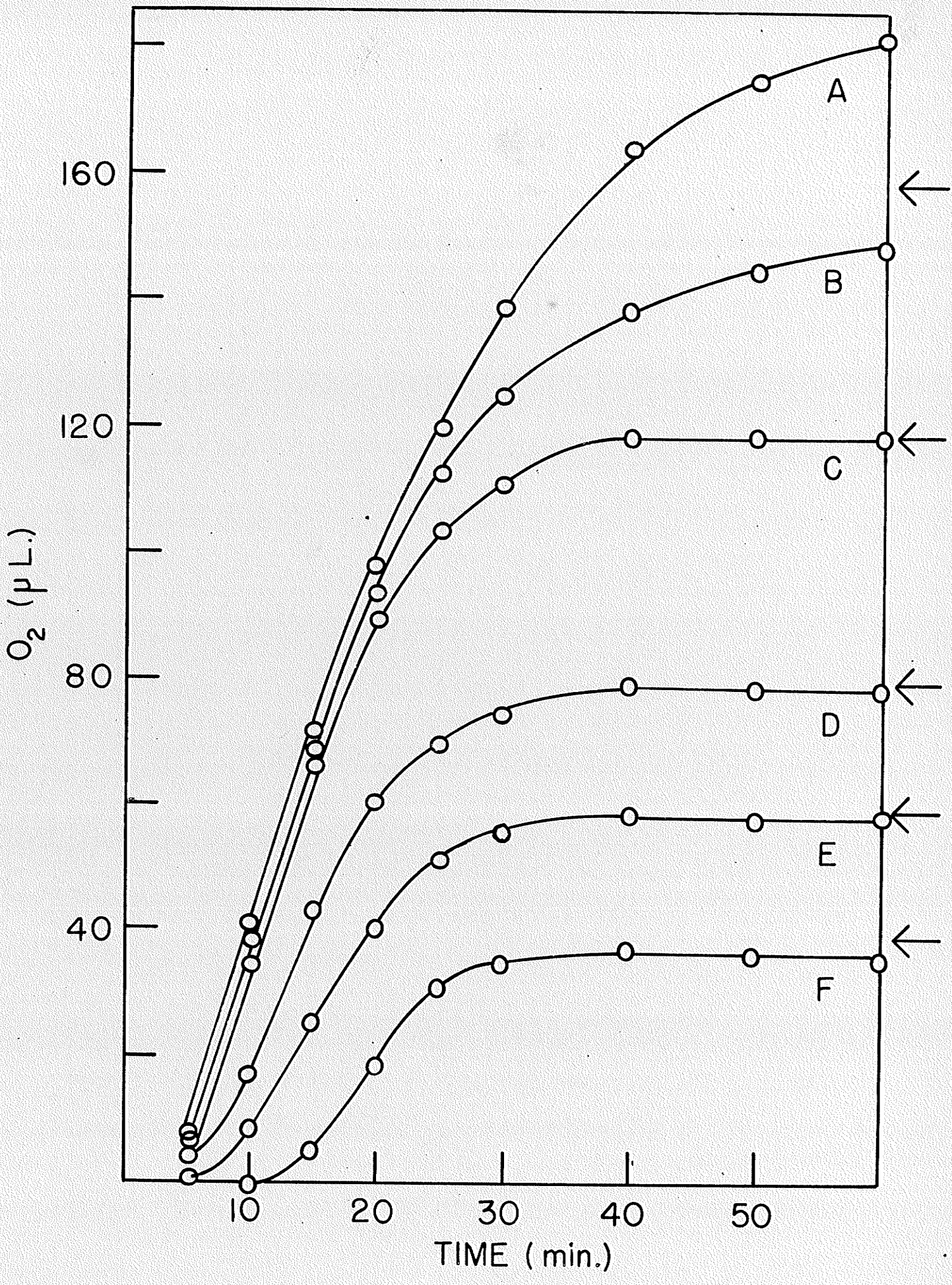

A
B
C
D
50
40
30
20
10
$V_{max.}$ (μL./ 5min.)
0.25
0.50
0.75
1.0
Vol. Enzyme (mL.)

Figure 7. Effect of indoleacetic acid concentration on the progress of oxygen uptake.

Systems standard with IAA in ugms.: A, 10.0; B, 6.67; C, 5.0; D, 3.33; E, 2.5; F, 167. Theoretical molar oxygen equivalences for curves B to F indicated by arrows.

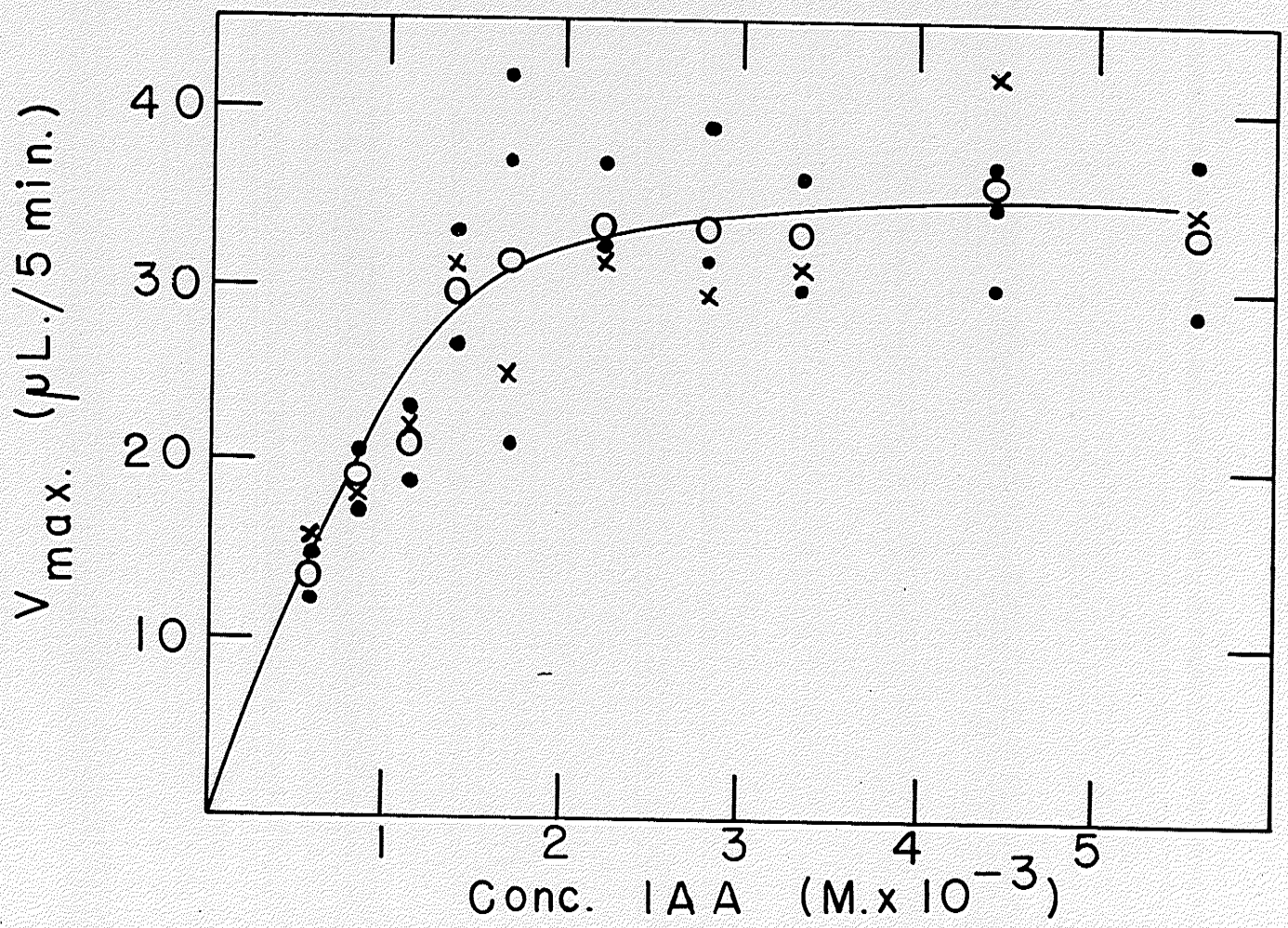

160
120
80
40
O_2 (μL.)
A
B
C
D
E
F
10
20
30
40
50
TIME (min.)

been dialysed for only 8 hours. The lag phase was longest in reaction mixtures containing the least amount of IAA thus implicating the substrate as a factor in overcoming inhibitors of its own oxidation. Secondly, the rates of oxygen uptake increased linearly with increasing concentrations of IAA up to about 1.7×10^{-3}M (cf. Fig. 8). Thirdly, theoretical oxygen uptake was attained at equilibrium only when 1.7×10^{-3}M IAA or less was used. Above this concentration the effects of enzyme inactivation were evident in the premature decline of reaction velocity (Fig. 7:A,B). Kenten (1955) has found similarly that horseradish peroxidase preparations oxidized small amounts of IAA (0.83×10^{-3}M) almost completely, but larger amounts (3.33×10^{-3}M) required further additions of enzyme before a comparable destruction of IAA took place.

In order to compare the initial velocities of reactions with lag phases of variable length, the maximum increments attained in any 5 minute period were chosen as the most reproducible feature of the progress curves. In most cases this was the increment from 5 to 10 minutes after oxygen uptake had commenced (Fig. 7). The IAA concentration/activity curves obtained in this manner (Fig. 8) were approximately hyperbolic, but variations within any single experiment precluded an accurate kinetic treatment of the data. When the experiment was performed in an oxygen atmosphere, the lag phase was almost overcome, similar initial velocities were obtained,

Figure 8. Effect of indoleacetic acid concentration on the oxidation rate.

Systems standard, allowed to react in atmospheres of air (solid circles) and oxygen (crosses). Average rates in air and oxygen shown as open circles.


40
30
20
10
V max. (μL./5 min.)
1
2
3
4
5
Conc. IAA (M.x 10⁻³)

but variability was still encountered. From the average of the maximum velocities found in the experiment in oxygen and three experiments in air (Fig. 8) a Michaelis constant of between 0.7 and 1.1 x 10^{-3}M was calculated.

3. The Effect of pH:

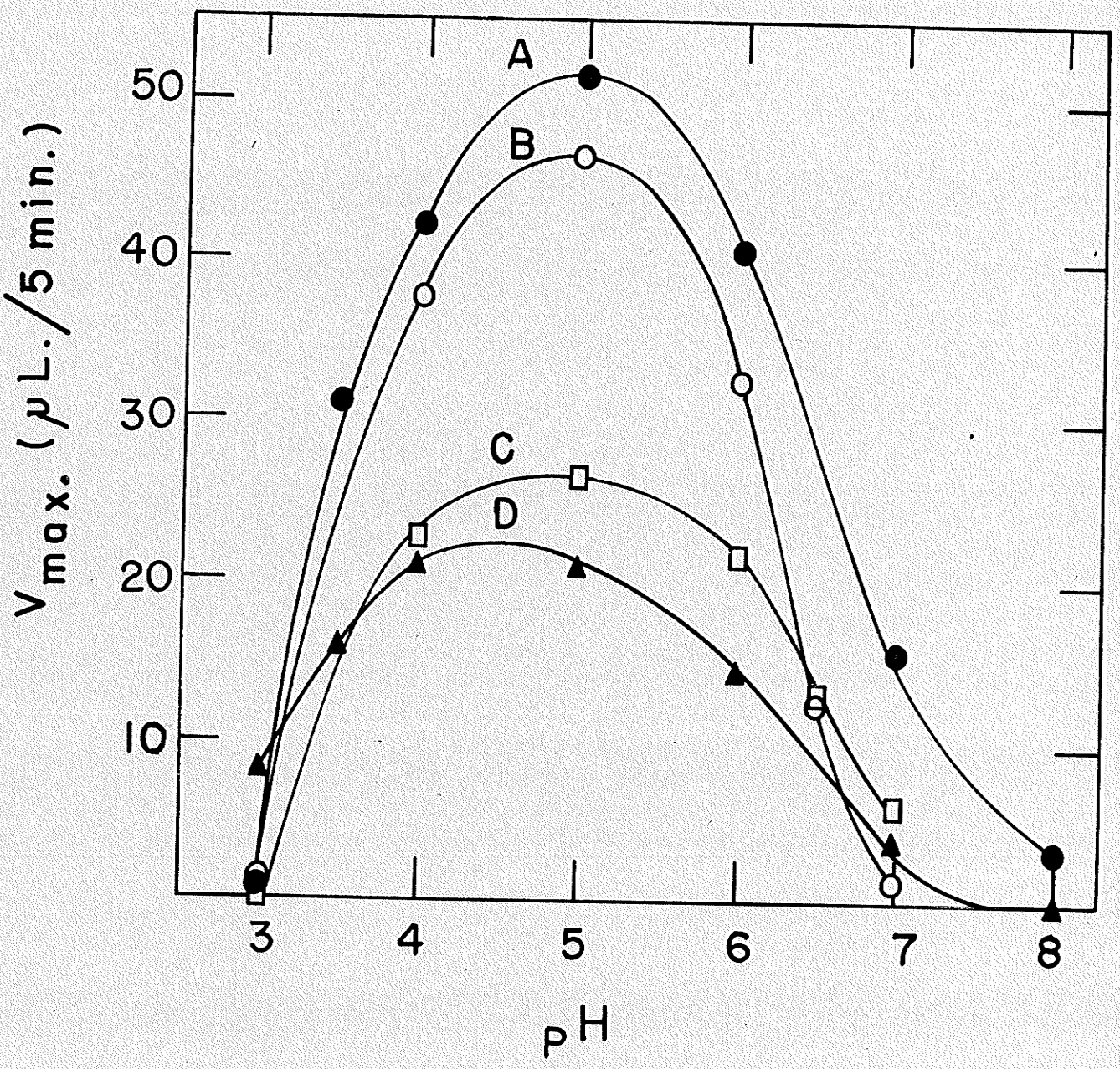
Hydrogen ions are not involved in the overall reaction of normal peroxidation (Chance, 1951) i.e.:

$$ROOH + AH_2 \longrightarrow ROH + H_2O + A$$

Hence the reaction velocity is constant over a wide pH range. This was not true of IAA oxidation by wheat leaf extracts. In the presence of resorcinol, DCP, maleic hydrazide or the natural factor the rate of oxygen uptake was greatest about pH 5.0 and was markedly inhibited above pH 6.0 (Fig. 9). At pH 4.0 and 6.0, where the rates of oxidation were suboptimum, the reactions nevertheless proceeded to molar equivalence without showing signs of enzyme inactivation. The rate of carbon dioxide release, measured in one experiment using resorcinol, was somewhat greater than the rate of oxygen uptake at pH 3.0, 4.0 and 5.0, but equal to it at higher pH values. The reaction product at each pH and with each cofactor was a soluble yellow substance. Precipitate formation, so marked in the non-enzymic system below pH 5.0 (Maclachlan and Waygood, 1956) was never observed.

Figure 9. Effect of pH.

Enzyme-buffer mixtures prepared as follows: 1.25 ml. wheat leaf enzyme plus 3.0 ml. 0.1 M phosphate adjusted to required pH and made to vol. 5.0 ml. Reaction systems contained: 2.0 ml. enzyme-buffer, 10^{-3}M $MnCl_2$; cofactor; 2.22 x 10^{-3}M IAA; final vol. 3.0 ml. Cofactors as follows: A, 5 x 10^{-4}M dichlorophenol; B, 5 x 10^{-4}M. resorcinol; C, 10^{-2}M maleic hydrazide; D, 0.15 ml. natural factor.


A
B
C
D
50
40
30
20
10
3
4
5
6
7
8
pH
Vmax. (μL./5 min.)

The pH optimum for IAA oxidation catalysed by catalase-resorcinol systems also lay close to pH 5.0 and the rate of oxidation decreased sharply below pH 4.0 and above pH 6.0. However quantitatively the pH optimum was more distinct for the catalase than for the wheat leaf system i.e.: the ratios of activities at pH 5.0/pH 6.0 were 3.3 and 1.4 respectively.

Inactivity of the systems above neutrality can not be due to enzyme inactivation, but more probably to the instability of an essential intermediate e.g. manganic ions or free radicals. It is also possible that insoluble manganese carbonate is formed at higher pH values (Hochster and Quastel, 1952) thus lowering the effective manganese concentration below optimum. In addition, the oxidation of manganese by cofactor radicals (RO·) involves the utilization of hydrogen ions i.e.:

$$Mn^{2+} + H^{+} + RO\cdot \longrightarrow Mn^{3+} + ROH$$

Since the reverse reaction can readily be demonstrated (see p.40) a high pH would be expected to retard manganese oxidation and hence IAA destruction by a mass action effect.

4. Temperature:

The rate of IAA oxidation by wheat leaf extracts was measured at four temperatures: 10°, 20°, 29.5° and $42.5^{\circ}C$, using either DCP, maleic hydrazide, the natural factor or resorcinol as cofactor, the latter in both air and oxygen atmospheres. The initial velocities were represented by the

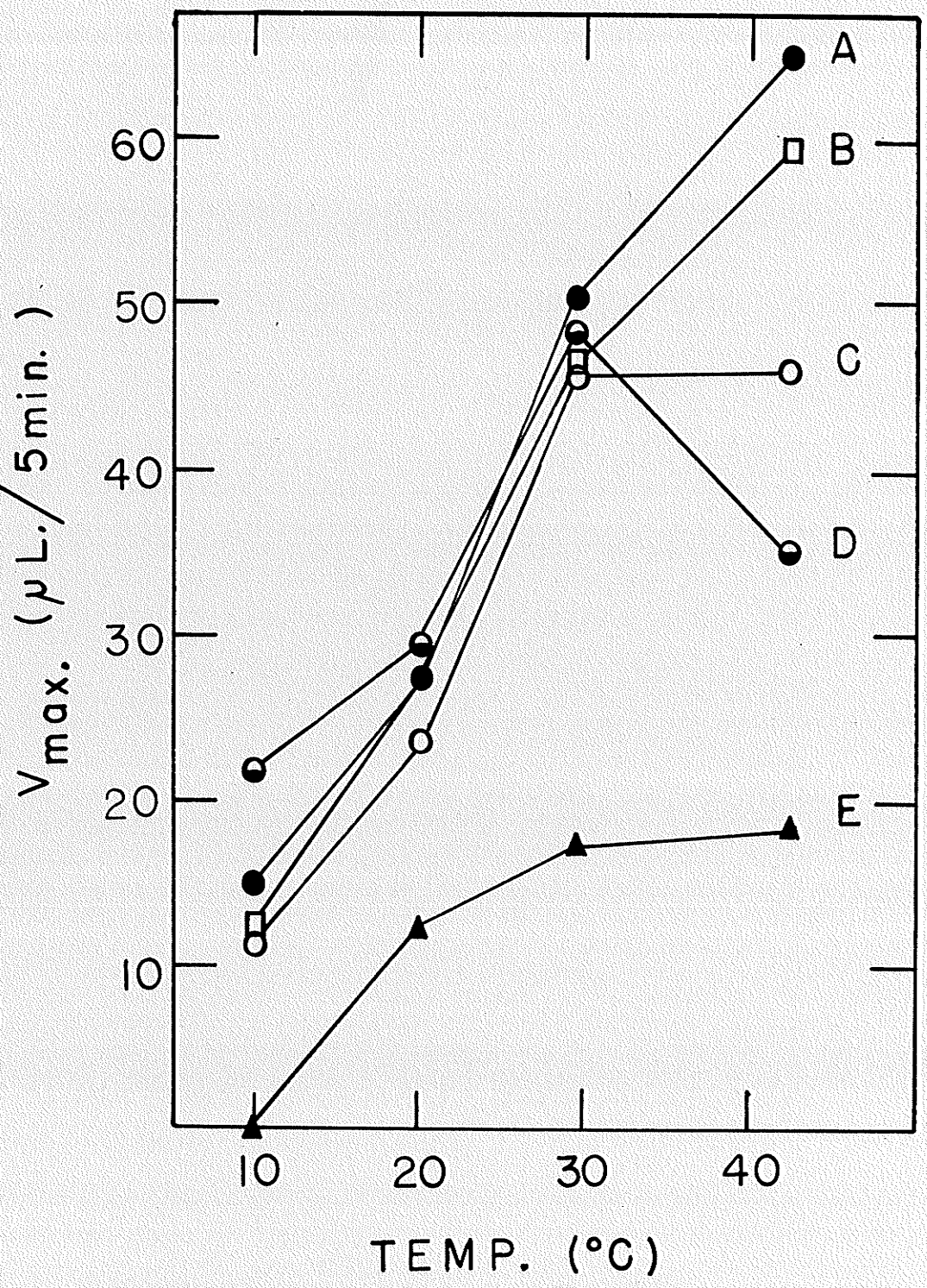
maximum increment of any 5 min. period rather than the net increment from zero time since with each cofactor the lag phase of at least 10 min. which occurred at the lowest temperature was almost overcome at the highest. Duplicate experiments were performed and the results, which agreed closely, were averaged to give the temperature/activity curves shown in Figure 10.

The rate of IAA oxidation increased with increasing temperature in the range 10°-29.5°, but only the systems containing DCP and maleic hydrazide (Figure 10:A,B) were significantly stimulated by a further temperature increase. At 42.5°C the progress curves of systems containing the natural factor or resorcinol in air were almost coincident with the corresponding curves at 29.5°C as indicated in Figure 10:C,E. When the oxygen tension was increased in the presence of resorcinol the rate of IAA oxidation (Fig. 10:C,D) was increased at 10°C , but was decreased at 42.5°C.

Thermal inactivation evidently began at temperatures above 30°C, particularly in the presence of resorcinol. However, according to the benzidine test, inactivation was not due to enzyme destruction. Instead, IAA autoxidation appeared to be interrupted at higher temperatures by side reactions e.g. cofactor polymerization. The brown color of oxidized resorcinol formed most intensely at high temperatures and in an oxygen atmosphere, prior to the addition of IAA.

Figure 10. Effect of temperature.

Systems standard with the following cofactors: A, 5×10^{-4}M dichlorophenol; B, 10^{-2}M maleic hydrazide; C, 5×10^{-4}M resorcinol in air; D, 5×10^{-4}M resorcinol in oxygen; E, 0.15 ml. natural factor.


A
B
C
D
E
60
50
40
30
20
10
10
20
30
40
$V_{max.}$ (μL./5min.)
TEMP. (°C)

Hence by promoting condensation of phenolic intermediates, oxygen and high temperatures diverted an essential intermediate from IAA oxidation i.e.: promoted termination reactions.

From the ratio of the velocities at different temperatures the energy of activation (Table II) was estimated by employing the Arrhenius equation:

$$E_a = \frac{T_1 T_2}{0.219(T_2 - T_1)} \log \frac{V_2}{V_1}$$

With the probability that IAA oxidation is a chain reaction, it must be taken into consideration that temperature would influence initiation and termination as well as propagation reactions. The fact that the lag phase was variable indicated that initiation reactions were temperature-controlled. Indeed, at $10^{\circ}C$ IAA was stable for a period of at least one hour in the presence of the natural factor (Figure 10:E) which, as used in these experiments, contained inhibitors of initiation as well as activators of propagation (see p.32). Accordingly, the activation energy for the natural factor-catalysed system would be infinitely large if calculated from the interval 10°-$20^{\circ}C$. On the other hand, with termination reactions prominent at higher temperatures, E_a values calculated from the interval 29.5°-$42.5^{\circ}C$ were low and even negative with resorcinol in oxygen.

Table II Arrhenius Activation Energy of Indoleacetic Acid Oxidation with Various Cofactors.

Temperature Interval	Activation Energy (cals./mole) in the presence of:				
	DCP	maleic hydrazide	resorcinol in air	resorcinol in oxygen	natural factor
10°-20° C	10,600	12,800	11,800	5,300	
20°-29.5°C	7,700	7,100	8,200	6,800	6,000

The activation energy of the system as a chain sequence i.e.: propagation reactions, should be most closely approximated by comparing the velocities at intermediate temperatures. From 20°-29.5°C the values for E_a in the presence of all cofactors were close and averaged 7160 cals/mole (Table II). This corresponds to a Q_{10} from 20°-30°C of about 1.5. It is interesting that the lowest E_a value (6000 cals/mole) in this temperature interval was obtained from the system containing the natural factor which on this basis may be considered the most efficient mediator. From 10°-20°C the E_a values were higher (averaging ca. 11,700 cals./mole) in the presence of DCP, maleic hydrazide and resorcinol in air, but lower (5,300 cals./mole) with resorcinol in oxygen. The high values probably reflect difficulty in overcoming natural inhibitors of the reaction that are destroyed by increasing oxygen tension thus resulting in a low E_a value.

5. Cofactor Concentration:

The oxidation of IAA catalysed by dialysed wheat leaf extracts and manganese was stimulated by the addition of certain organic cofactors e.g. resorcinol and monohydric phenols, however there was invariably some endogenous activity in the absence of a cofactor (Fig. 11, Table 1). This was also true of ammonium sulphate precipitates of wheat leaf extracts and horseradish peroxidase preparations but did not apply to catalase (Table 1). The endogenous activity could be decreased

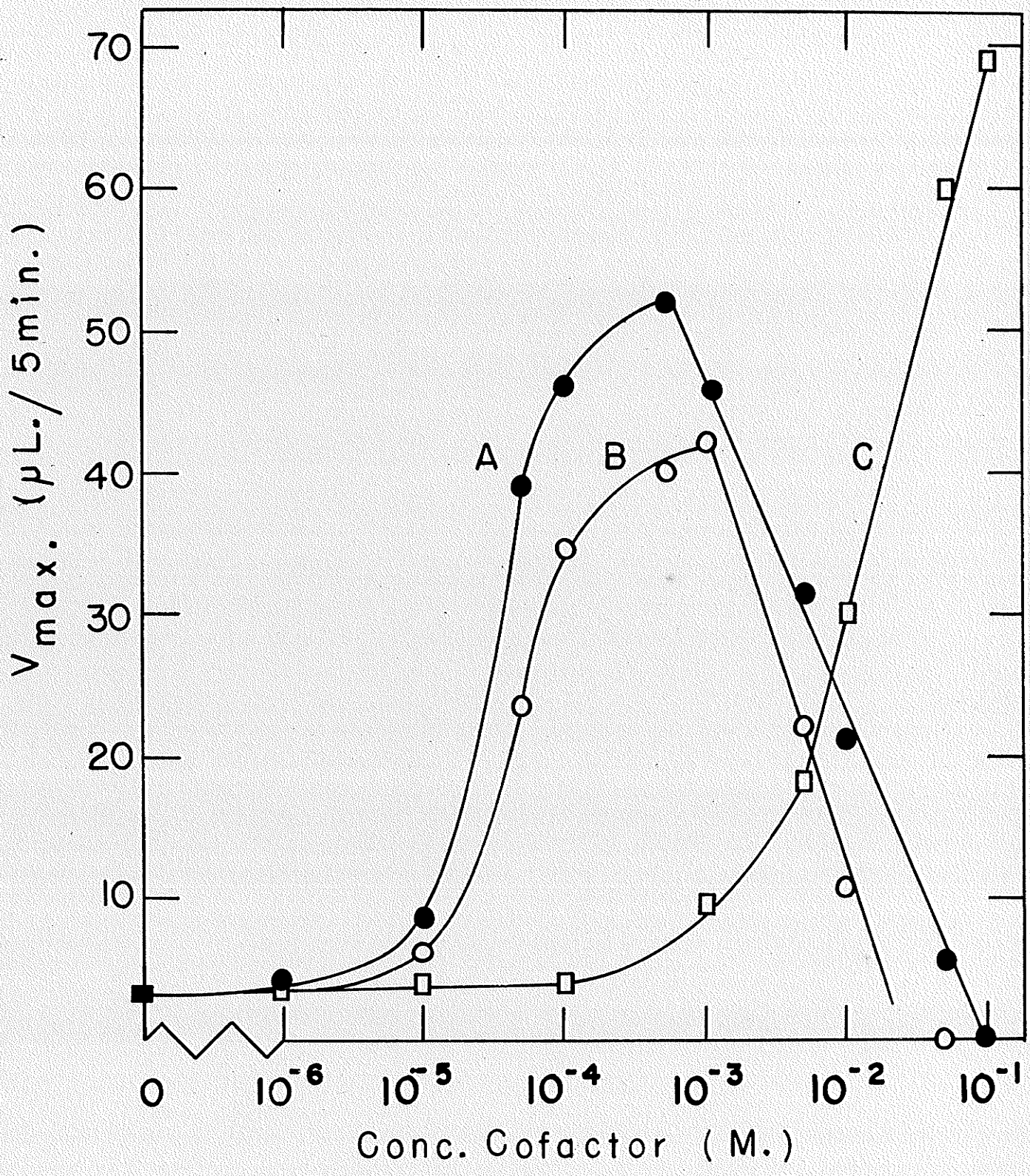
by extended dialysis, but peroxidase preparations which were completely inactive in the absence of an added cofactor were never obtained even after washing the protein with acetone followed by dialysis. It was concluded that peroxidase has a marked affinity for naturally-occurring substances which act as redox catalysts in the oxidation of manganese. Incomplete removal of such substances probably accounts for the IAA "oxidase" activity reported in extracts from various plant sources to which no cofactor was added.

The cofactor concentration/activity curves for resorcinol and DCP (Fig. 11:A,B) showed optima at 10^{-3}M and 5×10^{-4}M respectively in the presence of 10^{-3}M manganese. Above these concentrations the activity of the systems fell rapidly to zero. In contrast, higher concentration of maleic hydrazide i.e.: from 10^{-3}M to its maximum solubility (ca. 10^{-1}M), were not inhibitory, but continued to promote IAA oxidation (Fig. 1:C). Similar effects were observed in catalase systems except that in the presence of 10^{-3}M manganese the optimum resorcinol concentration was 10^{-4}M (See Table III). With horseradish peroxidase, as with the wheat leaf enzyme system, the resorcinol optimum was close to 5×10^{-4}M (see Table III), thus in this respect the catalase system differed quantitatively from both peroxidase systems.

It has been argued (Waygood et. al, 1956) that for

Figure 11. Effect of cofactor concentration.

Systems standard with the following cofactors: A, dichlorophenol; B, resorcinol; C, maleic hydrazide.


70
60
50
40
30
20
10
$V_{max.}$ (μL./5min.)
A
B
C
0
10^{-6}
10^{-5}
10^{-4}
10^{-3}
10^{-2}
10^{-1}
Conc. Cofactor (M.)

a substance to act as a cofactor it must be a substrate for peroxidase (or catalase) i.e.: reaction A, vide infra, and act as a redox catalyst for the oxidation of manganese i.e.: reaction B. The intermediate which enters into a peroxidatic oxidation with the cofactor (ROH) is considered to be the oxidized skatole radical ($SO_2\cdot$) and the resulting aryl radical intermediate (RO·) is then capable of oxidizing manganese.

Reaction A. $SO_2\cdot + ROH \xrightarrow[\text{or Catalase}]{\text{Peroxidase}} SO_2H + RO\cdot$

Reaction B. $RO\cdot + H^+ + Mn^{2+} \longrightarrow ROH + Mn^{3+}$

The reversibility of reaction B may be demonstrated by incubating resorcinol and DCP (ROH) with manganic ions in complex with versene and observing the rate of decolorization of manganiversene. Resorcinol forms a brown and DCP a white oxidation product both of which presumably result from condensation reactions i.e.: $2RO\cdot \longrightarrow$ products.

The abrupt decline shown in the concentration/activity curves for DCP and resorcinol systems (Fig. 11:A,B) resembles the fall-off in activity of most enzymic systems at the extremes of pH or temperature and which, in these cases, is due to enzyme inactivation. However, at high phenol concentrations there was little or no evidenceof enzyme inactivation although the progress curves for oxygen uptake attained lower than the theoretical equilibria. At equilibrium, benzidine tests were still positive and, no further IAA oxidation occurred if enzyme

were added (compare the reverse results at low phenol concentrations, Figure5.) Evidently a premature decline in reaction velocity can not be takenas necessarily diagnostic of enzymeinactivation.

Accordingly, inhibition of IAA oxidation by excess resorcinol and DCP may be explained by a mass action effect in reaction B. An excess of phenolic cofactor would compete with IAA for manganic ions. Thus, the equilibrium constant of the redox system: phenol/manganese controls the rate of the entire autoxidation sequence at supraoptimum levels of phenol, whereas the phenol/enzyme ratio controls at suboptimum levels.

On the other hand if manganic ions were not reduced by the cofactor i.e.: if reaction B were not reversible, then inhibition at high concentrations should not occur. Maleic hydrazide provides an example of this type of cofactor and its reaction with manganiversene has never been detected visibly or spectrophotometrically. Accordingly, maleic hydrazide does not compete with IAA for manganic ions and therefore in increasing concentrations continues to accelerate IAA oxidation. It is suggested that if a particular cofactor reacts with manganic ions it will exhibit an optimum concentration in its ability to promote IAA oxidation. From a brief survey of known Kenten-Mann cofactors this would apply to phenol, o-cresol, p-phenylenediamine, and p-chlorophenol, all of which decolorize manganiversene.

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The overall rate of IAA oxidation may be determined either by the equilibrium established in reaction B or by the rate of enzymic peroxidation (reaction A). For example, maleic hydrazide must be difficult to oxidize peroxidatically (reaction A) since considerably higher concentrations (100 fold)are required to produce an activity comparable to that of either resorcinol or DCP. When equimolar with resorcinol, DCP was a more efficient cofactor at all concentrations. Accordingly, either DCP was more efficiently oxidized by peroxidase than resorcinol (reaction A) or it reduced manganic ions more slowly (reaction B), or both factors contributed to the efficiency of DCP. Qualitative experiments with manganiversene showed that DCP reduced manganic ions more slowly than did resorcinol. Hence, in net effect the radical of DCP probably provided manganic ions to IAA at a faster rate than did the semiquinol of resorcinol because of a higher equilibrium constant in reaction B.

On the premise that an intermediate product of IAA degradation oxidizes resorcinol it would be expected that an increase in the concentration of IAA would increase the ratio oxidized/reduced resorcinol and thereby overcome inhibition by excess of the reduced form i.e.: due to reversal of reaction B. Thus the oxidation of 2.2×10^{-3}M IAA was completely inhibited by the presence of 5×10^{-2}M resorcinol(Fig. 11:B), but when the initial concentration of IAA was increased 12-fold to 2.67

x 10^{-2}M, oxygen uptake proceeded for at least one hour at an average rate of 27 µl/5minutes. Therefore, the system was potentially active in the presence of 5 x 10^{-2}M resorcinol, but its activity was dependent on the concentration ratio IAA/resorcinol. Accordingly, it might be more accurate to describe the optimum concentrations of resorcinol and DCP as relative to the IAA concentration i.e.: IAA/resorcinol = 2.2, IAA/DCP = 4.4.

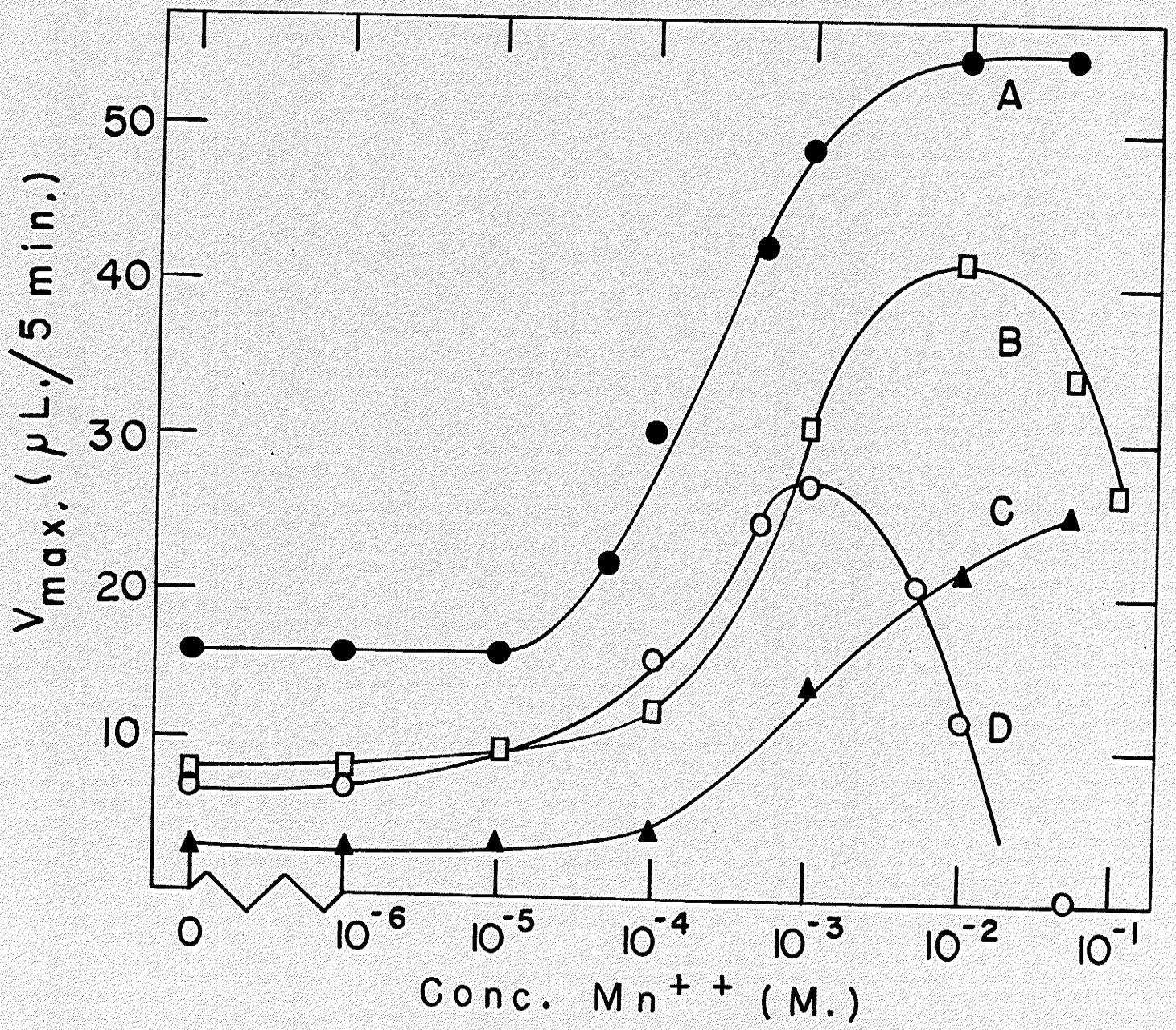
6. Manganese Concentration

The effect of varying the concentration of manganese from 10^{-6}M to 10^{-1}M on the rate of oxidation of IAA by wheat leaf extracts is shown in Figurel2. Succinate buffer was used throughout in order to avoid precipitation of insoluble manganese salts and consequent alteration in pH. The velocities at concentrations of manganese above 10^{-4}M are averages of at least two experiments.

In the absence of added manganese, oxidation of IAA proceeded slowly in spite of extensive dialysis of the enzyme preparation against water to remove endogenous manganese. The actual rate in the absence of manganese depended on the cofactor used. Of those tested DCP was the most effective yielding a maximum initial velocity of 16 µl O_2/5 min. (Fig. 12:A). After further dialysis of the enzyme against 2.5 x 10^{-4}M versene for 24 hr. the rate was decreased to 3.5 µl. O_2/5 min. Further

Figure 12. Effect of manganese concentration.

Systems standard with 5×10^{-2}M succinate buffer, pH 6.0, and the following cofactors: A, 5×10^{-4}M dichlorophenol; B, 10^{-2}M maleic hydrazide; C, 0.15 ml. natural factor; D, 5×10^{-4}M resorcinol.


$V_{max.}$ (μL./5 min.)
50
40
30
20
10
A
B
C
D
0
10^{-6}
10^{-5}
10^{-4}
10^{-3}
10^{-2}
10^{-1}
Conc. Mn^{++} (M.)

dialysis against water instead of dilute versene did not diminish the endogenous rate. This may be taken as evidence for the ability of the protein of these preparations to bind manganese and also the special properties of DCP as a mobilizing or coordinating agent of endogenous manganese.

In general, increasing the manganese concentration did not prolong the lag phase, but had either of two effects on the rate of oxygen uptake depending on the cofactor used. In the presence of the natural factor or DCP (Fig. 12: A,C) IAA was oxidized most rapidly at the highest concentration of manganese (10^{-1}M) although with DCP no further stimulation occurred beyond 10^{-2}M manganese. In contrast, with resorcinol and maleic hydrazide, distinct optimum manganese concentrations were found at 10^{-3}M and 10^{-2}M respectively (Fig. 12: B,D). With the catalase system the optimum manganese concentration was 10^{-2}M in the presence of 5×10^{-4}M resorcinol (Table III).

Inhibition by excess manganese does not involve an inactivation of the enzyme nor an interference in the sequence of reaction undergone by IAA since neither phenomenon takes place in the presence of DCP or the natural factor (Fig. 12). Furthermore, increasing the concentration of IAA twelve-fold failed to overcome manganese inhibition as it overcame inhibition by excessive resorcinol. Therefore it does not appear that manganous ions are protecting IAA by, for example,

displacing manganic ions from complex with IAA. Addition of cofactors also had no effect on manganese-inhibited IAA oxidation (see later Fig. 14) although this would not be expected from any mass action effect in reaction B (p.40.).

The fact that the optimum manganese concentrations (10^{-3}M and 10^{-2}M) were close to the concentrations of resorcinol (5×10^{-4}M) and maleic hydrazide (10^{-2}M) appeared to be coincidental. The optima remained unchanged when experiments were performed using less cofactor i.e.: 10^{-4}M resorcinol and 5×10^{-3}M maleic hydrazide (Table III). Accordingly, excess manganese cannot inhibit by reversing, but rather by interrupting an essential reaction e.g.: an oxidation, involving the organic cofactors. It is noteworthy that excess manganese also inhibited the oxidation of resorcinol (resorcinol "oxidase") which occurs independently of IAA in the presence of manganese and peroxidase (see later Fig. 14). It therefore appears justifiable to suggest that this occurs during IAA oxidation, but as yet the mechanism of manganese inhibition is not clear.

One further possibility should be noted in that excess manganese may have promoted the polymerization of skatole radical intermediates. This appears to have taken place in the non-enzymic system where less IAA was destroyed as the manganiversene concentration was increased (Fig. 2A).

Table III Rate of Indoleacetic Acid Oxidation. Manganese and Cofactor Optima in Various Enzyme Systems[γ]

Enzyme	Component constant	Component varying	$V_{max.}$/5 min. at molar concentrations of the varying component of:									
			10^{-6}	10^{-5}	5×10^{-5}	10^{-4}	5×10^{-4}	10^{-3}	5×10^{-3}	10^{-2}	5×10^{-2}	10^{-1}
Wheat Leaf	5×10^{-3}M Maleic hydrazide	$MnCl_2$						19.0	28.0	34.0	27.5	
	10^{-4}M Resorcinol	$MnCl_2$			11.5	14.0	27.0	30.0	26.0			
Horseradish peroxidase	10^{-3}M $MnCl_2$	Resorcinol	12.5	37.5		63.5		65.5		15.5		0
Beef liver catalase	10^{-3}M $MnCl_2$	Resorcinol	0.5	14.5		24.0		17.0		11.5		0
	5×10^{-4}M Resorcinol	$MnCl_2$				3.0		21.5		40.0		29.0

γConcentrations of cofactors and manganese as indicated, other components as in Table I.

Only a small fraction of intermediates polymerized in oxygen, but certainly less than detectable amounts would be sufficient to interrupt the propagation sequence and reduce the rate of IAA oxidation.

7. Initiation Reactions:

The lag or induction period which occurs in all progress curves for IAA decarboxylation and oxidation has been considered to represent the time necessary for the system to build up a threshold concentration of manganic ions (Waygood et.al., 1956). After induction the reaction exhibits autocatalytic characteristics which clearly indicates that the products of IAA breakdown contribute to initiation reactions. However, to account for the initiation of the reaction it is necessary to postulate that oxidizing power arose in the extracts prior to and independently of the process of IAA breakdown. Presumably this oxidizing power was effective in the form of manganic ions generated by reaction of the other system components i.e.: enzyme and cofactor. The independent reactions of resorcinol, enzyme and manganese were therefore investigated.

The ability of wheat leaf extracts to oxidize resorcinol was first noted in experiments performed in an oxygen atmosphere where, prior to adding IAA, the reaction mixtures developed the brown color characteristic of irreversibly

oxidized resorcinol. This was less evident in air, but final reaction mixtures always included the same brown substance which was presumably formed by semiquinol condensation. A similar "oxidase" reaction occurred with purified horseradish peroxidase or beef liver catalase and no color was formed in the absence of manganese. The oxidation cannot be due to polyphenol oxidase since this enzyme is not present in wheat leaves.

Oxygen consumption in this system was investigated by adding resorcinol at concentrations of between 10^{-1}M and 10^{-3}M to reaction mixtures containing wheat leaf extracts and 5×10^{-2}M manganese in succinate buffer. After a lag phase of 5-10 minutes duration, oxygen uptake commenced at rates proportional to the concentration of resorcinol (Fig. 13). This system will be referred to as the resorcinol "oxidase" system.

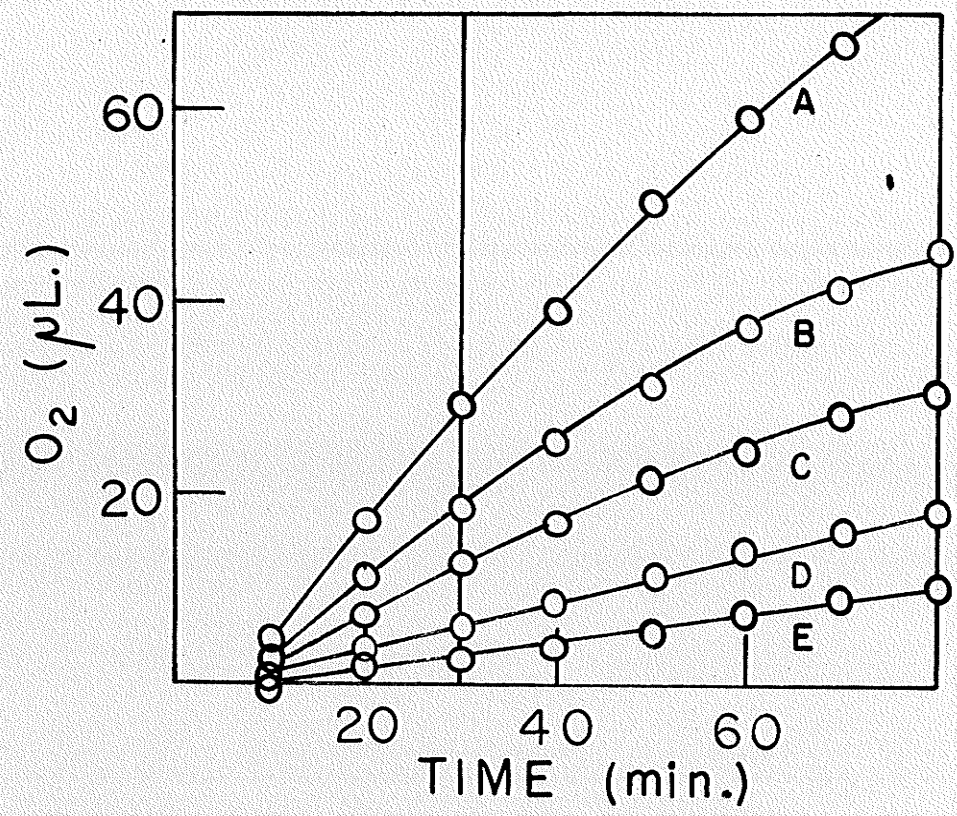
From previous kinetic data one would not expect IAA to be oxidized by these resorcinol "oxidase" systems owing to the high level of manganese (cf. Fig. 12:D). That this was the case is shown in Figure 13 where IAA, added after 30 min., had no effect on the progress of oxygen uptake. After two hours, Salkowski tests confirmed that IAA was not destroyed. Evidently inhibition of IAA oxidation by excess manganese was not overcome by raising the concentration of resorcinol.

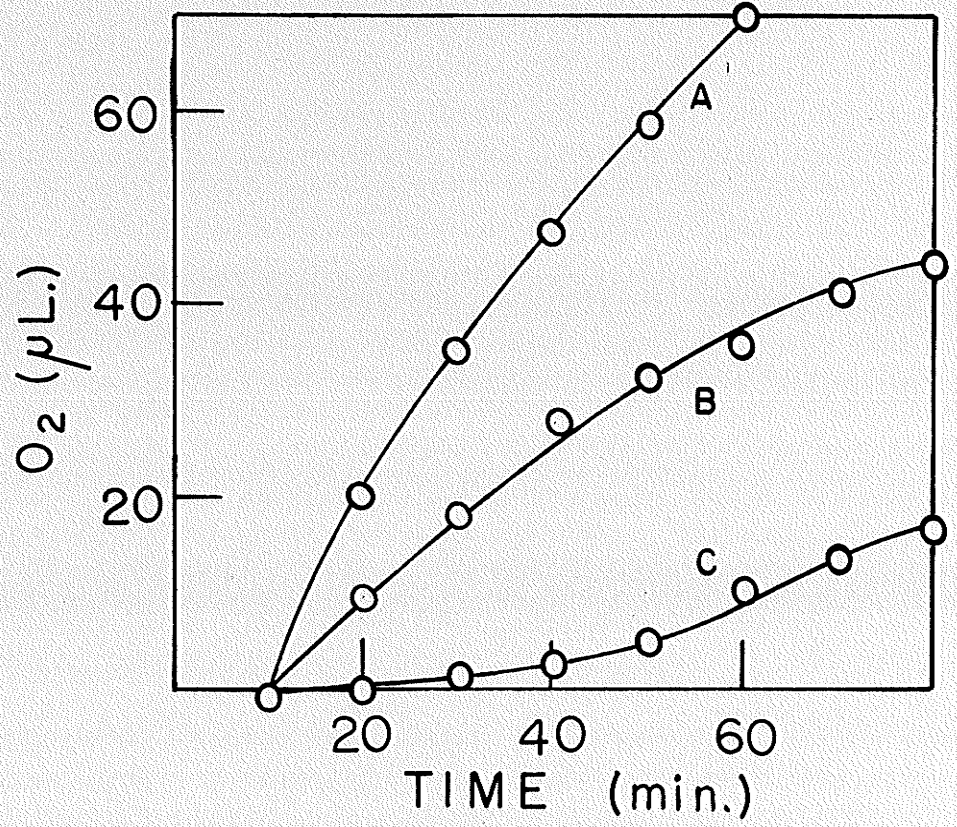
Figure 13. Effect of resorcinol concentration on its rate of oxidation by manganese-peroxidase systems.

Systems contained: 0.50 ml. wheat leaf enzyme; 5×10^{-2}M $MnCl_2$; 5×10^{-2}M succinate buffer pH 6.0; final vol. 3.0 ml. Resorcinol molarity as follows: A, 10^{-1}; B, 5×10^{-2}; C, 10^{-2}; D, 5×10^{-3}; E, 10^{-3}. 2.22×10^{-3}M IAA added to all systems at 30 min.

Figure 14. Effect of manganese concentration on resorcinol oxidation.

Systems contained 0.5 ml. wheat leaf enzyme; 5×10^{-2}M resorcinol; 5×10^{-2}M succinate buffer pH 6.0; final vol. 3.0 ml. Manganese molarity as follows: A, 5×10^{-2}; B, 10^{-1}; C, 10^{-2}.


60
40
20
O_2 (μL.)
A
B
C
D
E
20
40
60
TIME (min.)


60
40
20
O_2 (μL.)
A
B
C
20
40
60
TIME (min.)

The effect of the manganese concentration on the resorcinol "oxidase" system is shown in Figure 14. Manganese was most effective in promoting oxygen uptake when equimolar (5×10^{-2}M) with resorcinol (Fig. 14:A). At higher (10^{-1}M) or lower (10^{-3}M) concentrations of manganese (Fig. 14: B, C respectively) the system was considerably less active. In separate experiments it was again found that IAA was not oxidized at these cofactor concentrations.

The rate of oxygen consumption by resorcinol was greatly enhanced and the lag phase was decreased by replacing air in the reaction vessels with oxygen. The lag phase in air or oxygen was abolished by adding either riboflavin phosphate, hydrogen peroxide or manganiversene. Furthermore citrate and pyrophosphate completely inhibited resorcinol oxidation, all of which suggests that manganic ions were involved. Further studies on the system in an oxygen atmosphere have established that the optimum pH lay between 5.0 and 6.0 and that the oxygen consumed at equilibrium exceeded one mole per mole of resorcinol. The reaction appeared to be complex and to form a mixture of products in various states of oxidation.

Catalase or peroxidase, with manganese, were the only apparent requirements of the resorcinol "oxidase" system, but it is interesting to note that oxygen was consumed at a

very slow rate in the presence of riboflavin or its phosphate and manganese, but in the absence of enzyme. This non-enzymic oxidation required light unlike the resorcinol "oxidase" system.

Certain aspects of these systems resemble the system described by Andreae (1955a) which forms mangani-pyrophosphate from solutions containing catalase, riboflavin, a monohydric phenol or resorcinol, manganous ions and pyrophosphate. However, Andreae's system required light as well as each cofactor for maximum manganese oxidation. Light had no effect on resorcinol "oxidase" and riboflavin did not appear to be necessary. Nevertheless it is possible that both systems developed oxidizing power by similar mechanisms. Whether oxidized resorcinol or manganic ions were the end product appeared to depend only on the presence of an acceptor for manganic ions. In Andreae's system this was pyrophosphate or citrate. In the resorcinol "oxidase" system it is suggested that little irreversible resorcinol oxidation would occur and manganic ions would be reduced if IAA were added, provided the concentration of resorcinol were not high enough to successfully compete with IAA and also if manganous ions were not in excess. In the standard system for IAA oxidation these conditions are fulfilled. The relative concentrations of resorcinol and manganese (5×10^{-4}M:10^{-3}M) are such that some manganese oxidation would be expected

(cf. Figs. 13, 14).

Also since IAA is present at a high concentration relative to resorcinol (2.22×10^{-3}M: 5×10^{-4}M) it would tend to react preferentially with manganic ions (cf. Fig. 13). Thus the autocatalytic production of manganic ions via the resorcinol "oxidase" system, even if operating to an almost negligible extent in air, could be responsible for initiating IAA oxidation.

The apparent ease with which the semiquinol of resorcinol forms stable condensation products probably accounts for the ability of this phenol to consume oxygen in the above system. Other cofactors e.g.: phenol, o-cresol, DCP, maleic hydrazide, resulted in much less or no oxygen uptake under the same conditions. These substances have not been studied further but are known to produce manganic ions without the necessity of oxygen uptake (Andreae, 1955a).

8. Reaction Sequence

The enzymic oxidation of IAA has been investigated on the assumption that IAA decarboxylates and consumes oxygen via the same reaction sequence as occurs non-enzymically in the presence of manganiversene. The product and stoichiometry of the two reactions are the same but one significant difference has been found between the reaction conditions necessary to

achieve equivalence. As shown in the IAA concentration/activity curve (Fig. 8) and the temperature/activity curve (Fig. 10), increasing oxygen tension did not increase the Michaelis constant of the enzymic system nor the rate and amount of oxygen uptake at 29.5°C. In contrast, a high oxygen tension was essential in the non-enzymic system for complete oxidation (Fig. 2;A,B) even though complete decarboxylation may have taken place.

In the presence of manganiversene the decarboxylation product (skatole radical: S•) polymerized (reaction 1) more readily than it reacted with oxygen (reaction 2)i.e.:

1. $\begin{cases} S\bullet + S\bullet \longrightarrow \text{products} \\ S\bullet + IAA \longrightarrow \text{products} \\ S\bullet + MnV^{-} \longrightarrow \text{products} \end{cases}$

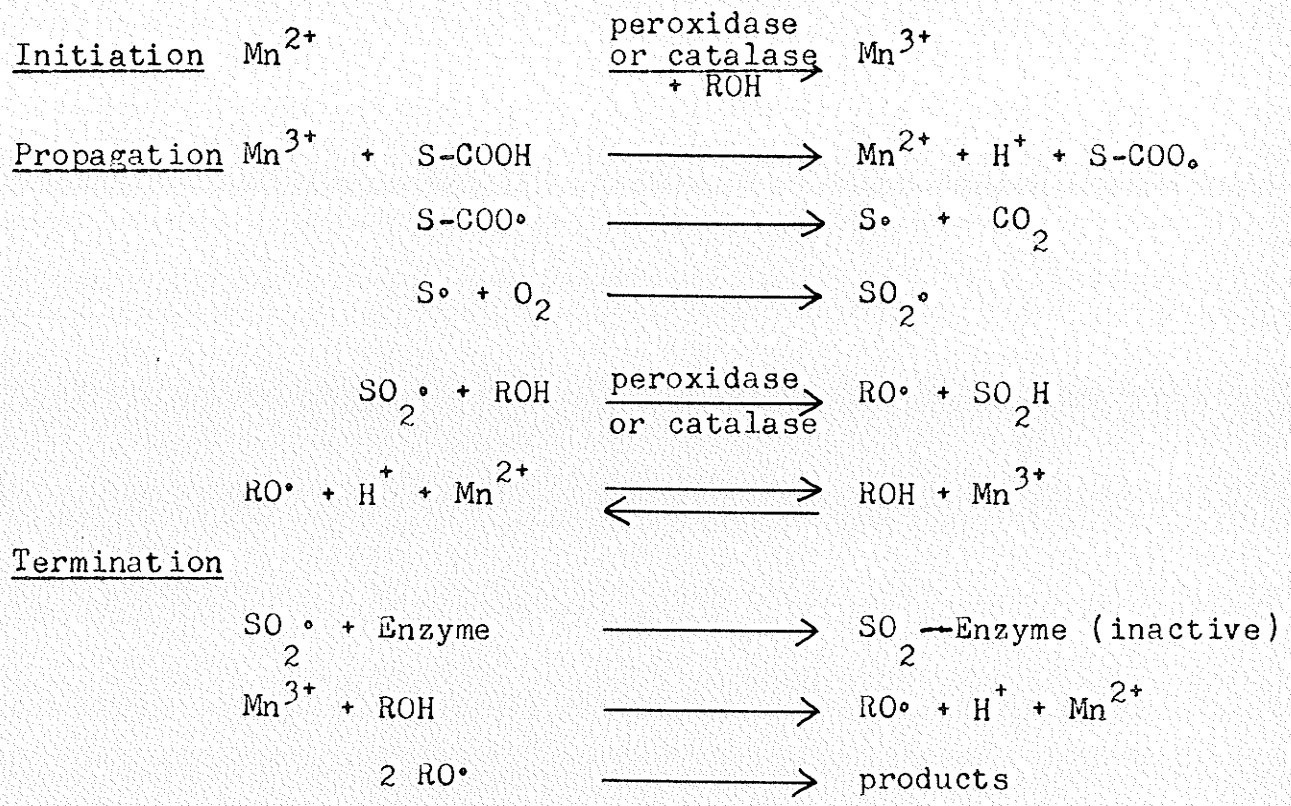
2. $S\bullet + O_2 \longrightarrow SO_2\bullet$

Polymerization has never been observed in the enzymic system. However, this can not be due to a slow rate of formation of the skatole radical (S•) since the most rapid rate of IAA decarboxylation observed non-enzymically (69 μl.O_2/5min.) was equalled by the rates of some enzymic systems e.g.: Fig. 11. Therefore, whether the skatole radical polymerizes or reacts with oxygen must depend on its rate of disposal which in turn will depend on the disposal rate of the oxidized skatole radical. Thus reaction (2) may be considered reversible and to proceed rapidly to the right only if a substance is

present which reacts with $SO_2\cdot$ more rapidly than S· polymerizes.

Evidently formation of the final yellow product from the oxidized skatole radical is more readily accomplished in the enzymic than in the non-enzymic system. The only consitutent of the former which is easily oxidized and not present in the latter is an organic cofactor e.g.: resorcinol. Therefore it is most probable that the oxidized skatole radical undergoes a direct and rapid enzyme-catalysed reaction with the organic cofactor rather than what must be a slower reaction with any substance common to both enzymic and non-enzymic systems. This conclusion renders a reaction between the oxidized skatole radical and water improbable in the enzymic system and may be used as evidence that hydroxyl radicals or hydrogen peroxide are not involved.

These conclusions and those arrived at within the text of the preceeding sections are summarized in the following reaction sequence for IAA autoxidation by a "standard" system i.e.: IAA = S-COOH; skatole radical = S·; skatole peroxide = $SO_2\cdot$ (oxidized skatole radical); product = SO_2H; resorcinol = ROH.



The propagation reactions allow for only one manganic ion produced per molecule IAA destroyed and thus the system would have to be 100% efficient if there were no other source of manganic ions or propagation pathway. Excess manganic ions could be generated by the resorcinol "oxidase" system. It is also possible that a direct reaction occurs between theoxidized skatole radical and IAA as has been observed in the non-enzymic system (see p.27). However, as long as the supply of reduced cofactor is sufficient, this reaction is improbable since SO_2· reacts with the cofactor more rapidly than with any other system component (see above). It is concluded that the system <u>can be</u> close to 100% efficient and

that less than complete oxidation occurs only if termination reactions are promoted by conditions which are not optimum.

(C) Inhibition and Retardation

1. General Characteristics:

The kinetics of IAA breakdown have demonstrated that the reaction sequence follows a cyclic course and hence the speed of the entire autoxidation depends on the efficient operation of each reaction in the chain series.

In order to inhibit any such autoxidation sequence a substance must interrupt the chain of interdependent steps by reacting with an essential intermediate of the system. Inhibition may be expressed in either of two ways: (a) as an extension of the induction period which occurs in all autoxidations, or (b) as a retardation of reaction velocity. As pointed out by Waters and Wickham-Jones (1951) these effects are caused by agents which interfere in the reaction sequence in fundamentally different ways and which may be described as chain-stopping and chain-transferring reagents respectively.

In the presence of a true inhibitor or chain-stopping agent the initiation of the autoxidation is prevented. However, at low inhibitor concentrations the reaction may commence abruptly after a long induction period and attain a rate equal to that of the control in the absence of inhibitor. Such temporary inhibition or "lag-extension" indicates that the inhibitor is altered or destroyed during the induction period to a product that is not inhibitory to the reaction. Inhibitor destruction must occur at

the expense of an essential intermediate of the system, but also may be aided by side reactions e.g. autodestruction. Thus, the system would never operate in the presence of excessive amounts of the inhibitor or if the latter were not destroyed.

On the other hand, in the presence of retarding agents, the reaction may proceed to completion without an induction period, but at a reduced rate. This could occur only if the retarder acted as a chain-transferring agent by substituting a sequence of slow reactions within the rapid sequence of the control. In order to exert its retarding effect continously, a substance must be reformed following reaction with an essential intermediate. Thereby the retarder inserts a "shunt" into the normal reaction sequence which slows down the speed of propagation.

The reaction sequence for IAA breakdown involves several unstable intermediates e.g. $RO\cdot$, Mn^{3+}, $S\cdot$, $SO_2\cdot$, with which many reducing agents would be expected to react and result in chain-stopping or chain-transferring. It should be noted that the destruction of IAA differs from standard autoxidation sequences by being dependent on an enzyme-controlled peroxidation. Consequently, substances that inhibit peroxidase or catalase e.g. cyanide, also inhibit IAA oxidation. Such enzyme poisons should be distinguished from inhibitors and retarders of the autoxidation of IAA and are not considered in the following discussion.

Typical progress curves of oxygen uptake during IAA

oxidation catalysed by extracts from winter-grown wheat leaves exhibited a very short induction period and a subsequent rapid rate of oxidation that gradually decelerated until theoretical molar equivalence was attained (Fig. 15:A). The system was inhibited by substances that either extended the induction period e.g. 10^{-5} M catechol (Fig. 15:B), or retarded the rate of oxidation from the outset e.g. 2.1 x 10^{-4}M riboflavinphosphate (Fig. 15:C) or 1.5 x 10^{-5}M hydroquinone (Fig. 15:D). The retarded system containing hydroquinone attained a low oxygen equilibrium prematurely. Thereby it differed from the system containing riboflavinphosphate which consumed oxygen up to and in some cases beyond the oxygen equivalence of indoleacetic acid. These examples illustrated "lag-extension" and retardation in the classical sense and may be regarded as a basis for considering the oxidation of IAA as a chain autoxidation.

The above experiments were performed using resorcinol as cofactor of the oxidation, but the effects of these inhibitors and retarders were qualitatively the same in the presence of other cofactors (Table IV). The length of the lag phase induced by 10^{-5}M catechol depended on the cofactor used. On the other hand, the degree to which hydroquinone and riboflavinphosphate retarded the oxidation of IAA was independent of the nature of the cofactor. The systems retarded by riboflavinphosphate but not those by hydroquinone were rendered fully active by illumination (Table IV). The effect of light will be discussed more fully in following

Figure 15. Retardation and inhibition of indoleacetic acid oxidation.

Systems standard with: A, no additions; B, 10^{-5}M catechol; C, 2.1×10^{-4}M riboflavinphosphate; D, 1.5×10^{-5}M hydroquinone.

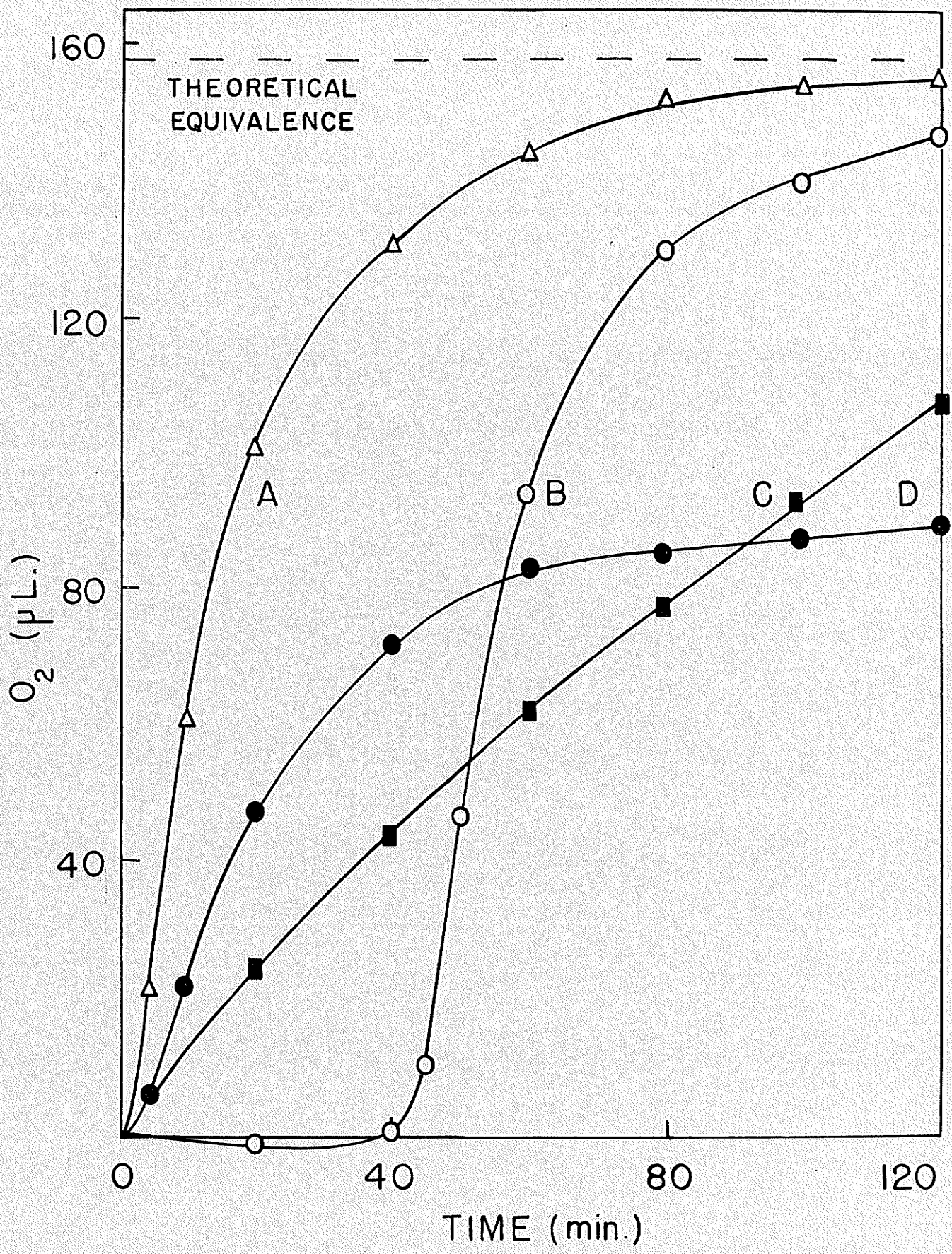

160
120
80
40
THEORETICAL
EQUIVALENCE
A
B
C
D
O_2 (μL.)
0
40
80
120
TIME (min.)

Table IV Inhibition and Retardation of Indoleacetic Acid Oxidation in the Presence of Catechol; Hydroquinone and Riboflavinphosphate[γ]

Cofactor	Induction period in the presence of catechol (min.)	% Inhibition in the presence of:		
		hydroquinone	riboflavinphosphate	
			Dark	Light[σ]
Resorcinol	46.5	55	35	100
Dichlorophenol	24.0	59	28	97
Phenol	26.5	45	31	91
Maleic hydrazide	30.0	53	33	94
Natural factor	41.0	42	40	147

[γ]Concentrations: catechol, 5×10^{-6}M; hydroquinone, 2×10^{-5}M; riboflavinphosphate, 1.1×10^{-4}M; resorcinol, DCP, phenol, 5×10^{-4}M; maleic hydrazide, 10^{-2}M; natural factor, 0.15 mL.

[σ]Blue light, 220 ft. candles.

sections which deal with the mechanism of inhibition and retardation.

All substances which have been found to retard or inhibit IAA oxidation by a "standard" system, do so at concentrations ranging from 10^{-4}M to 10^{-5}M. The following is a list of substances which, at 10^{-3}M, had no detectable inhibitory or retarding effects on the system: 2,4-dichlorophenoxyacetate 2,4,5-trichlorophenoxyacetate, indole, skatole, indolepropionate indolebutyrate, isatin, naphthaleneacetate, naphthoxyacetate, eosin'B', carotene (suspension), cytochrome c, adenosine-mono, and -triphosphate, menadione, uracil, glutathione, cysteine, versene, methylene blue, iodoacetate, potassium iodide, cobalt chloride, mercuric chloride.

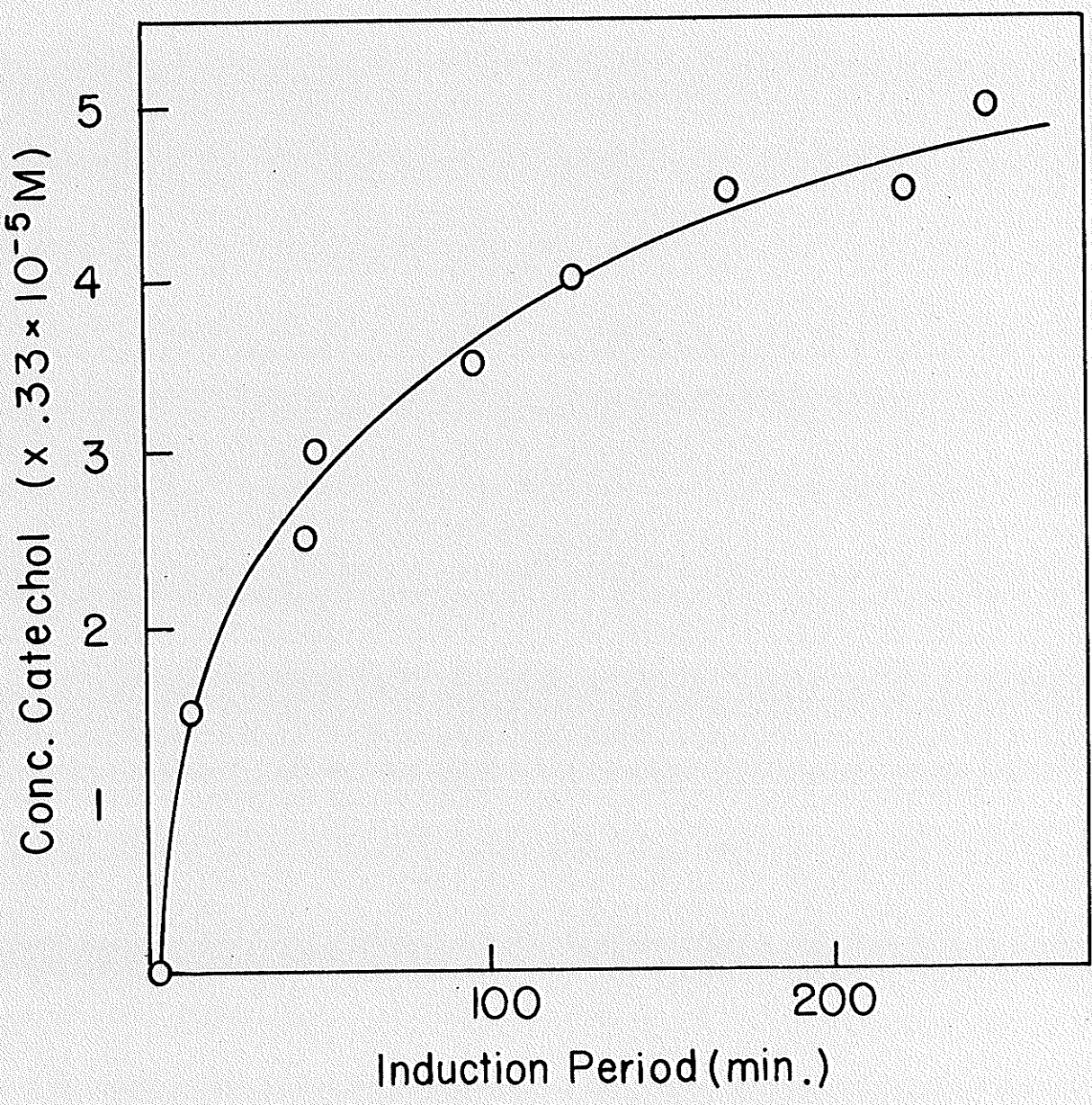
2. Inhibitors:

The relationship between the concentration of catechol in the system and the length of the induction period is shown in Figure 16. The data demonstrate that the oxidation of IAA would never occur above a catechol concentration of 1.9×10^{-5}M. However, this value can be regarded only as approximate since the age of the solutions and the enzyme used was found to influence the length of the lag phase induced by catechol.

A similar inhibition was caused by pyrogallol and the flavonoid pigment rutin, both of which extended the lag phase

Figure 16. Induction period in indoleacetic acid oxidation caused by catechol.

Systems standard, catechol added immediately prior to IAA.


Conc. Catechol (x .33 x 10⁻⁵M)
1
2
3
4
5
100
200
Induction Period (min.)

by 25 min. at concentrations of 1.7×10^{-5}M and 0.5×10^{-5}M respectively. Catechol and pyrogallol were reported previously to inhibit the oxidation of IAA catalysed by horseradish peroxidase (Kenten, 1955). It is noteworthy that to induce an appreciable lag phase, the concentration required of these inhibitors was in the neighbourhood of 10^{-5}M. Therefore it may be significant that commercial phloridzin (Nutritional Biochemicals Co.) and terramycin (Merck and Co.) extended the lag phase by 40 min. and 80 min., but only at relatively high concentrations of 1.7×10^{-3}M and 5.0×10^{-3}M respectively. It is improbable that the systems could overcome this amount of inhibitor and therefore it is suggested that the inhibitory effects were caused by impurities present at concentrations of 1.0 to 0.1% of the respective reagent.

3. Mechanism of Inhibition:

In general terms it may be assumed that the catechol-type inhibitor or chain-stopping agent is destroyed by an intermediate formed prior to or during IAA oxidation. Catechol, pyrogallol and probably rutin are all readily oxidized to quinones and could inhibit the oxidation of IAA by reducing an essential intermediate e.g. a cofactor radical, skatole radical, or manganic ions. Since all these intermediates would be present in higher concentration after the oxidation had commenced it is significant that the addition, during IAA oxidation, of an amount of catechol that would

Figure 17. Effect of natural inhibitors and light on indoleacetic acid oxidation by catalase.

System: as for Table I using catalase and dichlorophenol, IAA added at zero time.

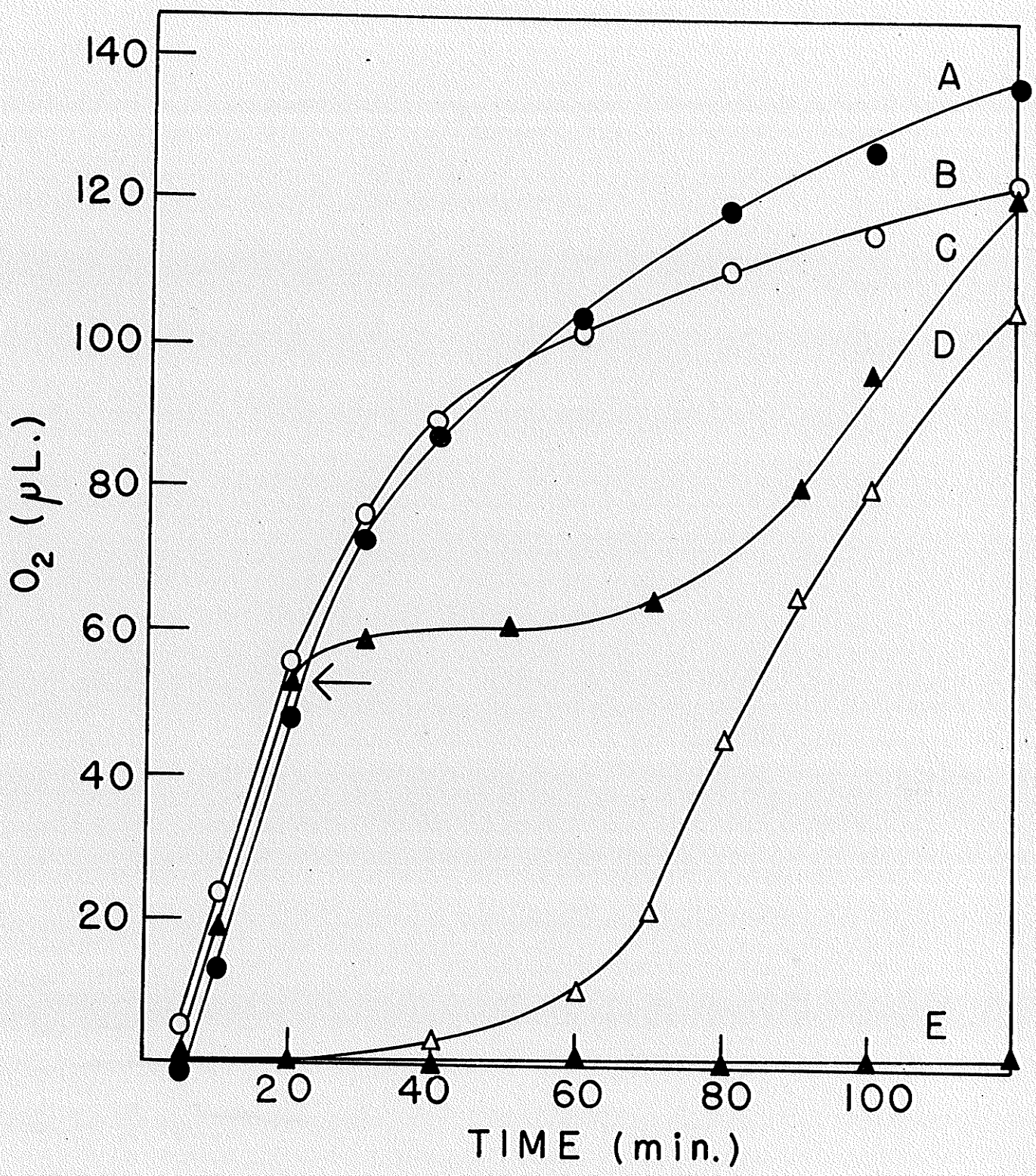
A. control in darkness

B. control in white light (1380 ft. candles)

C. control in darkness, boiled undialysed catalase (10 mgms. dry weight) added at arrow

D. control in light, boiled undialysed catalase added at zero time

E. same as D in darkness


140
120
100
80
60
40
20
O_2 (μL.)
A
B
C
D
E
20
40
60
80
100
TIME (min.)

light-activated intermediate formed from the inhibitor causes an additional production of manganic ions which brings about destruction of the reduced inhibitor. The inhibitor was removed with difficulty from catalase since the control system that had been dialysed for 72 hr. still showed a significant induction period (Fig. 17:A) which was almost overcome by illumination (Fig. 17:B). It is noteworthy that the rate of oxidation in light with or without inhibitor was no greater than the rate in darkness and therefore light cannot be regarded as an activator, but only as an agency overcoming natural inhibition in this case.

The question as to which intermediate is responsible for destroying catechol or the other inhibitors cannot be unequivocally answered on the basis of the present data. It is possible that manganic ions arising during initiation and propagation reactions preferentially oxidise catechol. This is supported by the findings that manganese dioxide, riboflavin or an oxygen atmosphere overcome catechol inhibition (Table V) since these agencies are known to lead to the formation of manganic ions (see Kenten and Mann, 1950; Andreae, 1955a). Preincubation of catechol with manganiversene or even manganous ions also destroys its inhibitory effect (Table V) presumably by causing oxidation or promoting autoxidation of catechol.

On the other hand, there is no reason to exclude the possibility that free radical intermediates are capable of

Table V Effect of Various Treatments on the Catechol-Induced Lag Phase in Indoleacetic Acid Oxidation.

System[γ] plus:	Lag phase (min.)
No additions	43
Illumination	41
Riboflavinphosphate (10^{-4}M)	18.5
$MnCl_2$	6.5
Oxygen atmosphere	5.5
MnO_2	5.0
Manganiversene	3.0

[γ]Standard wheat leaf system containing 10^{-5}M catechol. Riboflavinphosphate, MnO_2 and manganiversene added to catechol plus system 20 min. prior to IAA. $MnCl_2$ pre-incubated with catechol alone 105 min. prior to adding other constituents.

destroying catechol. The fact that the catechol-induced lag period varies in length with the cofactor used (Table IV) suggests that the cofactors may compete at different rates with catechol for the destroying agent. All artificial cofactors and inhibitors (with the exception of rutin, no data available) are known hydrogen donors in peroxidatic reactions. Hence it is possible that the inhibitors compete with the cofactors for the peroxidase substrate i.e. skatole peroxide ($SO_2^{\cdot}$)

Naturally occurring substances that extend the induction period in IAA oxidation have been found in many sources including extracts of beef liver catalase, wheat leaves, uredospores of wheat rust (Waygood and Dangerfield, 1956), pea epicotyls (Tang and Bonner; 1947) and pineapple tissue (Gortner and Kent; 1953). Indeed any crude plant extract would contain substances that are readily oxidised by manganic ions, peroxides or radicals. Unlike catechol some of these e.g. pea epicotyl inhibitor, (Galston and Baker, 1951), are less inhibitory after exposure to light and presumably these are destroyed photochemically. It is possible that such substances exert an effect on IAA oxidation <u>in vivo</u>, but since they are readily destroyed their effect could be only transitory.

(4.) Retarders

Typical of the progress curves of oxygen uptake at varying retarder concentration are those shown in Fig. 18, where riboflavinphosphate was progressively more inhibitory in darkness as its concentration was increased above 10^{-5}M. The progress curves for hydroquinone retardation were similar except that the systems attained a low equilibrium (Fig. 15). This was not due to enzyme or cofactor destruction, but apparently to the decline in the concentration of IAA, since the further addition of IAA at equilibrium caused a resumption in oxygen uptake. Both hydroquinone and its oxidation product p-quinone (para-benzoquinone) retarded the oxidation to the same extent. The coumarin derivative, scopoletin, had effects resembling riboflavinphosphate. Competitive inhibition (retardation) of IAA oxidation by scopoletin was reported previously by Andreae (1952).

The maximum initial rates of IAA oxidation in the presence of each of these retarders are compared as a function of retarder concentration in Fig. 19. Unlike the inhibition induced by chain-stopping agents, that caused by retarders did not appear to depend on the age of reagents, enzyme etc. Irrespective of the absolute rate of the unretarded control, close to 50% retardation occurred at the following concentrations: hydroquinone, 2.0×10^{-5}M; p-quinone, 2.5×10^{-5}M.; riboflavinphosphate,

Figure 18. Progress of indoleacetic acid oxidation in the presence of riboflavinphosphate in darkness.

Systems standard, riboflavinphosphate molarity $x10^{-4}$ as follows: A, 0; B, 0.42; C, 0.625; D, 1.25; E, 2.1; F, 12.5.

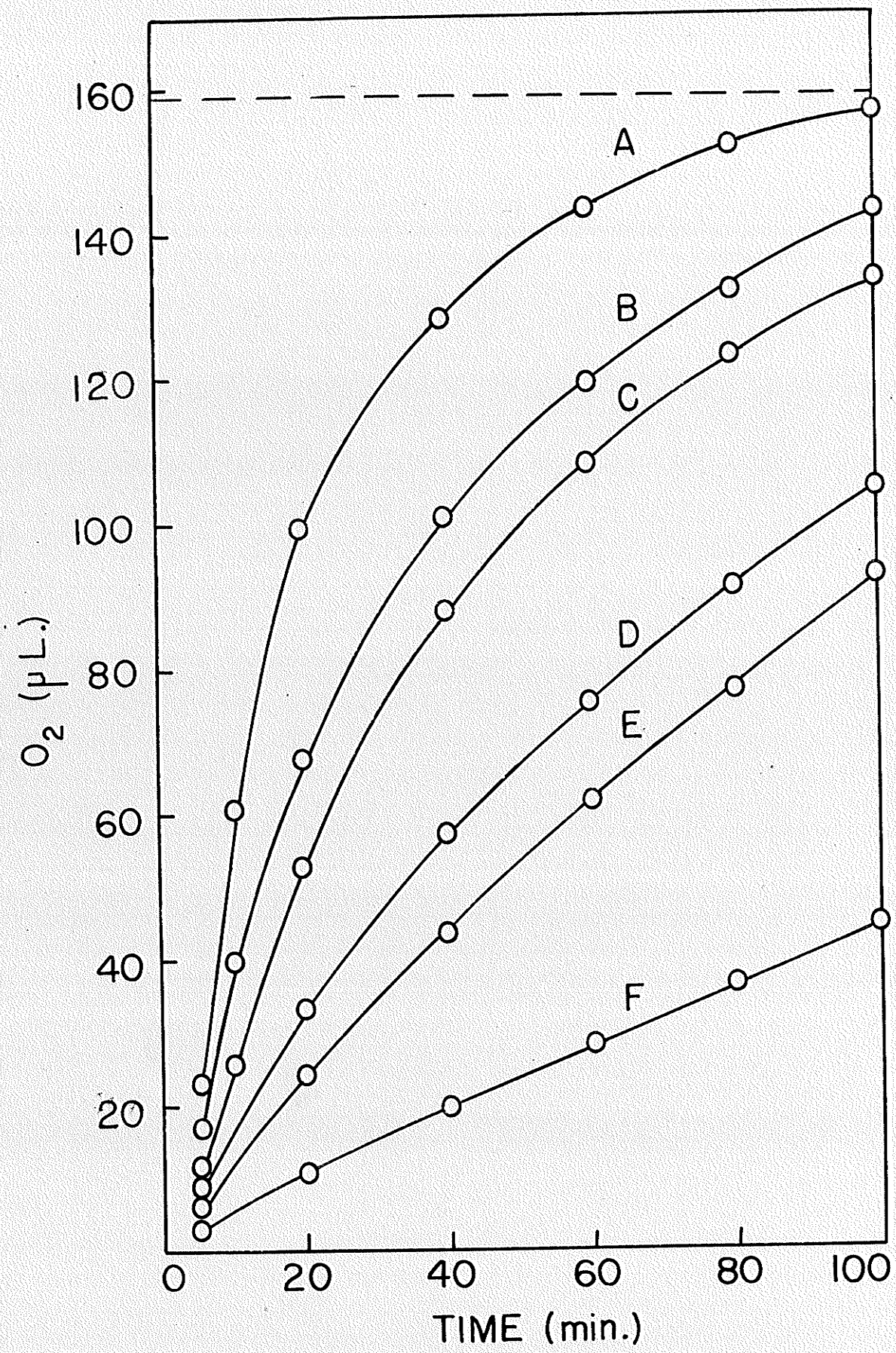
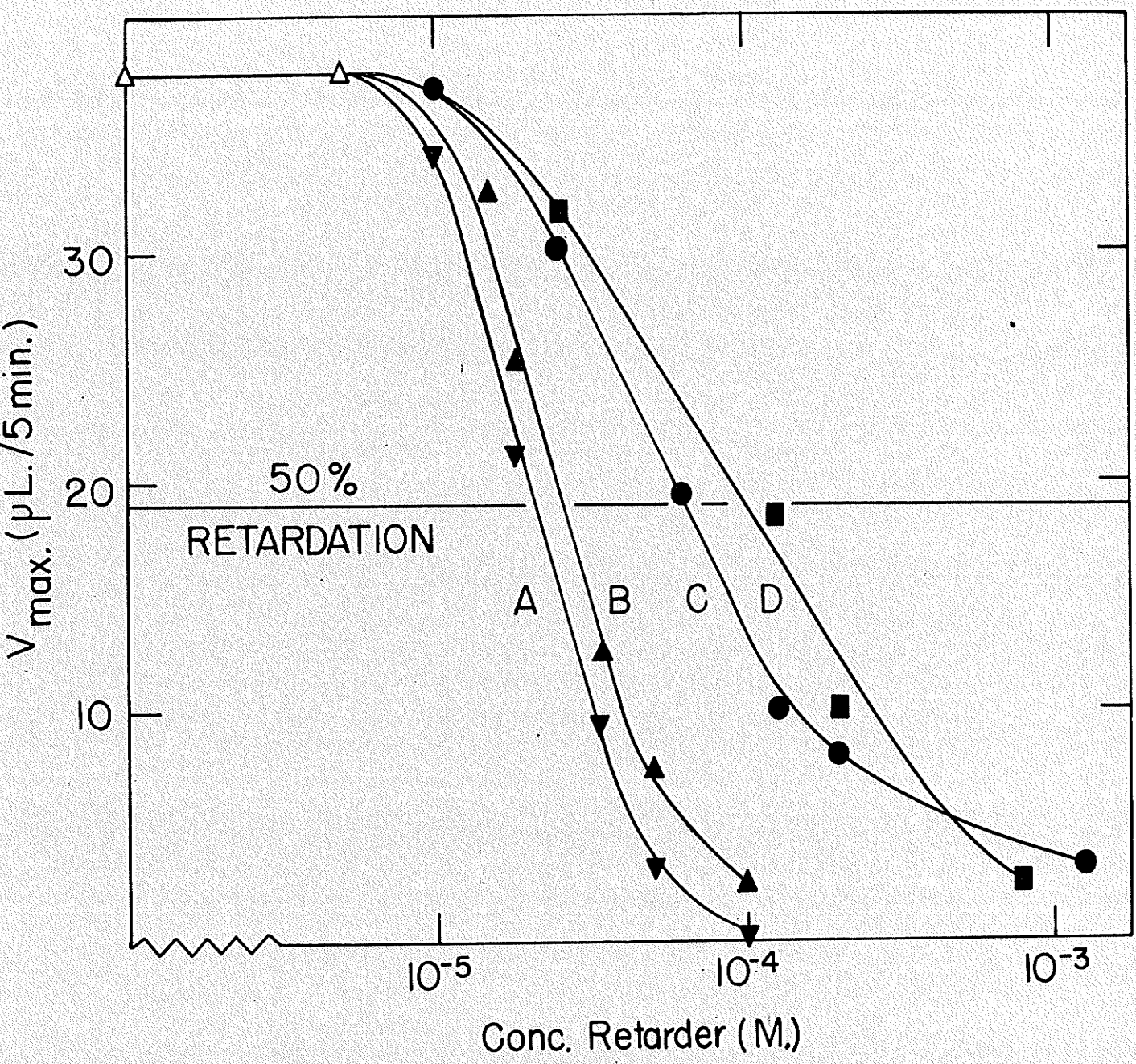

160
140
120
100
80
60
40
20
0
20
40
60
80
100
O_2 (μL.)
TIME (min.)
A
B
C
D
E
F

Figure 19. Relative rates of indoleacetic acid oxidation in the presence of various concentrations of retarders.

Systems standard containing retarders: A, hydroquinone; B, p-quinone; C, riboflavinphosphate; D, scopoletin.


30
20
10
50%
RETARDATION
A
B
C
D
$V_{max.}$ (μL./5 min.)
10^{-5}
10^{-4}
10^{-3}
Conc. Retarder (M.)

6.5×10^{-5}M.; scopoletin, 1.25×10^{-4}M. (see Fig. 19). Hydroquinone and p-quinone retarded the standard catalase-DCP system (see Table IV) by 50% at concentrations of 1.1 to 1.3×10^{-5}M. It should be added that these retarder concentrations apply only to the systems exactly as indicated. Variation in the concentration of any one component (see later, Table VII) altered the % retardation to a value which, however, was constant for the new set of conditions. As indicated previously (Table IV) the extent to which systems were retarded by hydroquinone and riboflavinphosphate was independent of the nature of the cofactors. These properties bear witness to the stability and persistence of retardation as opposed to inhibition of IAA oxidation, phenomena which may have physiological significance.

It is not known whether any of the natural inhibitors reported to be present in crude plant extracts are retarders. Tests of regions of chromatograms of various fractions from wheat leaves have revealed as yet only the presence of inhibitors. Nevertheless riboflavin and its phosphate are universally distributed in plants and both retard IAA oxidation equally at the same concentration. Similarly, scopoletin has been identified in many dicotyledonous plants and oat roots from which it has been isolated (Goodwin and Kavanagh; 1949). Hydroquinone is the aglycone of the naturally-occurring glycoside arbutin. An impurity in commercial streptomycin (Agristrep, 27%, Merck and Co.) caused a 75% retardation of the reaction at a concentration of 0.83×10^{-3}M streptomycin. Purified streptomycin (Merck and Co.)

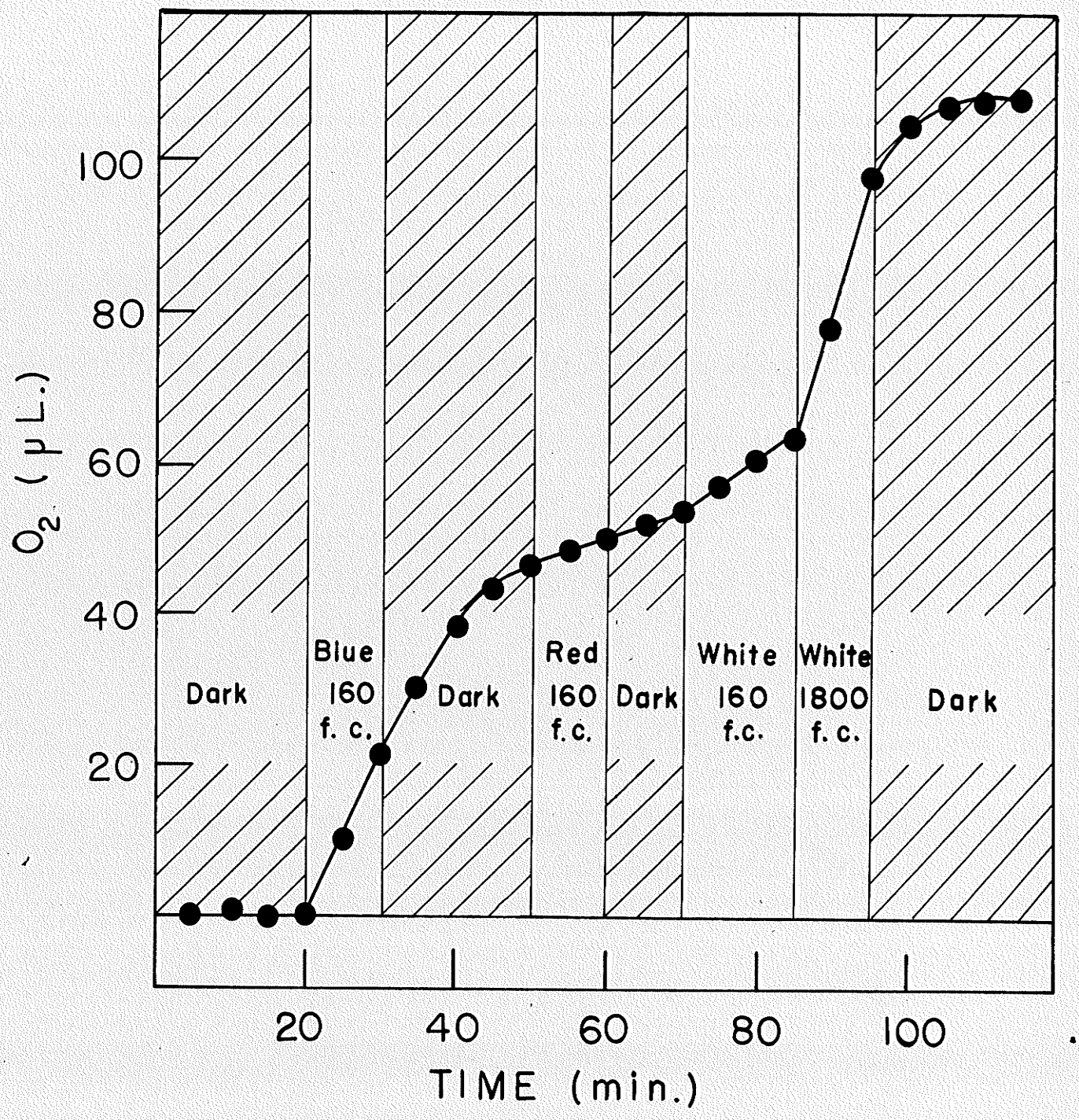
had no effect.

5. The Effect of Light and Riboflavin:

Blue light overcame the retarding effect of riboflavinphosphate on IAA oxidation by the wheat leaf system in the presence of any one of the cofactors (Table IV). The rate of oxidation of illuminated systems containing riboflavin was no greater than the rate in darkness without riboflavin except when the natural factor was used as organic cofactor. In the absence of riboflavin light had no effect on the system. The augmentation of the dark rate in the illuminated natural factor-riboflavin system may have been due to the combined effect of riboflavin and light in overcoming an inhibitor present in the partially purified extract of the natural factor.

The alleviation of the retardation by riboflavin depended on the quality and intensity of illumination. Figure 20 shows the progress of the oxidation under various kinds of illumination. As would be expected from the absorption spectrum of riboflavin blue light was most effective, whereas red light was totally ineffective and white light was required at a higher intensity to give the same effect as blue. It is noteworthy that after the blue light had been switched off (Fig. 20) rapid oxidation of IAA continued for some time indicating a residual effect of light.

Figure 20. Effect of quality and intensity of light on riboflavin-inhibited indoleacetic acid oxidation. Standard system with 2×10^{-3}M riboflavinphosphate. Color and intensity of light indicated in diagram (f.c. = ft. candles at surface of reaction vessel).


100
80
60
40
20
O_2 (μL.)
Dark
Blue
160
f. c.
Dark
Red
160
f. c.
Dark
White
160
f.c.
White
1800
f. c.
Dark
20
40
60
80
100
TIME (min.)

Riboflavin and riboflavinphosphate also retarded IAA oxidation in catalase-DCP systems and riboflavin at a concentration of 5×10^{-4}M completely inhibited the reaction in darkness (Fig. 21:E). At 2×10^{-5}M it was without effect in light or darkness, but at intermediate concentrations, the retardation which occured in darkness was overcome, as in the wheat leaf system, by white or blue light (Fig. 21:B,C) but not by red light (Fig. 21:D). Light had virtually no effect on the catalase system in the absence of riboflavin (Fig. 17).

In wheat leaf systems containing 1.1×10^{-4}M riboflavinphosphate, light saturation was attained at an intensity of 110 ft. candles, under which conditions the rate of oxidation attained a maximum equal to the dark rate without riboflavin. The intensity of white and blue light used in the catalase experiment i.e. white: 1380 ft. candles, blue: 220 ft. candles, was well above that required for light saturation. Clearly, illuminated systems containing riboflavin oxidize IAA at a rate which approaches but does not exceed the dark rate in the absence of riboflavin. Thus riboflavin can not be considered to activate or sensitize IAA oxidation but, on the contrary, protects the hormone in darkness. Illumination of riboflavin-containing systems has no effect other than to permit normal dark oxidation to take place.

One property of systems containing excess riboflavin deserves further comment. The oxygen consumed in the breakdown

Figure 21. Effect of riboflavinphosphate and light on indoleacetic acid oxidation by catalase systems.

Catalase-dichlorophenol system as in Table I

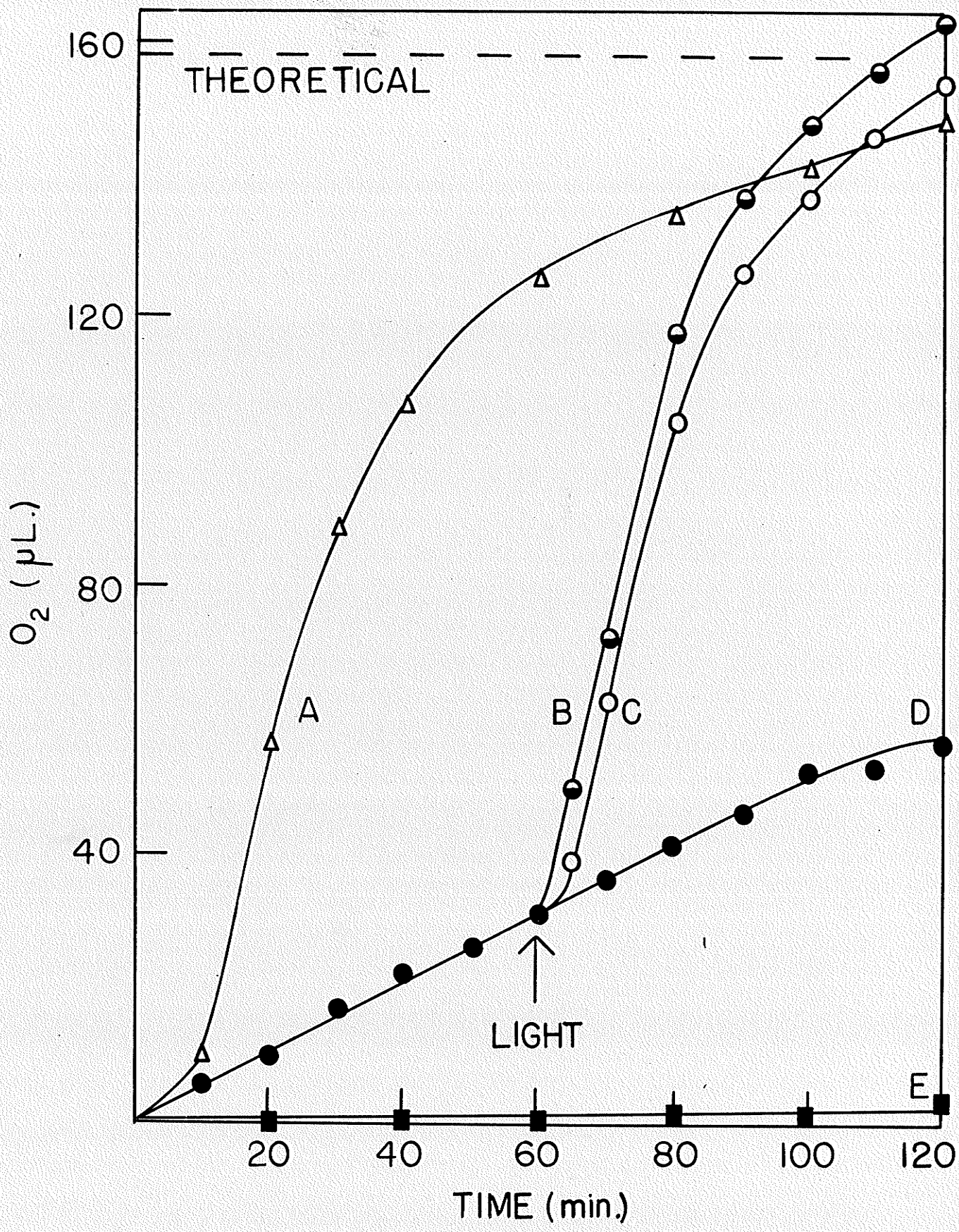
A. control in darkness

B. control + 10^{-4}M riboflavinphosphate. Illuminated with blue light (220 ft. candles) at 60 min.

C. same as B with white light (1380 ft. candles)

D. same as B with red light (960 ft. candles)

E. control in darkness + 5×10^{-4}M riboflavinphosphate


160
120
80
40
THEORETICAL
O_2 (μL.)
A
B
C
D
E
LIGHT
20
40
60
80
100
120
TIME (min.)

of IAA by catalase systems exceeded the theoretical molar equivalence of IAA when illuminated in the presence of riboflavin (Fig. 21:B,C). This also occurred with the wheat leaf systems (Fig. 22:A, Table VI). In an experiment carried on for 400 min., 199 uL. of oxygen were consumed by an illuminated standard system containing riboflavin and the equivalence of 79 uL. IAA (Table VI). The rate of oxygen uptake showed no signs of abating and similar results were obtained when DCP or maleic hydrazide were present in place of resorcinol (Table VI).

In the absence of IAA (Fig. 22:C), oxygen was consumed by these systems at a slower initial rate, but at the same final rate as with IAA (Fig. 22:A). The difference between the oxygen consumed with and without IAA (plotted in Fig.22:B) always exceeded at equilibrium the molar oxygen equivalence of IAA. The difference was greater at higher light intensities or when the riboflavin concentration was increased e.g. to equimolar with IAA (Table VI). Thus it is possible that IAA was oxidised directly by excess riboflavin as demonstrated by Galston, (1949b), in addition to being oxidised by manganic ions as in the riboflavin-free system.

6. Mechanism of Retardation:

The retarding effect on IAA oxidation of hydroquinone, p-quinone, riboflavin and its phosphate, and scopoletin persisted even though several hundred moles of oxygen had been consumed per mole of retarder present. The persistence indicates that, although the retarders must interfere in the reaction sequence, they are

Figure 22. Oxygen consumption of systems containing excess riboflavin with and without indoleacetic acid.

A. standard system with 2×10^{-3}M riboflavin phosphate in blue light (220 ft. candles). IAA concentration: 1.11×10^{-3}M = 79 μL.O_2.

B. A minus C

C. same as A minus IAA

D. same as C in darkness

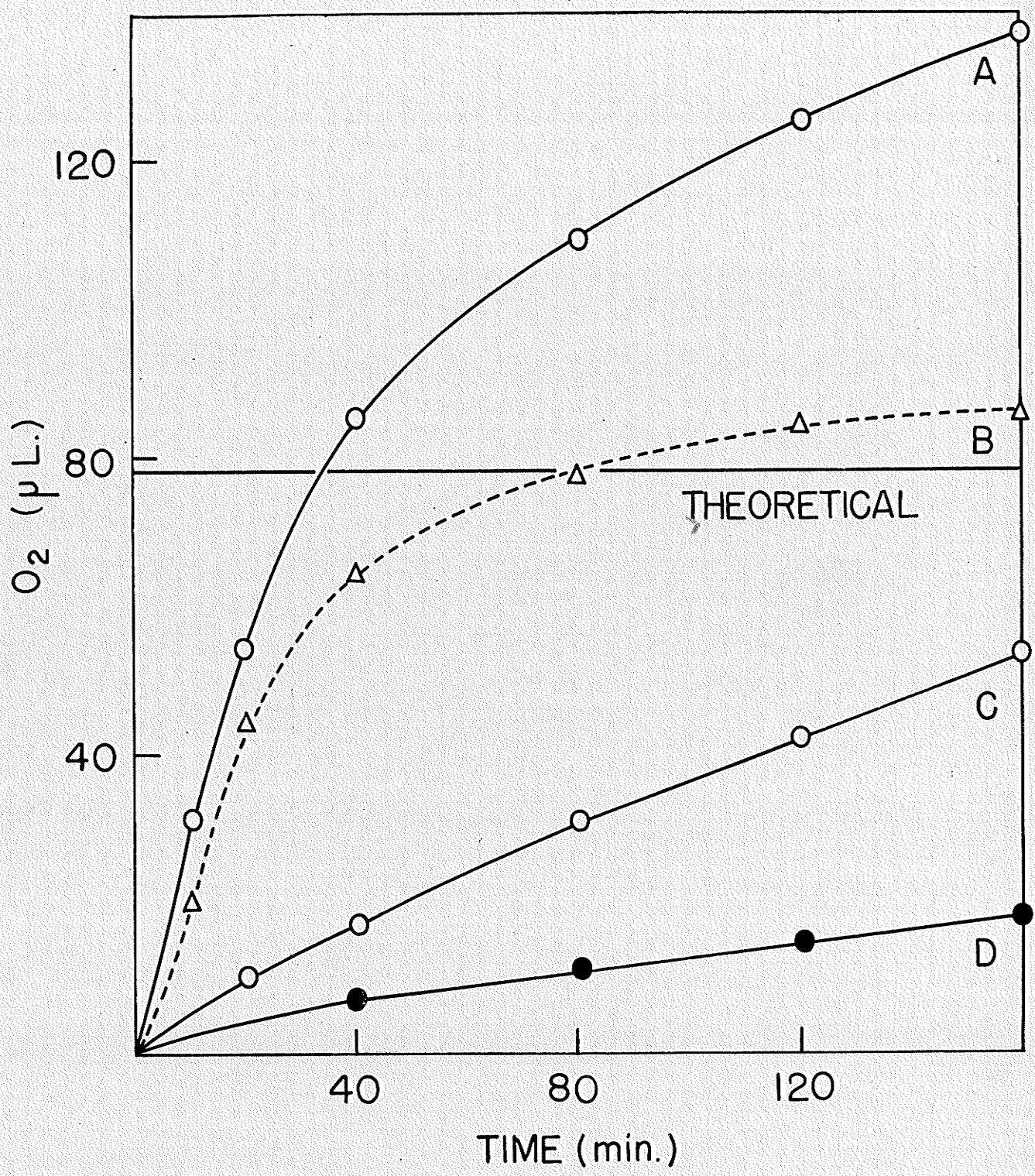

120
80
40
O_2 (μL.)
A
B
THEORETICAL
C
D
40
80
120
TIME (min.)

Table VI Oxygen Consumed by Illuminated Systems[γ] Containing Riboflavin.

Cofactor	Oxygen uptake (μL./400 min.)		
	Plus IAA	Minus IAA	Difference
Resorcinol	199	70	129
Dichlorophenol	181	53	128
Maleic hydrazide	149	32	117

[γ] standard wheat leaf systems containing: 3.33 μM IAA; 6.0 μM riboflavinphosphate (2×10^{-3}M); blue light, 220 ft. candles. Oxygen equivalence of: IAA = 79 μL.; resorcinol and dichlorophenol = 35.5 μL; maleic hydrazide = 712 μL.

also reformed and at the end of the experiment are essentially in the same form as they were at the beginning. All of these retarders share the property of readily participating in reversible oxidation-reduction reactions. Significantly, hydroquinone and its oxidation product p-quinone retarded the oxidation equally at the same concentration. The progress of oxygen uptake by reaction mixtures containing either the phenol or its quinone differed only in the initial velocity which was always slightly greater with the quinone. Thereafter the curves continued exactly parallel as would be expected if a steady state equilibrium were established between the oxidized and reduced forms. Therefore it is suggested that the retarders operate in the system as chain transfer agents by alternately oxidising and reducing essential intermediates. Evidence to support this conclusion is given by the following results.

There are at least three potential oxidants of reduced retarding agents among the reaction intermediates of IAA breakdown. However, the only readily oxidizable component that could take part in a reversible reaction is the organic cofactor e.g. resorcinol. Thus, to produce for example a hydroquinone/p-quinone equilibrium in the system, it should be possible to demonstrate a reaction between p-quinone and the organic cofactors. Resorcinol formed an insoluble brown oxidation product merely on standing with p-quinone. Dichlorophenol and maleic hydrazide did not show any visible reaction probably because their oxidized radicals (RO$^{\bullet}$) do not

condense readily to stable products (see p. 41). Nevertheless, when p-quinone was incubated in solutions of manganous ions and pyrophosphate, the addition of either maleic hydrazide or DCP resulted in the oxidation of manganese. Manganic ions were rapidly produced on warming and were detected as pink manganipyrophosphate or orange manganicitrate. This is essentially the Kenten-Mann reaction for the oxidation of manganese but differs in that quinone replaces peroxide plus peroxidase. Both systems involve the reversible oxidation of organic cofactors ($ROH \longleftrightarrow RO\cdot + \cdot H$) and with p-quinone ($Q(=O)_2$) as oxidant the reactions may be represented as follows:

$$Q(=O)_2 + 2ROH \longrightarrow Q(=OH)_2 + 2RO\cdot$$

$$RO\cdot + Mn^{2+} + H^{+} \longrightarrow ROH + Mn^{3+}$$

$$Mn^{3+} + H_4P_2O_7 \longrightarrow 3H^{+} + MnHP_2O_7$$

Thus, provided an acceptor for manganic ions is present e.g. pyrophosphate, citrate or IAA, p-quinone is capable of oxidizing manganese indirectly.

Analagous reactions have been demonstrated by Andreae (1955a) using riboflavin as initial oxidant. However, the mechanism whereby riboflavin promotes manganese oxidation appears to be more complex since in addition to a phenolic cofactor and a manganic-acceptor, light and catalase were required for maximum oxidation (Andreae, 1955a). The investigations into the gas

exchange of riboflavin systems provide correlative evidence for what may be called the "manganigenic" effects of riboflavin. Reference has been made to this with respect to the resorcinol "oxidase" system (see p. 47) in which solutions of riboflavin, manganese and resorcinol consumed oxygen non enzymically when the systems were illuminated. In darkness no reaction occurred. However when wheat leaf peroxidase was added, the dark rate was detectable (Fig. 22:C) and light enhanced the rate of oxygen uptake beyond that of the non-enzymic system (Fig. 22:D).

The oxygen consumption by these riboflavin systems in the absence of IAA could not have been due entirely to irreversible cofactor oxidation since some oxygen uptake occurred in the presence of maleic hydrazide which does not form a stable oxidation product. Furthermore oxygen uptake appeared to continue indefinitely and by 400 min. had exceeded the molar oxygen equivalence of resorcinol and DCP (Table VI). Since no oxygen uptake occurred in the absence of a cofactor or manganese, it is suggested that one product of the reaction must be oxidized manganese formed via reversible cofactor oxidation. From the work of Kenten and Mann (1952) it is known that manganic ions are to a small extent stable in orthophosphate solution, and there is also the possibility that manganese dioxide could be produced. A mechanism whereby riboflavin (Rb), a redox catalyst (ROH) and peroxidase could interact to oxidize manganese is summarized as follows:

$$Rb + 2ROH \longrightarrow Rb.2H + 2RO\cdot \qquad A$$

$$Rb.2H + O_2 \longrightarrow Rb + H_2O_2 \qquad B$$

$$H_2O_2 + 2ROH \xrightarrow[\text{catalase}]{\text{peroxidase}} 2H_2O + 2RO\cdot \qquad C$$

$$RO\cdot + Mn^{2+} + H^{+} \longrightarrow ROH + Mn^{3+}$$

$$2RO\cdot + Mn^{2+} + 2H^{+} \longrightarrow 2ROH + Mn^{4+} \qquad D$$

Reaction B is a well known spontaneous reaction (Fruton and Simmonds, 1953), reaction C is normal peroxidation and reaction D is the Kenten-Mann reaction. This reaction sequence is similar to that proposed by Andreae (1955a) except that peroxidase and catalase are considered to perform a peroxidatic reaction (C) and thus dispose of hydrogen peroxide which would otherwise speed the decomposition of oxidized manganese (Kenten and Mann, 1952). The enzyme is therefore not essential for oxygen uptake (see previously) or for the formation of manganic ions (Andreae, 1955a).

It is important to note that light absorbed by riboflavin is not an absolute requirement for, but merely an accelerator of, these reactions. In darkness manganic ions were still produced by Andreae's system and oxygen was consumed in the present system, albeit at a slower rate (cf. Fig. 22:C and 22:D). If riboflavin did not generate manganic ions in darkness it would be without effect on IAA oxidation. The ability of light to overcome retardation by riboflavin can therefore be explained by a photoinduced increase in manganigenesis which, it should be emphasized, is

a phenomenon independent of IAA breakdown.

With respect to the mechanism by which light activates the system tests were made on the effect of light on the oxygen uptake of riboflavin-resorcinol systems (reaction A), and on the spontaneous oxidation of reduced riboflavin in air (reaction B). Very little reaction was found to occur between riboflavin and resorcinol in light or darkness and hence an activation by light of reaction A as proposed by Andreae (1955a) appears unlikely. On the other hand, white light (980 ft. candles) almost doubled the rate of oxygen uptake by solutions of riboflavinphosphate artificially reduced by dithionite. Therefore, it is suggested that light acted on reaction B in both Andreae's system and riboflavin-retarded IAA oxidation. On this basis it is concluded that the effect of light was to maintain riboflavin in the oxidized state.

The significant point emerging from these experiments with respect to the retardation of IAA oxidation is that oxidized retarders e.g. p-quinone and riboflavin, generate manganic ions in the system. Evidently, the reduced form of the retarder, which is a necessary product of manganigenesis, must be the immediate cause of the retardation by being involved in a second redox system with an essential oxidized intermediate. Retardation therefore depends on the equilibrium established between the oxidized and reduced forms of the retarder, the latter diverting

an oxidized intermediate from its normal function as a chain reactant in IAA autoxidation. The concentration of the reduced retarder at equilibrium thus must differ for each retarder (Fig. 19) and set of reaction conditions (Table VII).

The intermediate with which the reduced retarders react is probably the manganic ion since reduced riboflavin, hydroquinone and scopoletin all decolorize manganiversene instantaneously, and indirect evidence suggests that no reaction occurs with other oxidized intermediates. For example, if the retarders reacted with the skatole peroxide they would necessarily compete with the cofactor which is the normal hydrogen donor to the peroxide. However, experimentally it was shown that the nature of the cofactor had no significant effect on the degree of retardation (Table IV) even though different cofactors react with the skatole peroxide at varying rates (see p.42). Thus the retarders do not appear to compete with the cofactor and accordingly do not react with the skatole peroxide.

A similar argument renders improbable any reaction of the oxidized cofactor with reduced riboflavin (reverse of reaction A, p. 71). This could occur only at the expense of the normal reaction of the cofactor radical with manganese. Since an increase in the manganese concentration failed to counteract the retardation by riboflavin (Table VII), manganese and riboflavin very probably do not compete for the cofactor

Table VII Retardation of Indoleacetic Acid Oxidation[γ] by Riboflavin. The Effect of Enzyme, Manganese, Dichlorophenol and Indoleacetic Acid Concentrations.

Concentration of varying component				% Retardation			
Enzyme (mL.)	Mn^{2+} (M)	DCP (M)	IAA (M)	Enzyme	Mn^{2+}	DCP	IAA
0.1	5×10^{-4}	1.67×10^{-4}	0.56×10^{-3}	10	75.5	24	42
0.25	10^{-3}	3.33×10^{-4}	1.11×10^{-3}	44	75	39	53.5
0.50	2×10^{-3}	5×10^{-4}	1.67×10^{-3}	52	76.5	59	60.5
0.75	10^{-2}	1.33×10^{-3}	2.22×10^{-3}	60	77	70	67.5
1.0	2×10^{-2}	2×10^{-3}	3.33×10^{-3}	58	69	77	75.5

[γ]Standard DCP-horseradish peroxidase systems containing 2×10^{-4}M riboflavinphosphate.

radical. By inference, these results implicate the manganic ion as the intermediate which reacts with retarders.

An increase in the concentration of either IAA or cofactor (DCP) in the system increases the degree to which riboflavin retards IAA oxidation (Table VII). These effects may be understood by considering the IAA-independent equilibrium attained by oxidized and reduced riboflavin. Excess IAA would dispose rapidly of manganic ions derived from the reactions of riboflavin and the cofactor (reactions A and D), thereby increasing the concentration of reduced riboflavin. Excess reduced cofactor promotes the reduction of riboflavin directly (reaction A, see also resorcinol "oxidase" system, Fig. 13). Thus any increase in the concentration of a reduced component of the system, aside from influencing the propagation of IAA oxidation, shifts the riboflavin equilibrium towards the reduced form and thereby increases the retardation.

Systems containing high concentrations of enzyme were also retarded more effectively by a given riboflavin concentration than systems in which the rate of IAA oxidation was enzyme-limited (Table VII). Variation in enzyme concentration would not be expected to alter the riboflavin equilibrium (reactions A and B) since in the absence of IAA the enzyme appears to act only in the disposal of hydrogen peroxide. However, the enzyme does control the rate of IAA oxidation and thereby the rate of

manganigenesis via IAA breakdown. Provided the rate of IAA oxidation was not enzyme-limited i.e. with 0.5 ml. enzyme or greater, riboflavin retarded the systems equally (Table VII). When the rate of IAA oxidation was low due to low enzyme concentration, riboflavin had less retarding effect probably because the ratio of mangic ions produced by riboflavin to that produced by IAA increased with decreasing enzyme.

Inhibitors of enzyme-controlled reactions are generally considered to be either competitive or non-competitive according to whether or not they compete for the active sites of the enzyme. With respect to IAA autoxidation, the enzyme controlled reaction is a peroxidatic step between the skatole peroxide ($SO_2^{\cdot}$) and a cofactor hydrogen donor (RO·) i.e.:

$$SO_2^{\cdot} + En \longrightarrow SO_2^{\cdot}\text{-}En$$

$$SO_2\text{-}En + ROH \longrightarrow SO_2H + RO^{\cdot}$$

In the classical sense, a competitive inhibitor of these reactions must contest the active sites of peroxidase or catalase with the skatole peroxide (substrate). From the foregoing results it is evident that none of the inhibitors or retarders discussed can be considered as competitive by this definition.

The difficulty in equating the types of inhibitors of IAA breakdown with inhibitors of other enzymic reactions lies in the cyclic nature of the former reaction. Since the rate of

IAA oxidation depends on the speed of each component reaction in the autoxidation sequence, inhibition may result from interference at any point. Such interference embodies preferential reaction of the inhibitor with an essential intermediate. Thereby the inhibitor competes with the substance with which the intermediate normally reacts. If the definition of a competitive inhibitor is widened to include substances which compete for any reactive intermediate, then both lag-extension and retardation of autoxidation sequences are expressions of competitive inhibition.

V DISCUSSION

The main body of this thesis has been concerned with ellucidating the biochemistry of IAA degradation and few conjectures have been made as to the physiological implications of the results. The study was undertaken with the supposition that external and internal factors influenced the growth patterns of higher plants by altering the process of IAA degradation. On this basis it should be possible to interpret well established physiological findings in terms of the biochemical findings provided IAA is degraded *in vivo* and *in vitro* via the same pathway.

At the present time there is a wide area of disagreement on the *in vitro* conditions required to effect IAA degradation (see literature review). Conflicting reports have appeared on the catalase effect and also on the pH optimum and cofactor, manganese and light requirements, all of which has led to the suggestion that IAA is degraded by different pathways and enzymes in plant tissues (Gortner and Kent, 1953). Such a conclusion may appear to be warranted by the discrepancies of the *in vitro* systems, but from a physiological viewpoint metabolic patterns are conveniently similar in different species and indeed in kingdoms. If then IAA catabolism is an important factor controlling auxin level and subsequent growth responses of higher plants it is probable that the metabolic pathway and the enzymes responsible are common to them all. However, before this probability receives experimental justification the conflicting results of the *in vitro*

studies must be reconciled.

The present results show that catalase, like peroxidase, catalyses the complete oxidation of IAA provided manganese and an appropriate cofactor are present. Earlier demonstrations that catalase inhibited the IAA "oxidase" from pea epicotyls (Goldacre, 1951) may possibly be explained by the presence in catalase preparations of a powerful, but easily destroyed inhibitor. The inhibitor is dialysable and thermostable but is destroyed by light. Inhibition of pea "oxidase" by catalase was also overcome by light (Galston et. al., 1953) and the illuminated catalase plus oxidase system showed some degree of activation. The activation in light would be caused by the peroxidatic effect of the uninhibited catalase.

It is interesting that preparations of 2,4-dichlorophenoxyacetic acid reduced the inhibition of pea "oxidase" by catalase (Goldacre, 1951), but the acid solutions were later shown to contain some dichlorophenol (Goldacre and Galston, 1953). It is generally agreed that dichlorophenol promotes IAA oxidation and although it appears to inhibit the catalatic action of catalase (Goldacre and Galston, 1953) its function here is undoubtedly as a redox catalyst since it can be replaced by classical peroxidase substrates, e.g. resorcinol, which are active as oxidants of manganese in the Kenten-Mann system.

With regard to reports of IAA "oxidase" systems which

show no apparent phenol (Tang and Bonner, 1947; Wagenknecht and Burris, 1950; Goldacre, 1951) or manganese (Tang and Bonner, 1947, Goldacre and Galston, 1953; Kenten, 1955) requirement, it is possible that the extracts used contained these cofactors endogenously. It has been found that even after prolonged dialysis of wheat leaf extracts and horseradish peroxidase, some residual activity towards IAA persisted in the absence of either cofactor. This does not apply to catalase. Peroxidase appears to possess an affinity for both manganese and naturally occurring cofactors and this would serve to explain previous reports of IAA oxidation in the absence of added cofactors. Because of these considerations it is concluded that IAA degradation in most of the systems studied in vitro proceeded via the same metabolic pathway and utilized peroxidase or catalase, organic redox catalysts and manganic ions.

The question remains as to whether this system operates in the intact plant. At present the evidence in favour of this is mostly indirect, but firstly it is clear that the experimental environment of the system could exist in vivo. Peroxidase and catalase are ubiquitously distributed in the plant kingdom and the former appears to have an affinity for a substance or substances which mediate IAA oxidation. These natural cofactors occur at least in wheat leaves and probably elsewhere (Goldacre and Galston, 1953; Kenten, 1955). Most probably such factors have a monohydric

phenolic group and it would not be surprising if they varied from plant to plant and set the pattern of the growth response to some auxins.

Secondly, maleic hydrazide, which promotes IAA oxidation by acting as a redox catalyst, is also a widely used growth inhibitor (No-Gro of commerce). It has been shown (Leopold and Klein, 1951) that stimulation by IAA of the growth of pea stem sections was prevented by maleic hydrazide provided the latter's concentration was at least ten times that of IAA. This is in accord with the results of the *in vitro* system in which a significant rate of IAA oxidation was attained only with a similar maleic hydrazide/IAA concentration ratio (see Fig. 11). Large excesses of maleic hydrazide continue to inhibit growth as would be expected since maleic hydrazide shows no optimum concentration for promoting IAA oxidation *in vitro*. Goldacre et. al. (1953) reported that dichlorophenol also inhibited the growth of pea stem sections but, in contrast to maleic hydrazide, it showed a distinct optimum concentration *in vivo* as it did *in vitro* (see Fig. 11).

The effects on growth of other mediators of the oxidation of IAA *in vitro* e.g. resorcinol and monohydric phenols, should be similar to those of either dichlorophenol or maleic hydrazide depending on the shape of their concentration/activity curves. If further studies confirm this prediction, the present system

for IAA oxidation offers a convenient bioassay for the possibility that a substance may act as a growth inhibitor.

The third main body of evidence that implies that IAA is degraded by manganic ions in the plant is found in the action on growth of substances which inhibit IAA oxidation in vitro. For example, scopoletin retards IAA oxidation (Fig. 19) and also stimulates the growth of pea roots at low concentrations and inhibits at high (Andreae, 1952). The latter effect was very probably due to such efficient protection of IAA that the hormone accumulated to levels which were inhibitory to growth (see Muir and Hansch, 1955).

As previously pointed out, the effect of riboflavin on the inactivation of IAA is of particular physiological interest since the action spectrum of phototropic responses and the absorption spectrum of riboflavin are closely related (Galston and Baker, 1949; Galston, 1950a). Furthermore, Galston and Baker(1949) demonstrated that riboflavin stimulated the growth of pea stem sections in darkness although it was somewhat inhibitory in light. Galston et. al. (1951, 1953) suggested that a light-activated flavoprotein participated in the reactions of IAA breakdown but this postulate was rendered unlikely by experiments performed in the absence of flavin by Kenten (1955) and Maclachlan and Waygood (1956).

The present results demonstrate that riboflavin neither

activates nor sensitizes IAA oxidation, but on the contrary protects the hormone in darkness and inhibits its breakdown. The inhibition by riboflavin is photoreversible and light has no effect other than to allow normal dark oxidation to take place. Galston et. al. (1951, 1953) reported that light stimulated IAA oxidation catalysed by crude undialysed extracts from peas. It is possible that naturally occurring riboflavin in Galston's system retarded the oxidation of IAA and light produced its effect by overcoming the inhibition. Thus the parallel which exists between the effect of light on growth and on riboflavin-retarded IAA oxidation in vitro supports the hypothesis that catabolism of IAA in the plant follows the in vitro pathway. It is in full accord with physiological data to regard riboflavin as continuously exerting a check on IAA destruction in vivo, the extent of which is determined by illumination.

It should be noted that both scopoletin and riboflavin, as retarders of in vitro IAA oxidation, operate persistently as opposed to inhibitors, e.g. catechol, which are easily destroyed to neutral products. Thus low concentrations of a retarder at the site of IAA activity in vivo would be expected to have a greater physiological effect than the equivalent of an inhibitor (lag-extender). The present system for IAA oxidation clearly distinguishes between inhibitors and retarders and could be used to indicate the potentialities of a substance as a growth stimulant.

In accordance with the results of Andreae (1955a), it has been shown that light-activated riboflavin oxidizes manganese via the Kenten-Mann system *in vitro*. There is also evidence that light promotes manganese oxidation possibly through riboflavin in the plant (Arens, 1933) and in isolated chloroplasts (Kenten and Mann, 1955). Evidence that plants do oxidize manganese was reviewed previously (Maclachlan and Waygood, 1956). There can be little doubt that manganic ions are generated *in vivo* by any peroxide-producing system coupled to phenol-peroxidase (the Kenten-Mann system). Thus manganic ions required for the initiation of IAA destruction *in vivo* could be derived either autocatalytically as they are *in vitro* or this source may be supplemented by other peroxigenic systems. The contribution of the latter would depend on the juxtaposition of cofactors and enzymes at the site of IAA activity. If peroxidase is distributed throughout the cytoplasm of plant cells whereas catalase is confined to the particulate fractions (see: Goldacre and Galston, 1953, Jagendorf and Wildman, 1954), it is possible that both enzymes catalyse IAA oxidation in different parts of the cell.

The destruction of indoleacetic acid by manganic ions raises the question of the physiological significance of the mangano-manganic equilibrium in the growth processes of plants. Such an equilibrium is known to exist in soil (Sherman and Harmer, 1942) and is probably due to the action of bacteria

(Bromfield and Skerman, 1950) or fungi (MacLachlan, 1941). Among many factors which could displace this equilibrium, light through its peroxigenic effects (Siegel and Galston, 1955) is one of the most important. Gerretson, (1950) has presented potentiometric evidence for the oxidation of manganese by illuminated chloroplasts and Kenten and Mann (1955) have demonstrated that manganic ions are formed by such preparations. Thus inhibition of growth by light could be regarded as an effect of illumination on the mangano-manganic equilibrium which, as evidence in this thesis suggests, is in turn under the control of riboflavin.

Perhaps the most significant finding of the present studies with respect to future investigations is the demonstration that intermediate radicals produced from IAA are extraordinarily reactive. In the presence of manganic ions IAA decomposes with the release of carbon dioxide leaving a free radical of empirical formula equivalent to that of the skatole nucleus. This radical has been shown to react with any of versene, oxygen, manganic ions, itself, another radical or even IAA. The intermediate product of its reaction with oxygen, a skatole peroxide, is capable of reacting in turn with versene, any of the cofactor hydrogen donors of the enzymic system, IAA, probably other radicals and phenolic inhibitors e.g. catechol, and, most significantly, with peroxidase or catalase.

In order to produce such striking effects on plant growth it is assumed that IAA acts through an as yet unidentified "growth centre" or "reactive entity" within the cell, (Veldstra, 1953; Muir and Hansch, 1955). There is no clear explanation available of how this "growth centre" controls growth. However, from kinetic data there is evidence (Foster et.al., 1952) which suggests that IAA is attached to this centre at a minimum of two points and that excess IAA or certain growth inhibitors, e.g. maleic hydrazide, interfere in the attachment and thereby render the complex inactive. In the in vitro system for IAA oxidation it has been shown that maleic hydrazide reduces a skatole peroxide-peroxidase or catalase complex. Without sufficient maleic hydrazide present, the complex becomes inactivated with respect to its ability to oxidize phenols. It may be, therefore, that the "growth centre" is equivalent to or resembles this reactive complex. The function of the haemoprotein may be to stabilize and direct the non-specific reactivity of the skatole radical towards, for example, specific hydrogen donors. This, of course, is speculative, but the fact remains that the skatole peroxide-enzyme complex is the only demonstration of a reactive association between a protein and an auxin derivative, and as such deserves further attention.

VI SUMMARY

I. The complete decarboxylation and oxidation of indoleacetic acid (IAA) is catalyzed by peroxidase or catalase systems containing manganese and a monohydroxy phenol or resorcinol. The kinetics of the systems indicates that the reaction sequence is a cyclic autoxidation as follows: (S-COOH = IAA; S· = skatole radical; SO_2· = skatole peroxide; SO_2H = end product; ROH = phenolic cofactor; RO· = phenolic radical; Enzyme = peroxidase or catalase.)

Initiation: $Mn^{2+} \xrightarrow[+\ ROH]{Enzyme} Mn^{3+}$

Propagation:

$$Mn^{3+} + S\text{-}COOH \longrightarrow S\cdot + CO_2 + Mn^{2+} + H^+$$

$$S\cdot + O_2 \longrightarrow SO_2\cdot$$

$$SO_2\cdot + ROH \xrightarrow{Enzyme} SO_2H + RO\cdot$$

$$RO\cdot + Mn^{2+} + H^+ \longrightarrow ROH + Mn^{3+}$$

Termination:

$$Mn^{3+} + ROH \longrightarrow Mn^{2+} + H^+ + RO\cdot$$

$$2RO\cdot \longrightarrow \text{products}$$

$$SO_2\cdot + Enzyme \longrightarrow SO_2\text{-}Enzyme$$

2. In the presence of manganic ions, IAA decomposes non-enzymically with a gas exchange and product at pH 6.0 equivalent to that of the enzymic system. The stoichiometry of the system indicates the following reaction sequence: (symbols as above, MnV^- = manganiversene)

<u>Initiation</u>: $MnV^{-} + S\text{-}COOH \longrightarrow S\cdot + CO_2 + MnV^{-} + H^{+}$

<u>Propagation</u>: (at pH 6.0)

$S\cdot + O_2 \longrightarrow SO_2\cdot$

$SO_2\cdot + S\text{-}COOH \longrightarrow SO_2H + S\cdot + CO_2$

<u>Termination</u>: (below pH 5.0 and anaerobically)

$S\cdot + MnV^{-} \longrightarrow$ products

$2S\cdot \longrightarrow$ products

3. Inhibition of enzymic IAA oxidation occurs in such a way that the induction period is extended in the presence of catechol, pyrogallol, rutin and dialysable inhibitors in wheat leaf extracts and beef liver catalase. These substances stop the chain sequence by competing favorably with IAA for manganic ions e.g.

<u>Chain stopping:</u> $2Mn^{3+} + \text{catechol} \longrightarrow 2Mn^{2+} + H^{+} + \text{o-quinone}$

Thus initiation of IAA oxidation is prevented until the inhibitor is oxidized, in which form it has no further effect on the system.

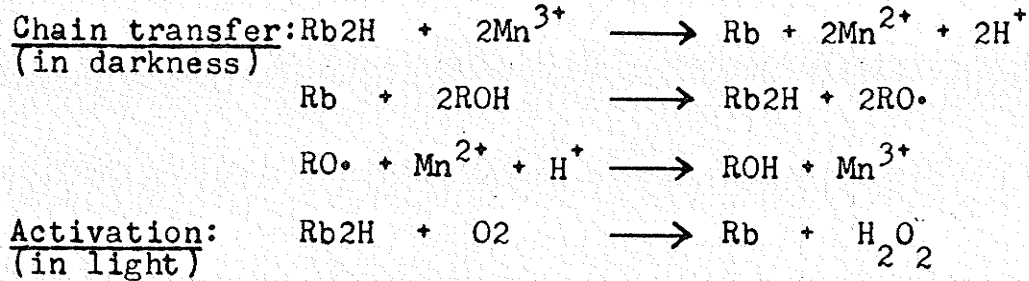
4. The enzymic system is retarded by hydroquinone, p-quinone, riboflavin and scopoletin which act as chain transfer agents. The reduced form of these substances inhibits the system by reacting with manganic ions but the oxidized form indirectly generates manganic ions. Thus the nature of the redox equilibrium established determines the degree of retardation. The reactions of hydroquinone ($Q(-OH)_2$) and p-quinone ($Q(=O_2)$) are summarized as follows:

<u>Chain transfer</u>: $Q(-OH)_2 + 2Mn^{2+} \longrightarrow Q(=O)_2 + 2Mn^{2+} + 2H^{+}$

$Q(=O)_2 + 2ROH \longrightarrow Q(-OH)_2 + 2RO\cdot$

$RO\cdot + Mn^{2+} + H^{+} \longrightarrow ROH + Mn^{3+}$

5. The retardation of IAA oxidation caused by riboflavin (Rb) is overcome by blue light which promotes the spontaneous oxidation of reduced riboflavin (Rb2H). When riboflavin is maintained in the oxidized form it has no effect on IAA oxidation.



6. Evidence for the participation of manganic ions in IAA oxidation in the plant falls into three main sections: (a) The reaction environment <u>in vitro</u> could exist <u>in vivo</u>; manganese is oxidized by plant cells and intracellular particles. (b) Substances which accelerate IAA oxidation <u>in vitro</u> occur naturally, and two artificial cofactors (dichlorophenol and maleic hydrazide) are known to inhibit growth. (c) Riboflavin and scopoletin occur naturally, retard <u>in vitro</u> IAA oxidation and have been reported to stimulate growth under certain conditions. The effects of riboflavin on growth and IAA oxidation are reversed by light.

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