STUDIES ON THE α-DECARBOXYLATION OF OXALOACETIC ACID IN WHEAT

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ABSTRACT

The α-decarboxylation and oxidation of oxaloacetic acid to malonic acid is catalysed by extracts from both wheat leaves and commercial horseradish peroxidase. The oxidation shows an absolute requirement for manganese, and is enhanced by the presence of either 2,4-dichlorophenol or resorcinol. The effects of substrate concentration, enzyme concentration, cofactor concentration, pH, anaerobosis, and hydrogen peroxide, on this system have been examined.

During the oxidation of oxaloacetate a brown colour appears and disappears in the reaction system. A spectral analysis of this colour phenomenon showed an absorption band at 310 mμ. The factors influencing the formation of this colour have been investigated.

An attempt has been made to explain the mechanism of this oxidative α-decarboxylation of oxaloacetic acid.

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DHF	dihydroxyfumaric acid
DHM*	dihydroxymaleic acid
DCP	2,4-dichlorophenol
DPNH	reduced diphosphopyridine nucleotide
EDTA	ethylenediaminetetraacetic acid
HRP	horse radish peroxidase
IAA	indoleacetic acid
OAA	oxaloacetic acid
TPNH	reduced triphosphopyridine nucleotide

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* Originally it was believed that the substrate was dihydroxymaleic acid, but it has since been shown (Gupta, 1953) that this compound is the 'trans' isomer-dihydroxyfumaric acid.

I INTRODUCTION

Many investigations have reported examples of the oxidation of indoleacetic acid in which peroxidases appear to act as oxidases; that is, to catalyse oxidations by molecular oxygen. These oxidations are influenced by several external agencies, including hydrogen peroxide, manganese, and by certain hydrogen donors capable of undergoing oxidation by normal peroxidase systems.

De Vellis et al. (1963), Vennesland et al. (1946), and Kenten and Mann (1953) have demonstrated that oxaloacetic acid can be oxidized to malonic acid in the presence of peroxidases. Similarly, this oxidation is affected by hydrogen peroxide, manganese, and certain phenolic compounds.

The present study was undertaken to demonstrate whether oxaloacetic acid oxidase is present in wheat leaves, and if so, to compare its reaction mechanism with that of the indoleacetic acid oxidase as demonstrated by Maclachlan and Waygood (1956a). Also the enzymes extracted from wheat were compared to peroxidase enzymes obtained from other sources.

Recent spectrophotometric analysis (Ricard and Nardi, 1967, Yamazaki and Piette, 1963) have led to a reinterpretation regarding the mechanism of oxidase-peroxidases. In the light of this re-evaluation, classical procedures were

employed with the hope of assessing the mechanism of oxaloacetic acid oxidation.

II LITERATURE REVIEW

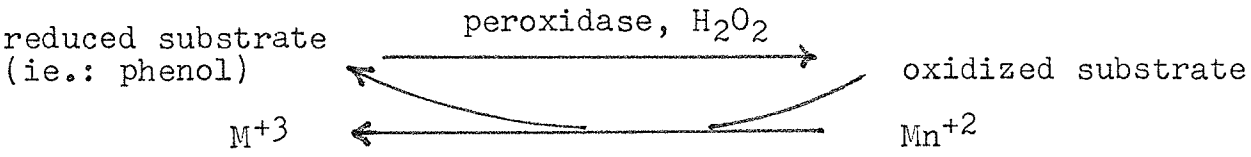
Peroxidases have been studied extensively since the early 1900's when Willstatter and his co-worker (1923) succesfully isolated and purified these enzymes. However, in spite of the enormous quantity of data acquired (Saunders, Holmes-Siedle, and Stark, 1964), the physiological role of peroxidases has remained obscure. Two factors in particular have contributed to this dilemma. Firstly, the number and variety of the compounds whose peroxidation is catalysed by these enzymes 'in vitro' is large, and secondly the substrate, hydrogen peroxide, probably does not accumulate to any significant extent in the living cell.

The possibility that the peroxidases may act as oxidases was recognized when Swedin and Theorell (1940) reported that Horse Radish peroxidase catalysed the oxidation of dioxymaleic acid (dihydroxyfumarate) by molecular oxygen. Since then peroxidases have been found to catalyse the oxidation of a variety of compounds in the absence of hydrogen peroxide. These include phenylacetaldehyde (Kenten, 1953), phenylpyruvate (Conn and Seki, 1957), dicarboxylic acids (Kenten and Mann, 1953), indoleacetic acid (IAA)(Kenten, 1955a, Maclachlan and Waygood, 1956b), DPNH, TPNH, ferrocytochrome, reduced glutathione (Akazawa and Conn, 1958), and 2-methyl, 1:4 naphtohydroquinone (Klapper and Hackett, 1963a). All these reactions show a requirement for Mn^{++} or a stimulation

by this ion. In many cases the presence of catalytic amounts of various phenols has a great influence on the rate of oxidation.

This review traces the development of current theories put forward to explain the mechanisms by which peroxidases are able to oxidize indoleacetic and certain dicarboxylic acids. These compounds were grouped together on the basis that peroxidases, in the presence of the appropriate co-factors, catalyse the 'oxidative-decarboxylation' of these acids.

Kenten and Mann (1949) found that extracts from horse radish roots in the presence of H_2O_2 oxidized Mn^{++} to MnO_2. In addition to peroxide, a thermolabile and thermostable factor, which Kenten and Mann (1950) later demonstrated to be peroxidase and a phenolic compound respectively, were necessary for this oxidation. From these results a scheme for manganese oxidation was proposed:



Further work by Kenten and Mann (1952a) showed that manganese oxidation could be brought about by coupling enzyme systems which produced H_2O_2 with peroxidase systems. If they added oxalate to such systems there was anincrease in oxygen uptake. This was attributed to a manganese oxidation-reduction

cycle whereby the acid would reduce the Mn^{+3}. This meant that both the phenol and manganese would act in a cyclic fashion and therefore be required only in catalytic amounts.

It was found (Kenten and Mann, 1952a) that while reaction mixtures containing oxalate and Mn^{+2} alone showed no oxygen consumption, the addition of peroxidase with p-cresol caused O_2 uptake to occur after an initial lag period. The fact that the oxygen uptake was dependent on the presence of peroxidase and p-cresol suggested that H_2O_2 formation and manganese oxidation were involved in the reaction mechanism. This production of trivalent manganese by a manganese-phenol-peroxidase system has come to be known as the Kenten-Mann system.

On the basis of their results, Kenten and Mann (1953) have suggested that the previously reported oxidations of dihydroxymaleate (Swedin and Theorell, 1940) and oxaloacetate (Vennesland, Evans, and Francis, 1946) which were dependent on the presence of both Mn^{+2} and peroxidase, or compounds with peroxidatic activity, involves the oxidation of manganese.

It is of interest to note that in those experiments with oxalate and high concentrations of manganese, Kenten and Mann (1953) noticed that a faint pink colour was present in the reaction mixture at the end of the lag period. This colour became more intense during the period of rapid oxygen uptake. They attributed this colour to the probable presence

of a Mn^{+3}-oxalate complex.

Kenten and Mann (1953) found that Mn^{+2} in the presence of peroxidase or peroxidase systems can catalyse the 'oxidative-decarboxylation' of oxaloacetic, ketomalonate, dihydroxymaleate, and dihydroxytartrate. Later,Kenten (1955) reported the oxidative-decarboxylation of IAA, stimulated by the presence of Mn^{++} and monophenols; and implied that a similar mechanism was operating. However, unlike oxalate, none of these acids gave any visual evidence as to the accumulation of manganic complexes.

Of significance is the fact that the oxidative-decarboxylation of these acids can proceed, after a lag period, without the addition of hydrogen peroxide. Positive benzidine tests (except with dihydroxymaleate) coupled with the fact that addition of H_2O_2 shortened the lag period, while the addition of catalase lengthened the lag period (Kenten, 1953, Kenten and Mann, 1952b, Kenten, 1955b), provided the basis for the assumption made by Kenten and Mann (1952a, 1955) that hydrogen peroxide was being produced during the course of the reaction. They suggested that H_2O_2 was produced by an auto-oxidation of the acid substrates. This has been demonstrated in the case of oxalate, but with dihydroxymaleate Hartree (1953) was able to abolish auto-oxidation completely without affecting the rate of the enzyme catalysed reaction. However, Kenten (1955) maintains that it is possible that

extremely slow auto-oxidation of these substrates could provide the traces of H_2O_2 whereby the reaction is initiated, and that further H_2O_2 could arise if the oxidation product, produced by the action of the peroxidase, auto-oxidizes readily with the production of peroxide.

Waygood et al. (1956a, 1956b), using wheat leaf extracts, observed similar promotive effects of Mn and phenols on IAA oxidation. The hypothesis that manganic ions are involved in the oxidative-decarboxylation of IAA was substantiated further by the discovery (Maclachlan and Waygood, 1956a) that non-enzymatically produced Mn^{+3} (EDTA-complex) will cause a destruction of IAA accompanied by CO_2 production and O_2 consumption. Maclachlan and Waygood (1956b) suggested a refinement of the Kenten-Mann scheme by proposing that destruction of IAA occurs by a chain auto-oxidation initiated and propagated by two enzyme controlled peroxidations:

initiation $Mn^{+2} \xrightarrow{\text{peroxidase, } H_2O_2} Mn^{+3}$

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

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

$\text{S}\cdot + O_2 \longrightarrow SO_2^{\cdot}$

$SO_2^{\cdot} + \text{ROH} \xrightarrow{\text{peroxidase}} \text{RO}\cdot + SO_2H$

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

Where S-COOH = indoleacetic acid; S· = skatole radical; $SO_2^{\cdot}$ = oxidized skatole radical or indole peroxide; SO_2H =

final reaction product; ROH = phenolic cofactor; RO• = semiquinol or aryl radical.

In spite of the evidence favoring hydrogen peroxide formation and utilization, Waygood et al.(1956b) suggest that a free radical intermediate (S•) reacts with O_2 to form an organic peroxide or radical. Such a radical could react with the phenolic compound (ROH) in a peroxidase catalysed reaction withdrawing an electron and hydrogen ion to produce an aryl radical (RO•) capable of oxidizing manganese. Waygood et al (1956b) further speculated that manganic ions initiated by a relatively slow oxygen-dependant 'oxidase'-type of reaction with an appropriate phenol would start the over-all reaction sequence. Once the reaction was initiated, the peroxide produced as a result of the oxidation of IAA ($SO_2^{\bullet}$) would oxidize the phenolic compound more effectively than the 'oxidase' reaction.

As was the case with the Kenten-Mann scheme, the above mechanism offers a suitable explanation as to the function of the Mn and phenol cofactors. Although the two schemes are comparable with regard to the Mn^{+2} oxidation occurring via an oxidized phenolic compound, the latter hypothesis (Maclachlan and Waygood) is better able to explain some known experimental results. The fact that CO_2 evolution preceeds O_2 consumption, and that this gaseous exchange occurs on a mole-to-mole basis with IAA, is appropriately illustrated in

the Maclachlan-Waygood mechanism. Their initiation step, presented as a slow enzymatic oxidation, which occurs in the absence of H_2O_2, affords a feasible explanation for the inherent lag period found in IAA oxidations. A critical analysis of both the Kenten-Mann and Maclachlan-Waygood schemes regarding IAA oxidation has been published (Ray, 1958).

It should be mentioned that Maclachlan (1956), during his studies on IAA degradation under high oxygen tensions, observed, prior to the addition of IAA, that the reaction mixture developed a brown colour. This colour, being less evident under normal atmospheric conditions, was attributed to oxidized resorcinol (RO·) with presumably some semiquinol condensation. Apparently, however, he neglected to mention whether or not this colour intensified during the period of rapid O_2 uptake, or if it remained visible at the completion of the reaction.

The above mentioned 'free radical' mechanism which involves the oxidation of manganese is but one of the current theories available to explain the oxidative reaction of peroxidases. With the ever increasing sophistication of analytical techniques (Piette, Yamazaki, and Mason, 1961), researchers have been able, as of late, to approach this problem at a near molecular basis. This has resulted in both a refinement of the 'free radical' hypothesis; and the development of a new

concept which advocates the participation of the iron prosthetic group.

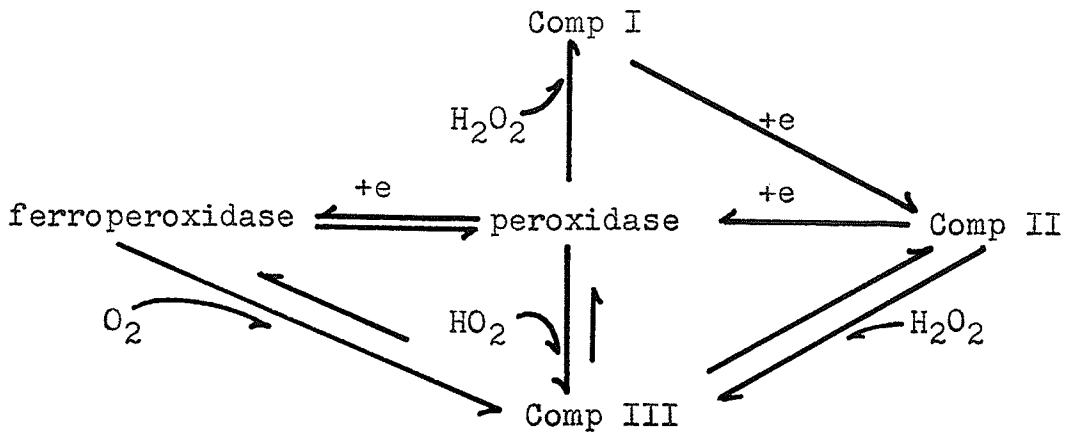
Actually, the possibility that iron might be the functional component of peroxidase enzymes is by no means a recent theory. Willstatter and Pollinger (1923), finding iron to be a prosthetic group in purified peroxidase, suspected that a relationship between the activity of the enzyme and its iron content existed. Later (1931) it was shown that the activity of peroxidase was proportional to the absorption by the enzyme preparation at 420 mu (the Soret band, characteristic of ferroporphyrins). Swedin and Theorell (1940), while studying the oxidation of dioxymaleic acid, were able to demonstrate the reduction of this iron prosthetic group from the ferric to the ferrous state. The absorption bands obtained during the oxidation belonged to the Fe^{+3}-peroxide-peroxidase (compound II) or to the hypothetical Fe^{+2}=oxygen-peroxidase (now known as Compound III). When carbon monoxide replaced air, the appearance of Fe^{+2}-carbon monoxide-peroxidase bands confirmed their findings that a valency change, ferric $\longrightarrow$ ferrous, was involved in the reaction of the enzyme with dioxymaleic acid.

Various other workers, however, were unable to confirm this iron valency change. Chance (1952a) found no effect of CO upon the absorption spectra of HRP in the presence of dioxymaleic acid, and little CO inihibition of the Mn^{++}-acti-

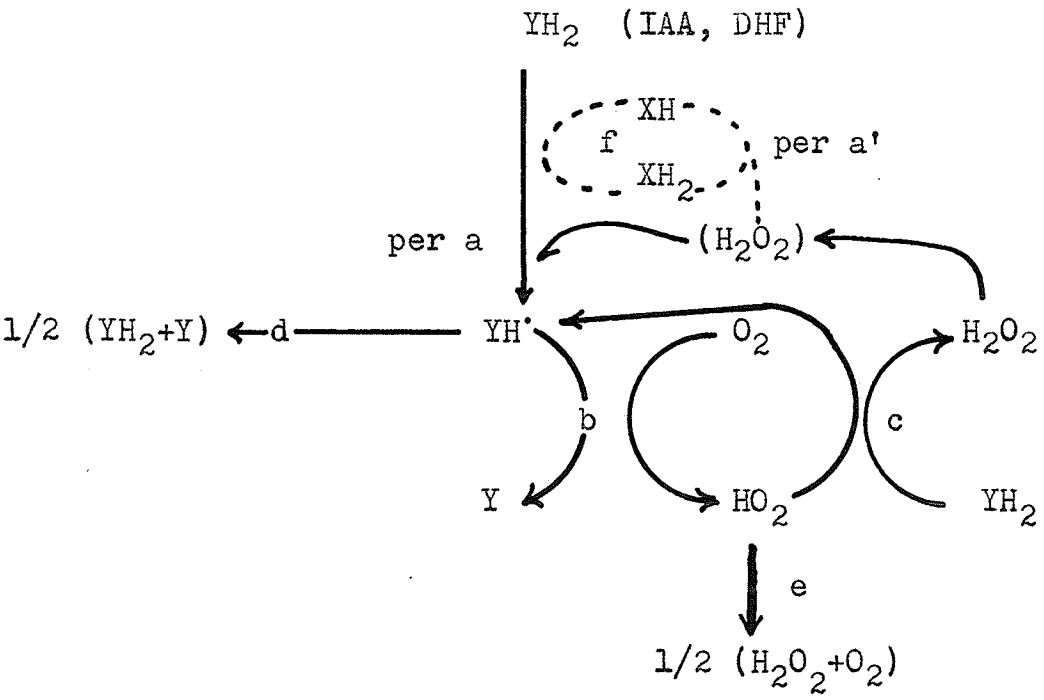
vated oxidase activity. He proposed that the catalyst for the oxidase reaction is a Mn^{++}-activated peroxidase-peroxide complex. It has been suggested (Yamazaki and Piette, 1963) that this contro versy over the effect of CO may be due merely to the differences in the reaction systems studied; since more recent work (Klapper and Hackett, 1963b)has confirmed the findings of Swedin and Theorell.(1940).

Supporting the idea of an iron valency change, Lemberg and Legge (1949) proposed a mechanism by which ferroperoxidase would act as an oxygen-binding oxidase with H_2O_2 participating in the formation of the ferroperoxidase.
The observation that peroxidase could be rapidly transformed into compound III or a substance spectroscopically similar, suggested to Mason and Anan (1958) that this compound III might be an active intermediate in the peroxidase-oxidase reaction.

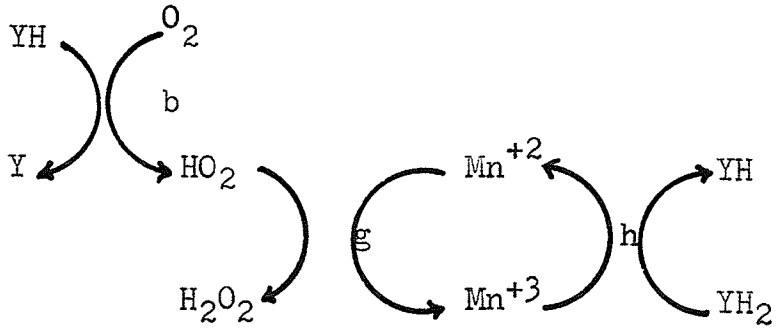
A tentative scheme for the relationship between peroxidase and derivatives which appear during peroxidase-oxidase reactions is illustrated below. This scheme is the culmination of considerable research (Chance, George, Keilin, Mason, Theorell, Saunders, Yamazaki) and the details can be found elsewhere (Saunders, Holmes-Seidle, and Stark, 1964).



Yamazaki and Piette (1963) expanding upon the earlier findings of George (1953) and Chance (1952b), proposed another type of free radical mechanism. In this extremely lucid hypothesis, it is suggested that peroxidase catalyses the formation of free radicals from hydrogen donors (substrates, ie., IAA, DHF) in the presence of H_2O_2; and that these free radicals can reduce molecular oxygen.



This mechanism is unique in that it is able to incorporate a variety of previously conflicting data. The requirement in peroxidase-oxidase systems of the cofactors Mn^{++} and monophenols has been a constantly disputed facet among many workers in this field. Yamazaki attempts to rectify this by saying that, while trace amounts of H_2O_2 are necessary (reaction per a), the phenol (reaction f) and Mn^{++} (reaction b) will act as stimulators only under certain conditions. If reaction per a' is faster than reaction per a, XH_2 will promote the peroxidatic disappearance of YH_2 and the formation of $YH^{\cdot}$. As for the manganese, it will catalyse the oxidation of the hydrogen donor by the perhydroxyl radical (reaction b see below) in the presence of both monophenol and Mn^{++}, Yamazaki does not rule out the possibility that the manganese oxidation suggested by Kenten and Mann (1950) and Maclachlan and Waygood (1956b) could occur.



Yamazaki and Piette (1963) demonstrated that although the radicals of dihydroxyfumarate and indoleacetic acid could reduce peroxidase to ferroperoxidase, the main reaction, at

least with dihydroxyfumarate, was the reduction of molecular oxygen. If however, CO was present in the system, any ferroperoxidase produced through this side reaction would be converted to the CO-complex and thereby be removed from the reaction. Thus the controversy of CO inhibition could be explained by the nature of the peroxidase.

In summation, Yamazaki and his co-workers (1965) postulate that the peroxidase-oxidase reaction begins with the peroxidatic reaction which forms free radicals of the donor substrates. Then, after this initial reaction, there are three possible paths of successive reactions which could occur. (a) The free radical reduces oxygen to perhydroxyl radical, which oxidizes the substrate and forms the free radical of the substrate again. This, he suggests is the case with dihydroxyfumarate oxidation. (b) Perhydroxyl radical reacts with peroxidase to form Compound II, which does or does not oxidize the donor substrate (the latter being the case in dihydroxyfumarate oxidation). (c) The free radical reduces peroxidase to ferroperoxidase, which reacts with oxygen to form Compound III. This, he implies, is the mechanism in IAA oxidation.

By contrast, Klapper and Hackett (1963b) found that certain quinols and naphtoquinols would reduce ferriperoxidase, directly, without the necessity of peroxide. The resulting ferroperoxidase reacts with O_2 to form Compound III.

A similar situation has been reported to occur with indoleacetic acid. Ricard and Nari (1967) showed that, under certain experimental conditions, horse radish peroxidase is reduced by indoleacetic acid and then reacts with oxygen to form a compound (Compound III) endowed with reactivity towards IAA, catechol, and probably other substances.

III METHODS AND MATERIALS

Plant Material Primary leaves of wheat, Triticum aestivum L. var. Selkirk, were harvested after growing 12 to 15 days in the greenhouse.

Extraction and Isolation of Peroxidases Leaves (20-30 g) were excised and chilled by washing under cold tap water and cut into small pieces. All subsequent procedures were performed either in the cold room, or with the enzyme preparation in packed ice. The leaves were ground in a cold mortar with one and one half parts of chilled acetate buffer, pH 5.3, and an abrasive (acid washed sand or carborundum). The resulting slurry was pressed through 4 layers of cheese cloth and the filtrate was centrifuged at 20,000 x g for 30 minutes at 4° C. The supernatant (ca. 25 ml) was dialysed against 2.5 litres of distilled water for 72 hours with continual stirring in the cold room. The dialysate was centrifuged as before and the supernatant was stored at 15°. Repeated thawing and freezing sometimes produced additional coagulation. In these cases the solutions were centrifuged as before and the protein determined. Although the enzyme preparations remained active for longer periods of time, no preparations older than 6 weeks were used in the experiments.

Unless otherwise stated, all experiments were performed

using an enzyme prepared by the above method. Other enzyme solutions were obtained by using different preparatory techniques and alternate sources. An enzyme preparation from wheat leaves was extracted according to the method described by Shannon et. al. (1963). However, the procedure was not carried beyond the heat-cold precipitation step.

Commercially available horse radish powder (Heinz and Co.) was also used as a source of enzyme. The method of preparation was similar to that described for the wheat leaf extract. Commercially purified 'Peroxidase' (Type II) was obtained from the Sigma Chemical Co., St. Louis, Mo. It had the following specifications: Rz 1.5 approx. (20 sec) 110 purpurgallin units/mg. A few milligrams of this 'Peroxidase' were dissolved in a small amount of 0.05M acetate buffer, pH 5.3. The protein concentration was 800 µgm/ml.

Protein Determination Protein was determined by the modified method of Warburg and Christian (1941). Enzyme preparations were usually visibly clear. The final preparations contained between 0.7 - 2.0 milligrams of protein per millilitre.

Manometric Analysis The decarboxylation and oxidation of OAA was followed by measuring the gas exchange by the direct method of Warburg in standard respirometers. Flasks of ca. 20 c.c. contained the components in a liquid volume of

3.0 ml with 0.2 ml of 10% KOH and a filterpaper wick in the central well. The concentrations of the components of a 'standard' system for OAA oxidation were as follows: enzyme preparation 0.25 - 1.5 mg protein; acetate buffer, pH 5.3, 100 umoles; manganese chloride, 3.0 umoles; dichlorophenol, 3.0 umoles; oxalacetic acid, 20.0 umoles. The OAA solution was prepared immediately before each experiment, and was adjusted to pH 3.2 - 3.5 with 1N NaOH before being placed in the sidearm. The tipping in of the substrate was designated as zero time. The flasks were shaken continuously while in a constant temperature water bath at 28°. Readings were taken every 5 minutes.

For anaerobic experiments, the flasks with the reaction solutions, while being shaken, were flushed with oxygen-free nitrogen for 10 - 15 minutes at the start of the experiment. Then the taps were closed and the system allowed to equilibrate for 10 minutes before the reaction was started.

Chromatographic Analysis In order to obtain an identifiable amount of product, slightly greater amounts of OAA were used (25-30 umoles) in an otherwise standard system. At the termination of oxygen uptake, the contents of the main chamber were transferred to a test tube containing 0.3 grams of dry 50-8X Dowex. The tube was shaken vigorously for several minutes, then the Dowex was centrifuged off. The supernatant was dried to ca. 0.3 ml. Of this 100 ul was spotted on a

sheet of Whatman No. 1 filterpaper. The chromatogram was run in an ascending manner for 6 to 8 hours.

The solvent used was ethanol:ammonium hydroxide:water (180:10:10), prepared by adding 10 ml of concentrated ammonium hydroxide to a solution of 180 ml of 95% ethanol and 10 ml water.

The aromatic amine-reducing sugar solution, used as the developing agent, was prepared as follows: to 100 ml of 50% ethanol was added 5 ml of analine and 5 g of D-xylose (Smith and Spriesterbach, 1954). It was prepared fresh for each use, since this developing agent lost some of its effectiveness if allowed to stand for any length of time.

The chromatogram was air dried, sprayed with the developing agent and dried again at 150° until the spots appeared (5-10 min.). The colour of the spots intensified after exposure to the air for 24 hours.

Spectrophotometric Analysis A Unicam SP 800 ultraviolet and visible recording spectrophotometer, with standard 3 ml (1 cm light path) cuvettes, was used to study the formation of the brownish colour that appeared in the Warburg flasks during the oxidative decarboxylation of OAA.

Disc Electrophoresis on Polyacrylamide Gel A comparison was made between an enzyme preparation from wheat leaves and

the commercial 'Peroxidase' (Type II) using disc electrophoresis on 7 1/2% polyacrylamide gel according to a modified method of Reisfield et al.(1962). The protein concentrations involved were 1.1 mg/ml and 0.8 mg/ml respectively. Approximately 0.2 ml of each preparation was subjected to the electrophoresis described.

The gels were fixed in 12.5% trichloroacetic acid (TCA) for 30 minutes. The proteins were visualized by staining with 0.025% solution of Comassie Brilliant Blue R 250 in 12.5% TCA for two hours. The destaining of the background was accomplished by the continual washing of the gels in 10% TCA for 24 hours.

IV EXPERIMENTAL RESULTS

1. General Characteristics of OAA Degradation Oxalacetic acid auto-decarboxylates to produce pyruvic acid and carbon dioxide. This auto-decarboxylation of the β-carbonyl group is enhanced considerably by the presence of metallic ions; especially by manganese (Mn^{++}). Since the standard assay system contained Mn^{++}, some of the OAA would be non-enzymatically converted to pyruvate. The number of controls required to estimate the actual amount of OAA remaining available for enzymatic degradation, at any particular time, made this aspect of the problem impractical. It was assumed, therefore, that the extent of this non-enzymatic decarboxylation was the same in comparible systems. Henceforth, when discussing the products of OAA assimilation, pyruvate will not be mentioned although it is invariably present. Only those products resulting from enzymatic oxidative α-decarboxylation of OAA will be considered.

When OAA was added to a dialysed wheat leaf preparation in acetate buffer at pH 5.3, no enzymatic destruction of the acid took place. However, enzymatic destruction of OAA would occur at a very slow rate in the presence of manganous ions. When, in addition to the manganese, 2,4-dichlorophenol (DCP) was present, OAA again was destroyed but at a much greater velocity. The rate of oxygen consumption with the complete system and in the absence of various components is shown in Figure 1.

Figure 1. Effect of cofactors on enzymatic oxidation of OAA.

System: enzyme, 0.42 mg protein; dichlorophenol, 3.0 µmoles; $MnCl_2$, 3.0 µmoles; acetate buffer, 100 µmoles, pH 5.3; OAA, 20 µmoles, pH 3.4 (side arm) added at time zero. Complete, (O–O); minus DCP, ▲–▲; minus Mn, ■–■; minus enzyme, ●–● .

FIGURE 1

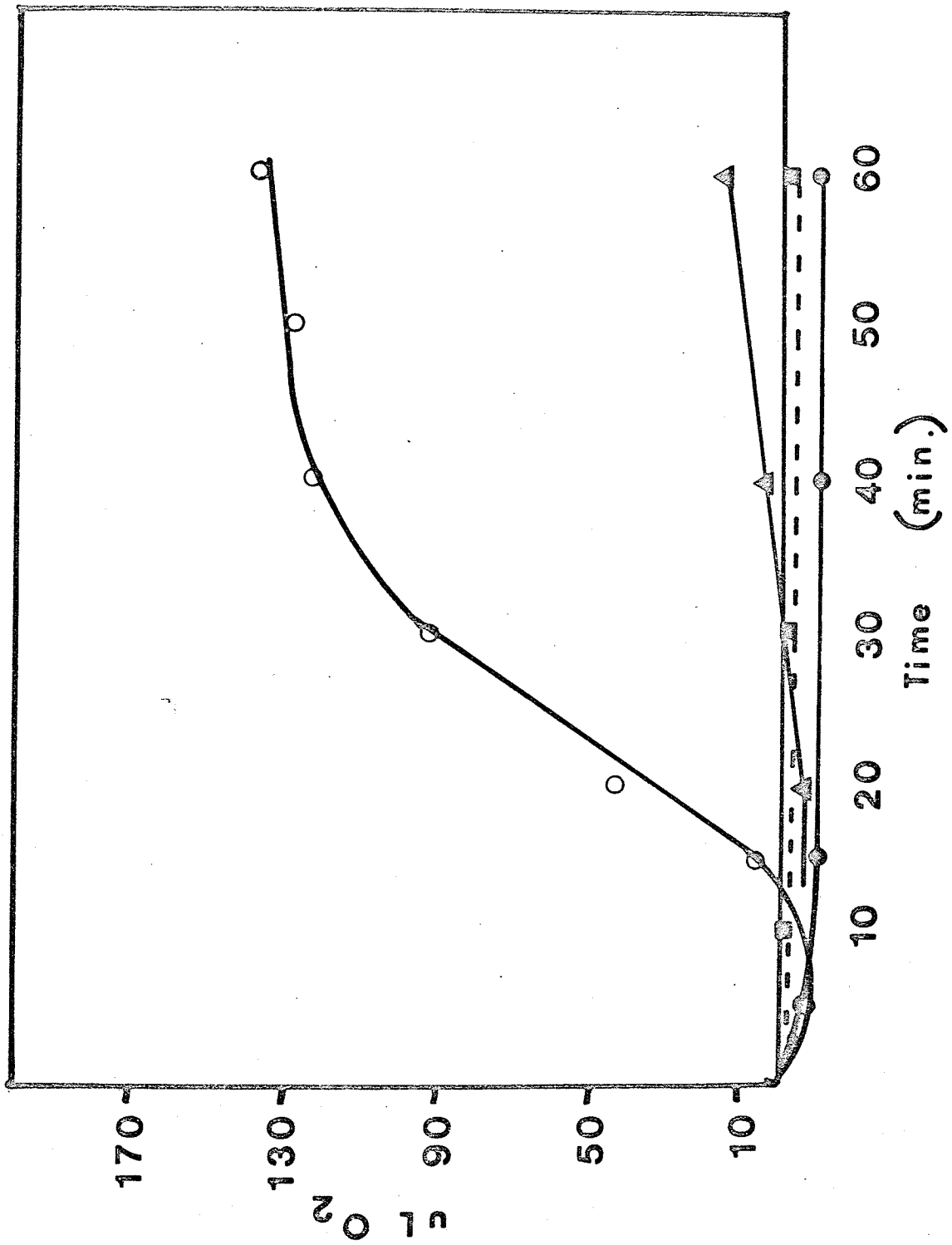


Figure 1 shows that a gas was evolved at the beginning of the reaction (i.e., when the OAA was tipped in from the side arm). Experiments were performed in which both KOH and pyrogallol were present in the central well, but the gas still appeared. Since the amount of gas given off was directly related to the concentration of Mn^{++} employed, it was believed that this gas was CO_2 with possibly some H_2. High concentrations of Mn^{++} caused such a rapid non-enzymatic decarboxylation of OAA that it is possible that the KOH solution was unable to absorb it all in the first few minutes. Where equilibrium was not regained (i.e., the unknown gas was never fully absorbed) it was assumed that the gas was H_2; again resulting from the rapid decarboxylation. The problem was never investigated beyond this point. It should be stated, however, that when low concentrations of Mn^{++} (10^{-5} - 10^{-6}M) were used no gas was detected. A similar occurrence of gas evolution when adding OAA has been previously reported (Kenten and Mann, 1953).

A lag period that lasted from 10 to 20 minutes was a characteristic feature of the progress curve for oxygen uptake in complete systems. This gave the curves a sigmoidal appearance. Although the lag period never could be eliminated completely, it could be varied by changing the concentrations of some of the reacting components.

A most interesting phenomenon occurred during the oxidative decarboxylation of OAA. Immediately prior to the up-

take of oxygen, a faint brown colour appeared in the reaction vessel. This signalled the termination of the lag phase. During the period of rapid oxygen consumption, the colour intensified. The subsequent fading of this brown colour coincided with that portion of the curve which signified that oxygen uptake had ceased. At the end of a 60 minute experiment the system in the flask was again clear.

2. Enzyme Concentration The velocity and total amount of oxygen uptake were found to be closely related to the enzyme concentration (Table 1). At the lowest protein level the total oxygen consumption was one quarter that at the highest concentration. The velocities (Table 1, Fig.2) showed the same 1:4 relationship.

TABLE I. EFFECT OF ENZYME CONCENTRATION ON THE VELOCITY AND TOTAL AMOUNT OF OXYGEN UPTAKE

Enzyme (mg protein)	Total oxygen uptake (µls)	Velocity (µl/5 min)
0.98	130	35
0.56	134	33
0.28	110	28
0.14	83	21
0.07	56	17
0.03	31	8

SYSTEM: acetate buffer, 100 µmoles, pH 5.3; dichlorophenol, 3.0 µmoles; $MnCl_2$, 3.0 µmoles; OAA, 20 µmoles, pH 3.2 (side arm) added at time zero.

Figure 2. Effect of enzyme concentration on OAA oxidation with optimum cofactors.

SYSTEM: enzyme, 1.1 mg/ml; dichlorophenol, 3.0 µmoles; $MnCl_2$, 3.0 µmoles; acetate buffer, 100 µmoles, pH 5.3; OAA, 20 µmoles, pH 3.2 (side arm) added at time zero.

Figure 3. Effect of enzyme concentration on the length of the lag period.

SYSTEM: enzyme, 0.77 mg/ml; dichlorophenol, 3.0 µmoles; $MnCl_2$, 3.0 µmoles; acetate buffer, 100 µmoles, pH 5.3; OAA, 20 µmoles, pH 3.2 (side arm) added at time zero.

FIGURE 2

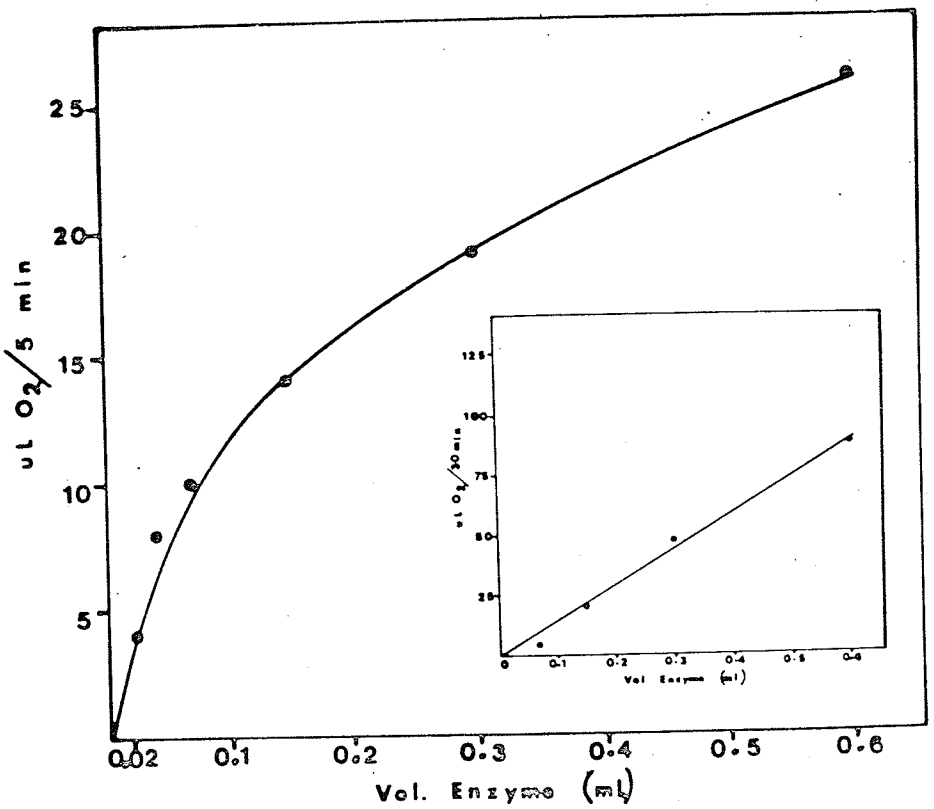
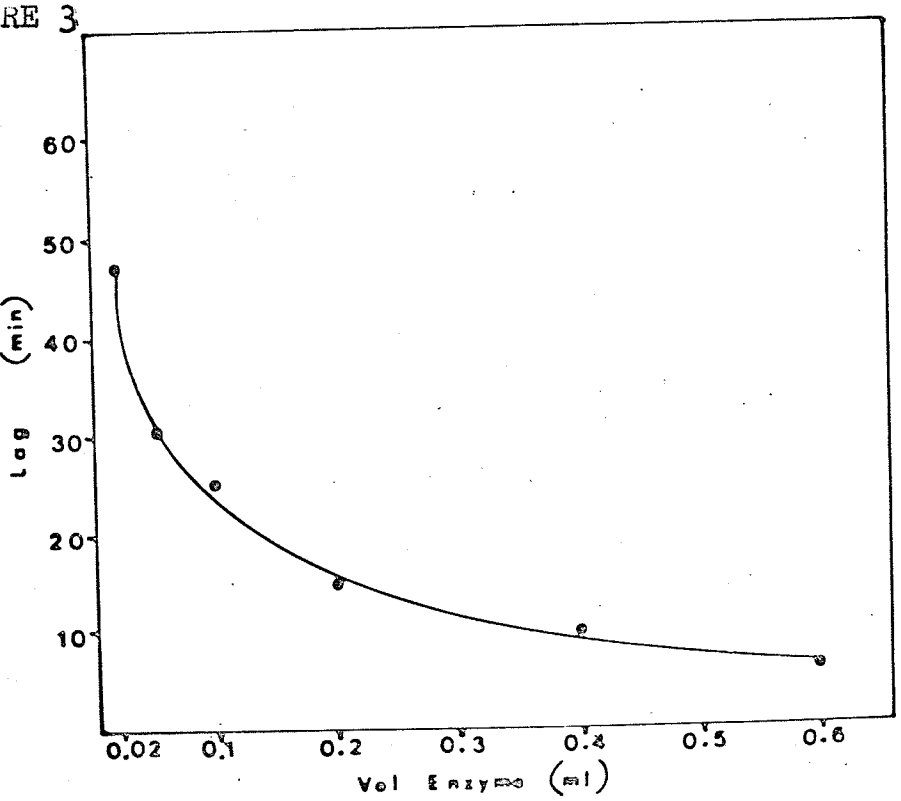


FIGURE 3



Variation of the wheat leaf enzyme concentrations produced substantial differences in the length of the lag period. When the enzyme concentration was low (0.01 mg protein), the lag phase (Fig.3) lasted 10 times longer then when enzyme concentration was high (0.49 mg protein). In the standard assay system, the enzymatic reaction competes with the non-enzymatic reaction for the available OAA molecules. When dealing with low enzyme concentrations and therefore longer lag periods, the amount of α-decarboxylation is greater. As a result the amount of OAA remaining after a 30 min. lag is considerably lower than after a 5 min.lag. This would explain both the reduced velocities and lower total oxygen uptake which occur at low enzyme concentrations.

The onset of 'browning', which occurs immediately prior to measurable oxygen uptake, appeared earlier and with greater intensity when higher enzyme concentrations were employed.

To ascertain whether or not the dialysed wheat leaf enzyme oxidized the OAA in the same manner as the enzymes prepared from other sources (see METHODS AND MATERIALS), these alternates were used for the purpose of comparison. The various preparations were tested manometrically with the standard reaction system. These preparations (including purified 'Peroxidase') demonstrated the same pattern of oxygen uptake as did the wheat leaf enzyme preparation. The lag

phases lasted 10 to 20 minutes, and the brown colour appeared and disappeared in the same manner as previously described (see General Characteristics).

3. Substrate Concentration Neither oxygen uptake, nor the browning phenomenon occurred in the absence of OAA. The amount of OAA used influenced the intensity of the brown colour. At low substrate concentrations (below ca. 10 μmoles) the colour was absent or faint. As the OAA concentration was increased, corresponding increases occurred both in the colour intensity and in the length of time the colour remained visible. But, as previously mentioned, the colour remained only so long as oxygen uptake was occurring.

TABLE II. EFFECT OF OAA CONCENTRATION ON THE LAG PERIOD AND VELOCITY

OAA conc. (μmoles)	Time of Browning (min.)	Time of O_2 uptake (min.)	Velocity (μl/5 min)
100	11	15	33
80	11	20	30
60	11	20	28
40	11	15	22
20	11	15	17

SYSTEM: enzyme, 0.7 mg protein; dichlorophenol, 3.0 μmoles; $MnCl_2$, 3.0 μmoles; acetate buffer, 100 μmoles, pH 5.3; OAA, varied, pH 3.5 (side arm) added at time zero.

When the OAA concentration was greater than 10 µmoles, the brown colour would appear, and the time of appearance was not influenced by the OAA concentration (Table II). At OAA concentrations of 20 and 40 µmoles, the colour intensity began to increase earlier than at higher OAA levels. Actually, the higher concentrations of OAA delayed the uptake of oxygen. It is doubtful that the OAA causes inhibition through the normal manner of substrate inhibition, since the velocities increase.

Substrate concentration experiments were performed in which the OAA was incubated, for 20 minutes, with the standard components; and the DCP was placed in the side arm. Time zero marked the point at which the DCP was tipped. Table III summarizes these results.

TABLE III. EFFECT OF OAA CONCENTRATION ON THE LAG PERIOD AND VELOCITY WHEN THE SUBSTRATE HAS BEEN PRE-INCUBATED

OAA conc. (µmoles)	Time of browning	Time of O_2 uptake (min.)	Velocity (µl/5 min.)
100	21	25	36
80	14	20	30
60	11	15	33
40	6	10	32
20	2	10	21
10	-	5	18

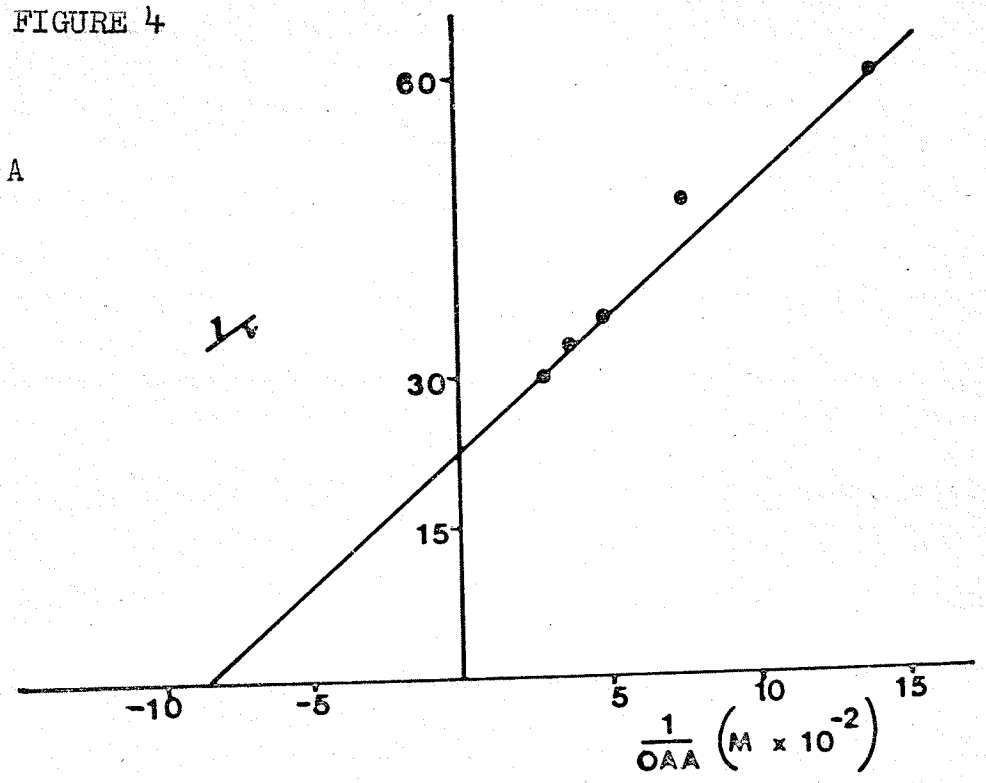
SYSTEM: enzyme, 0.7 mg protein; $MnCl_2$, 3.0 µmoles; acetate buffer, 100 µmoles, pH 5.3; OAA, varied, pH 3.5; dichlorophenol, 3.0 µmoles (side arm) added at time zero.

Figure 4. Lineweaver-Burke plot of OAA (a) and ketomalonate (b) with optimum cofactors.

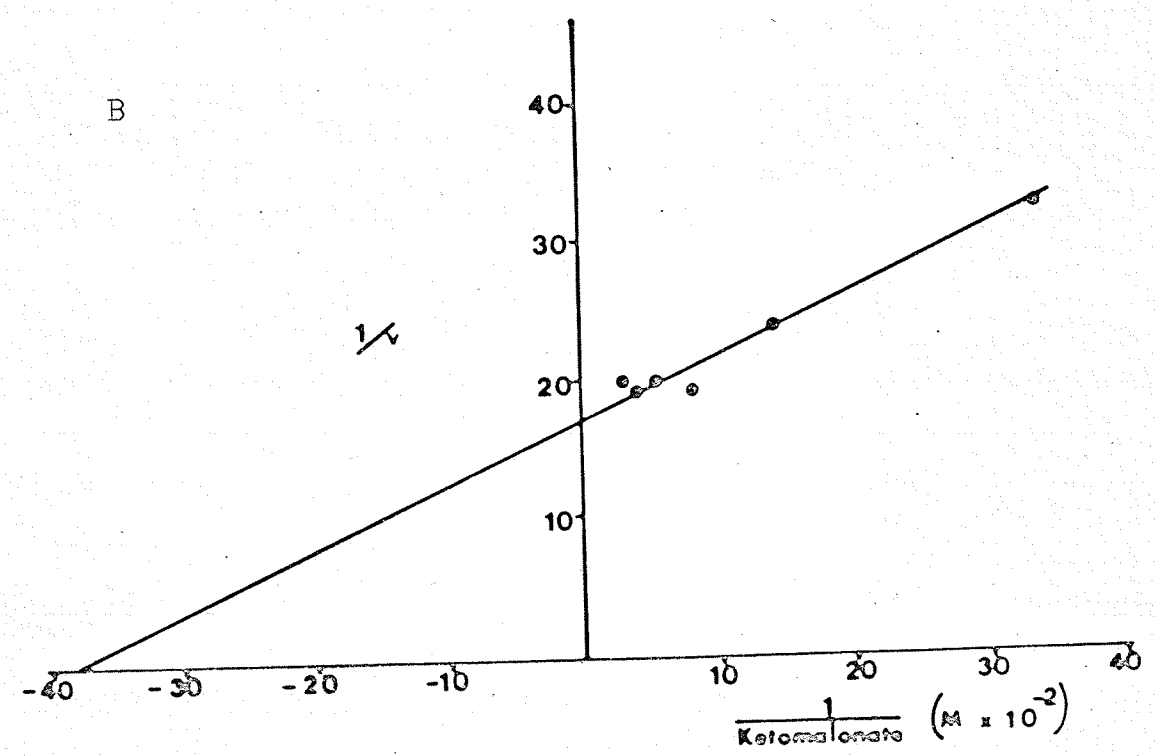
SYSTEM: enzyme, 0.7 mg protein; dichlorophenol, 15 μmoles; $MnCl_2$, 3.0 μmoles; acetate buffer, 100 μmoles, pH 5.3; substrates varied, added from side arm.

FIGURE 4

A


60
30
15
1/v
-10
-5
5
10
15
1/OAA (M x 10^-2)

B


40
30
20
10
1/v
-40
-30
-20
-10
10
20
30
40
1/Ketomalonate (M x 10^-2)

With pre-incubation, the higher levels of OAA delayed both the onset of browning and oxygen uptake. At low amounts of OAA the appearance of the brown colour was earlier (2 min.) than if the system described in Table II was employed.

Other organic acids with structures similar to OAA were tried in the standard system with the wheat leaf enzyme preparation. This was done as a possible means of determining a reaction mechanism. Of those acids used (Table IV), only IAA exhibited both oxygen uptake and the browning phenomenon. Oxygen uptake occurred in the presence of ketomalonate but the brown colour was not observed.

TABLE IV. EFFECT OF OTHER ORGANIC ACIDS IN THE STANDARD ASSAY SYSTEM.

Organic acid	Browning	Oxygen uptake
pyruvate	-	-
malonate	-	-
α-ketoglutarate	-	-
oxalate	-	-
ketomalonate	-	+
glyoxalate	-	-
glycollate	-	-
indoleacetate	+	+

In each case 20 µmoles of the acid was tipped in from the side arm. Although no browning was visible with ketomalonate as the substrate, the reaction mixture did become cloudy. The lag period at all concentrations of this acid,

was invariably 5 minutes. The Km for OAA (Fig.4a) was calculated to be between 0.8 and 1.1 X 10^{-3}. This is very close to the Km value of IAA as found by Maclachlan (1956). The Km for ketomalonate (Fig.4b) was approximately 2.6 X 10^{-4}.

4. Manganese Concentration Manganese was found to be essential for oxygen uptake. Occasionally a small amount of oxygen uptake occurred when manganese was omitted. This could be attributed to endogenous manganese not removed by the dialysis (Maclachlan and Waygood, 1956b). The effect of varying the manganese concentration from 10^{-6} M to 10^{-1} M on the rate of oxidation of OAA by wheat leaf extracts is shown in Figure 5.

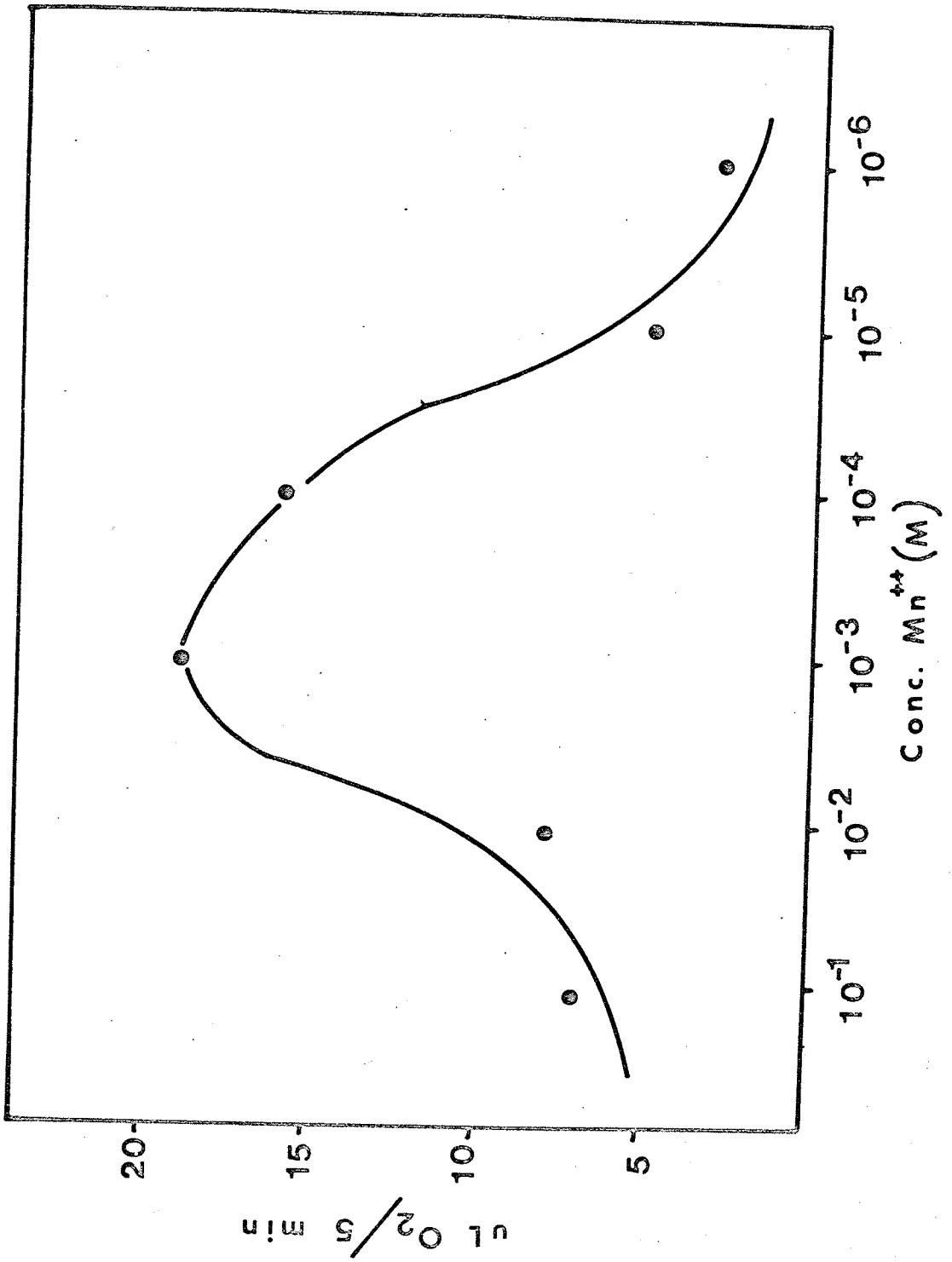
The decarboxylation of OAA to pyruvate is greatly accelerated by the presence of Mn^{++}. As a result, it is suggested that the manganese competes with the enzyme for the available OAA molecules. This would explain the increased lag periods and reduced velocities found when Mn^{++} exceeded 10^{-3} M. The optimum Mn^{++} concentration was between 10^{-3} M and 10^{-4} M. However, because of the rapid non-enzymatic decarboxylation incurred at higher Mn^{++} concentrations, the actual enzymic effect at these levels of Mn^{++} was difficult to determine.

It was of interest, when considering mechanism, to design experiments for the purpose of showing whether or not the enzyme and Mn^{++} were binding (interdependant). At low enzyme concentrations (Figure 6a), the progress curves of the reaction with 10^{-4} M and 10^{-3} M Mn^{++} appeared to be similar.

Figure 5. Effect of manganese on the rate of OAA oxidation.

SYSTEM: enzyme, 0.3 mg protein; dichlorophenol, 3.0 μmoles; acetate buffer, 100 μmoles, pH 5.3; $MnCl_2$, varied; OAA, 20 μmoles, pH 3.2, added from the side arm.

FIGURE 5



The velocities were comparable, with the 10^{-3} M being only slightly greater. If, however, a high concentration of enzyme was used (Figure 6b), the progress curves showed a marked difference. The velocity attained with the higher (10^{-3} M) Mn^{++} level was much greater. Figure 6 also demonstrates the extent to which the Mn^{++} concentration affects the evolution of the 'unknown' gas and the lag period.

When Mn^{++} (10^{-4} M) was the limiting factor, increased enzyme concentration would not increase the rate of oxidation (Figure 6c). If, however, Mn^{++} was present in the excess (10^{-3} M) the rate of oxidation increased with increasing enzyme concentration. The rate of oxidation appears, therefore, to be dependent upon the interaction and probable binding of the metal and the enzyme.

Some workers (Shannon et. al., 1963) have found that by the incubation of the enzyme with Mn^{++}, prior to the addition of the substrate, the lag period could be eliminated. This could not be verified by our procedures.

Additional experiments were performed in which the Mn^{++} was placed in the side arm and the OAA incubated with the enzyme. No matter how long the OAA was incubated with the enzyme, a lag phase of 7 to 10 minutes occurred after the Mn^{++} was added. Both the OAA and the Mn^{++} must be present with the enzyme before the reaction complex responsible for oxygen uptake can be formed (see Reaction Sequence).

Figure 6 Oxidation of OAA at varying concentrations of enzyme and manganese

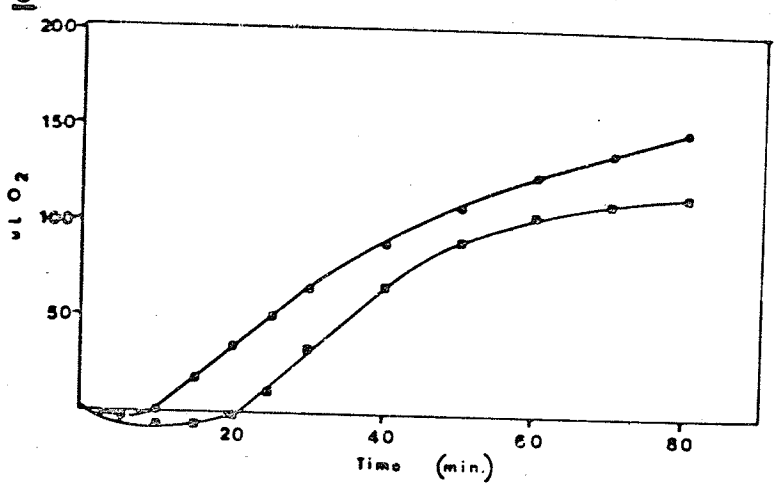
SYSTEM: A. enzyme, 0.28 mg protein; dichlorophenol, 3.0 µmoles; $MnCl_2$, 1 X 10^{-3} M, (■-■); 1 X 10^{-4} M, (●—●); acetate buffer, 100 µmoles, pH 5.3; OAA, 20 µmoles, pH 3.5, added from side arm at time zero.

SYSTEM: B. enzyme, 0.7 mg protein; dichlorophenol, 3.0 µmoles; $MnCl_2$, 1 X 10^{-3} M (■—■), 1 X 10^{-4} M, (●—●); acetate buffer, 100 µmoles, pH 5.3; OAA, 20 µmoles, pH 3.5, added from side arm at time zero.

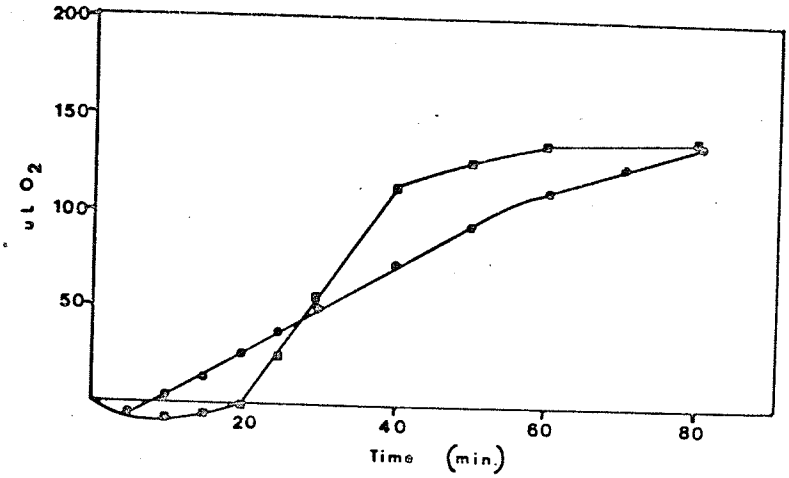
SYSTEM: C. enzyme, conc. varied; dichlorophenol, 3.0 µmoles; $MnCl_2$, 1 X 10^{-3} M (■—■), 1 X 10^{-4}, (●—●); acetate buffer, 100 µmoles, pH 5.3; OAA, 20 µmoles, pH 3.5, added from side arm at time zero.

FIGURE 6

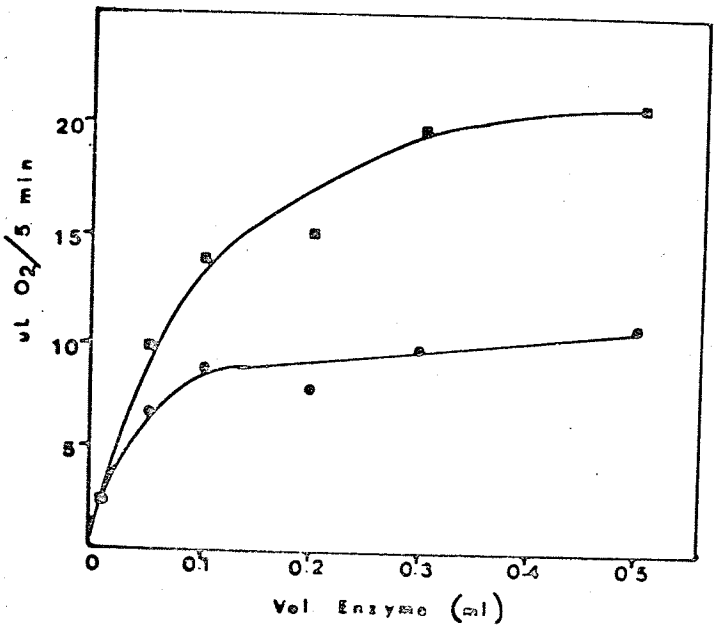
A


200
150
100
50
ul O2
20
40
60
80
Time (min.)

B


200
150
100
50
ul O2
20
40
60
80
Time (min.)

C


20
15
10
5
ul O2/5 min
0
0.1
0.2
0.3
0.4
0.5
Vol Enzyme (ml)

5. Dichlorophenol Concentration The enzymatic oxidation of OAA in the presence of manganese was stimulated by the addition of DCP (Fig.1). If DCP was omitted from the standard system, the lag period prior to oxidation was increased substantially. The effect of varying the DCP concentration from 1 X 10^{-5} M to 1 X 10^{-3} M on the rate of OAA oxidation is shown in Figure 7. In the concentration range 10^{-2} M to 5 X 10^{-5} M there was no variation in the duration of the lag. However, the velocities of the reaction at this range of co-factor did vary (Fig.7). The higher the concentration of DCP (up to 5 X 10^{-3} M) the greater the rate of oxidation became. The lag period increased by ca. 5 minutes when the DCP concentration fell below 5 X 10^{-5} M.

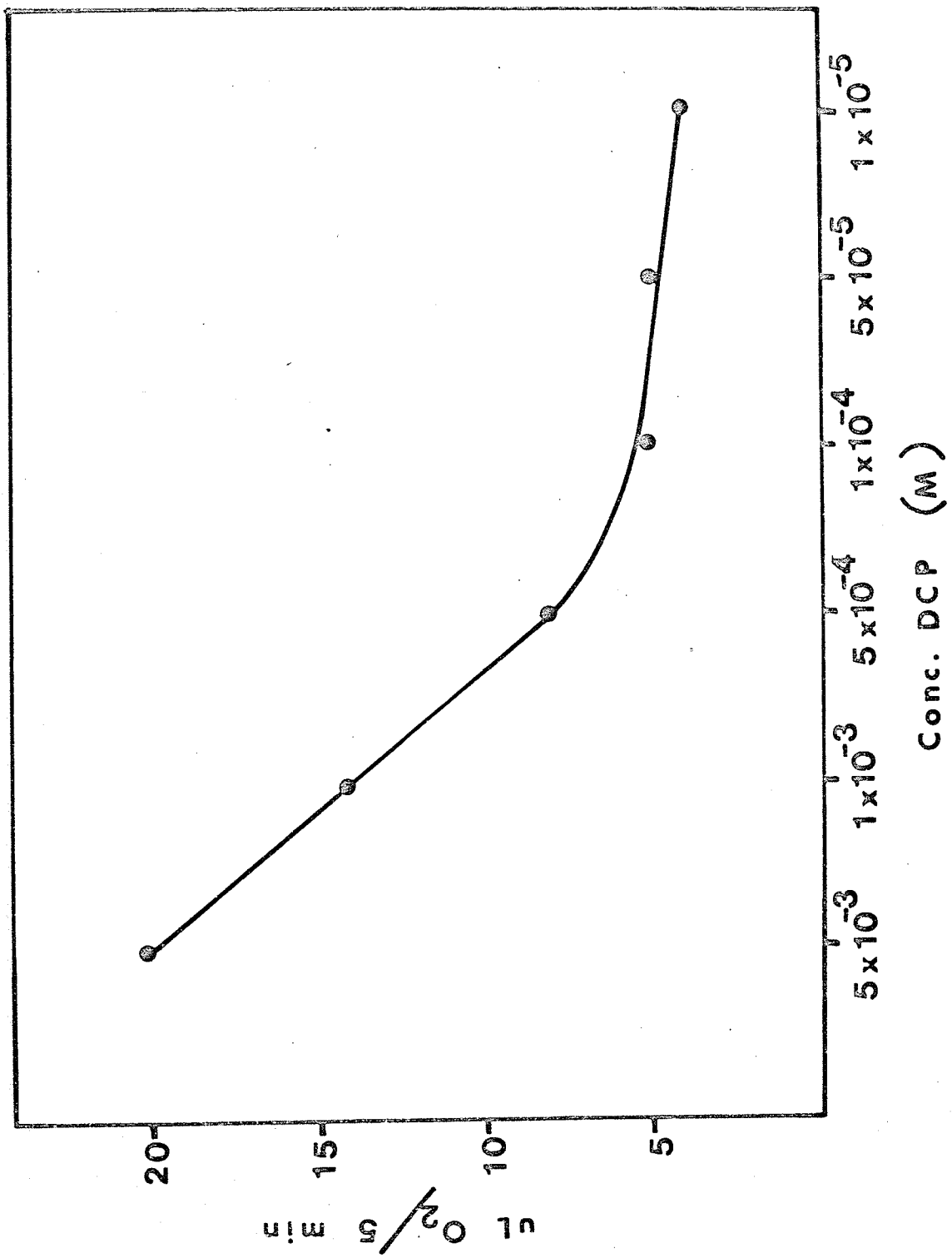
High concentrations of DCP (10^{-2} M and over) produced a whitish floculation which appeared even before the OAA was added. Usually, when this occurred, no oxygen uptake took place. Occasionally, even with the floculant, the oxidation commenced after the normal lag period. In these cases, the rate of oxidation was lower than that occurring at 10^{-3} M DCP, but the lag periods were the same. The reaction rates, in the presence of this precipitate, were compariable to those produced by a low enzyme concentration. It is likely, therefore, that partial (or total) enzyme denaturation results at the higher concentration of DCP.

When the 'standard' concentration of Mn^{++} (10^{-4} M) was

Figure 7. Effect of 2,4-dichlorophenol on the rate of OAA oxidation.

SYSTEM: enzyme, 0.3 mg protein; dichlorophenol, varied; $MnCl_2$, 3.0 μmoles; acetate buffer, 100 μmoles, pH 5.3; OAA, 20 μmoles, pH 3.2, added from side arm.

FIGURE 7


20
15
10
5
µL O2/5 min
5x10-3
1x10-3
5x10-4
1x10-4
5x10-5
1x10-5
Conc. DCP (M)

present, the intensity of the browning was related to the concentration of the DCP. If the Mn^{++} concentration was halved, the intensity was again proportional to the DCP concentration; but was less than when the higher concentration of Mn^{++} was used. At 1.5 umoles Mn^{++}, the velocity of oxygen uptake was also less (Fig.5). Therefore the browning, although accentuated by the DCP, is not necessarily related to the concentration of this cofactor.

As a comparison, resorcinol was substituted for DCP. The progress curves were similar to those of DCP, and the lag was terminated by the onset of the browning phenomena.

If the DCP was placed in the side arm, and the OAA incubated with the other components in the main chamber of the flask, no browning occurred. However, if after this incubation (ca. 15 min.) the DCP was tipped in, the brown colour appeared almost immediately (see Table III).

6. Decarboxylation When measuring the decarboxylation of OAA, precautions were taken to distinguish between the amounts of enzymatic and non-enzymatic CO_2 evolved. Before the lag period ended, the combined decarboxylation of enzymatic and non-enzymatic reactions was greater than the non-enzymatic alone (Figure 8). This would imply, even though the time difference was small, that enzymatic decarboxylation occurs before oxygen uptake. Waygood et al. (1956b), using indoleacetic acid as the substrate showed that CO_2 evolution occurred

Figure 8. The ratio of decarboxylation and oxidation with OAA as substrate.

SYSTEM: enzyme, 0.5 mg protein; dichlorophenol, 3.0 µmoles; $MnCl_2$, 3.0 µmoles; acetate buffer, 100 µmoles, pH 5.3; OAA, 20 µmoles, pH 3.4, (side arm) added at time zero. Non-enzymatic had water replacing enzyme.
CO_2 evolution, (○-○); O_2 uptake, (●-●)
non-enzymatic CO_2 evolution, (■-■).

Figure 9. A comparison of decarboxylation and oxidation of the substrates OAA and ketomalonate.

SYSTEM: enzyme, o.5 mg protein; dichlorophenol, 3.0 µmoles; $MnCl_2$, 3.0 µmoles; acetate buffer, 100 µmoles, pH 5.3; OAA, or Ketomalonate, 20 µmoles, pH 3.2 (side arm) added at time zero.
CO_2 evolution, (○-○); O_2 uptake, (●-●).

FIGURE 8

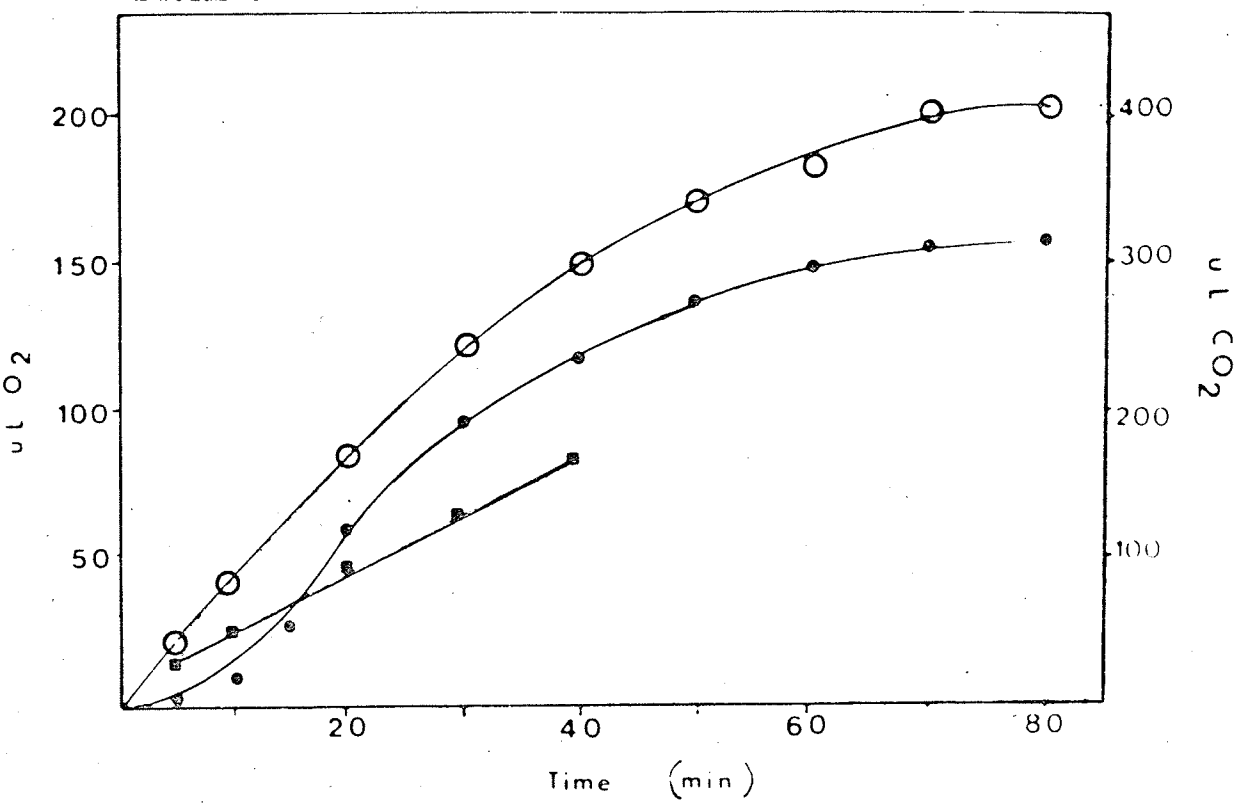
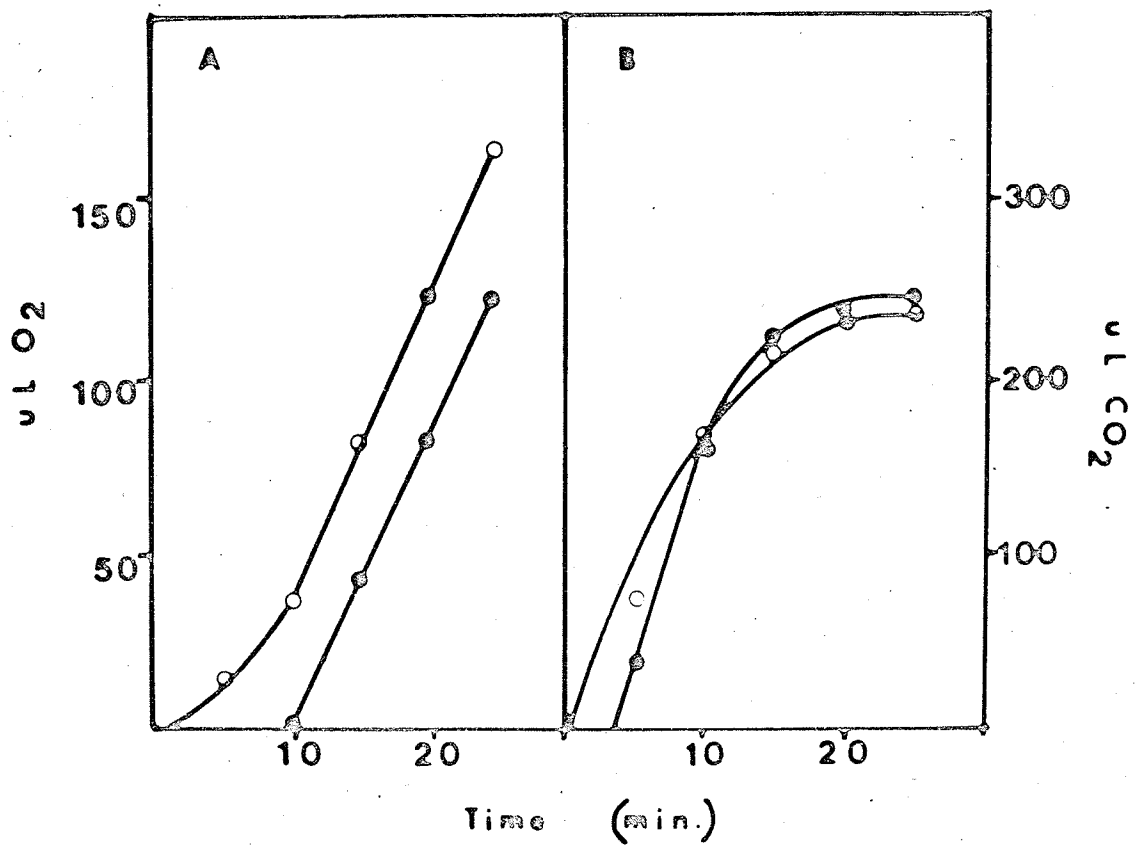


FIGURE 9



prior to O_2 consumption.

The total amount of CO_2 given off during the metabolism of 20 umoles of OAA was 395 ul. The non-enzymatic reaction accounted for about 100 ul (Figure 8). The total O_2 uptake after 80 minutes was 160 ul. These figures closely fit the ratio of 1/2 O_2 consumed per CO_2 evolved per mole of OAA as previously reported (Vennesland et al., 1964; Shannon et al., 1963). But they differ from the 1:1 ratio that Waygood et al. (1956a) found with IAA.

Ketomalonate, which does not auto-decarboxylate, was tested in a similar manner. This led to a more concise picture of the events at time zero (Figure 9). As with OAA, enzymatic decarboxylation of ketomalonate precedes its oxidation. The ratio of 1 CO_2 per 1/2 O_2 was more precise and suggests a similar reaction mechanism. The enzymatic decarboxylation occurred more rapidly with ketomalonate than with OAA and showed a shorter lag period (Fig.9) and a lower Km (Fig.4b).

7. Hydrogen Peroxide The simultaneous addition of H_2O_2 and OAA to the reaction system greatly reduced the lag period. When H_2O_2 was added to the standard system, the brown colour appeared immediately. Therefore, the oxidation of OAA in the presence of H_2O_2 was studied with, and without, the various components. Table V summarizes the results.

TABLE V. THE EFFECT OF HYDROGEN PEROXIDE ON THE OXIDATION OF OAA IN THE PRESENCE AND ABSENCE OF THE COFACTORS

System	Time of O_2 uptake (min.)	Total ul oxygen
complete	less than 10	165
- DCP	15	125
- Mn	-	-
- OAA	-	-
- enzyme	-	-
- H_2O_2	15	175

SYSTEM: enzyme, 0.45 mg protein; dichlorophenol, 3.0 µmoles; $MnCl_2$, 3.0 µmoles; acetate buffer, 100 µmoles, pH 5.3, OAA, 20 µmoles, pH 3.2 (side arm a); H_2O_2, 5-10 µmoles, (side arm b). Tipping of OAA and H_2O_2 at time zero.

Browning colouration did not occur if either enzyme or DCP were omitted. In the absence of Mn^{++}, the reaction mixture became cloudy and only faintly brown. In all other cases the brown colour appeared immediately upon the tipping in of the peroxide.

8. Spectrophotometric Analysis Since the browning phenomenon appears to be of significance in the oxidative decarboxylation of OAA, a more detailed study of it might result in a better understanding of the enzyme mechanism involved. Therefore, the following series of spectrophotometric analysis were performed.

Figure 10. Spectrum of the appearance and disappearance of the brown colour during the oxidation of OAA.

SYSTEM: enzyme, 0.5 mg protein; dichlorophenol, 15 µmoles; $MnCl_2$, 3.0 µmoles; acetate buffer, 100 µmoles, pH 5.3; OAA, 20 µmoles, pH 3.4 added at time zero. Total volume 3.0 ml with water replacing enzyme in blank.

A. scan at time 2 min.
B. scan at time 15 min.
C. scan at time 17 min.
D. scan at time 20 min.
E. scan at time 25 min.
F. scan at time 30 min.

Figure 11. Spectrum of enzyme in the presence of some cofactors.

SYSTEM: A. enzyme, 0.75 mg protein; acetate buffer, 100 µmoles,,pH 5.3. Total volume 3.0 ml.

SYSTEM: B. enzyme, 0.75 mg protein; acetate buffer, 100 µmoles, pH 5.3; $MnCl_2$, 3.0 µmoles. Total volume 3.0 ml.

SYSTEM: C. enzyme, 0.75 mg protein; acetate buffer, 100 µmoles, pH 5.3; OAA, 20 µmoles, pH 3.4. Total volume 3 ml.

B and C were scanned twenty minutes after the constitutents were mixed.

FIGURE 10

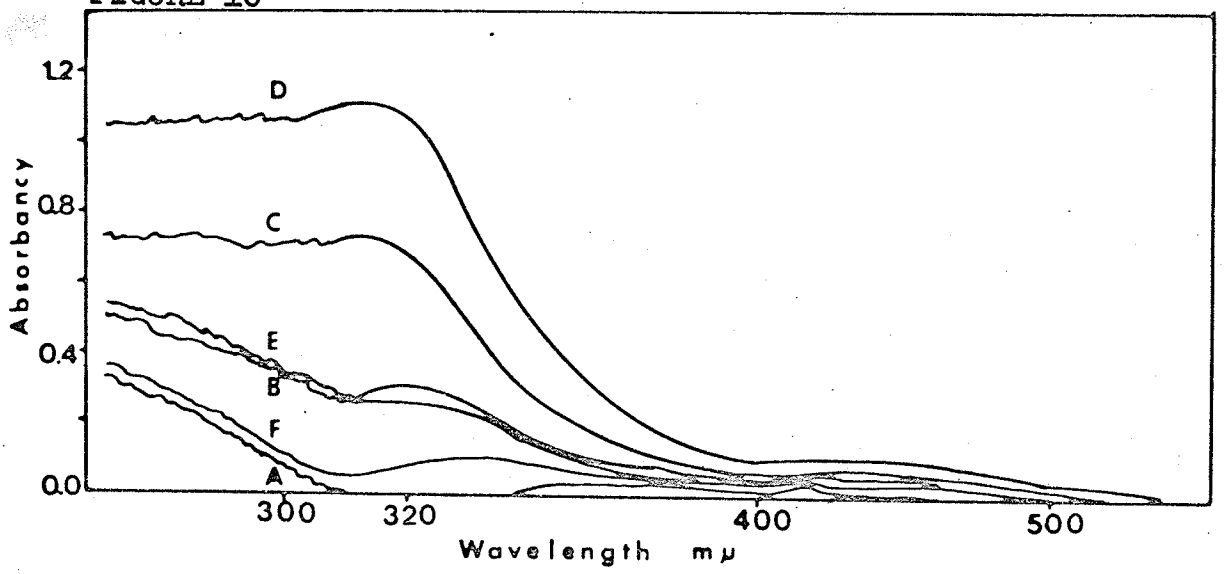
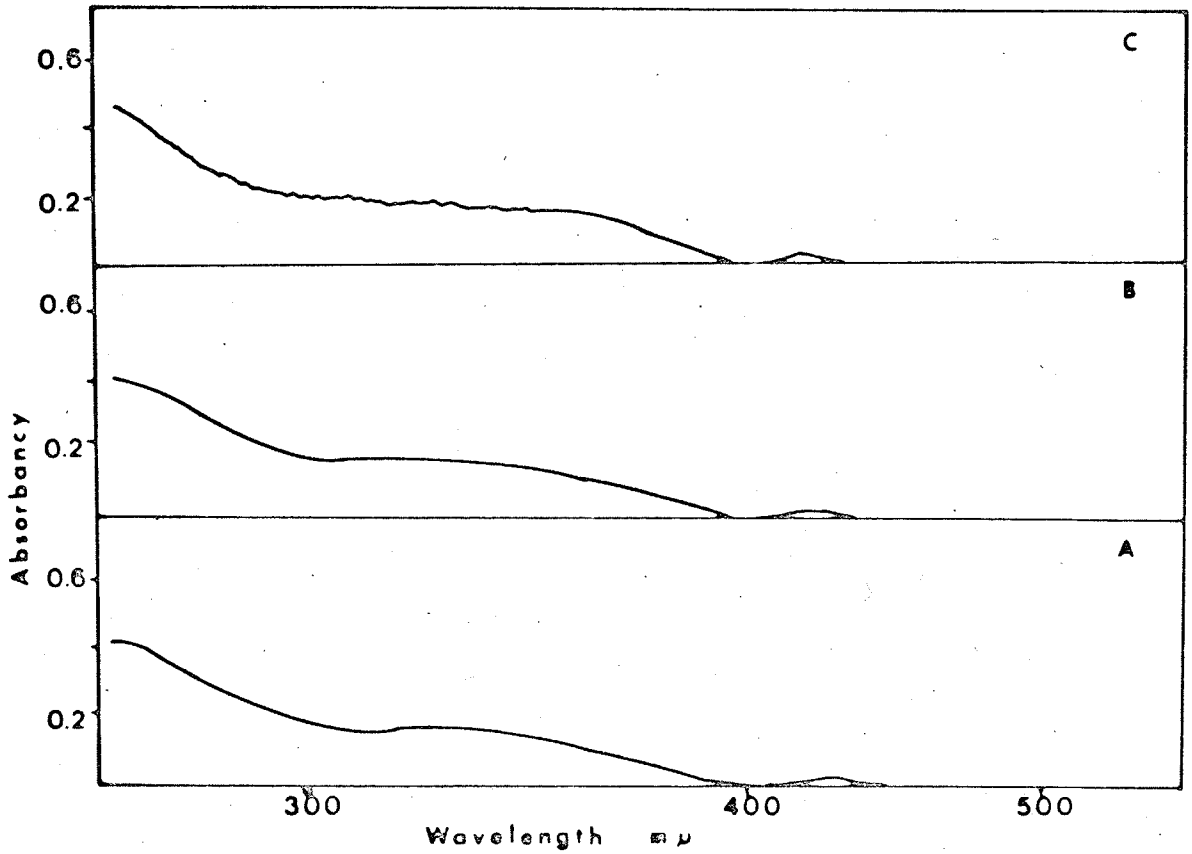


FIGURE 11



Preliminary experiments showed that, with the exception of a small peak in the Soret region (410-420 mμ), no absorption occurred in the visible-light range of the spectrum. The peak at 420 mμ indicated the presence of a ferroporphyrin structure characteristic of peroxidases.

The appearance and disappearance of the brown colour during the oxidative decarboxylation of OAA was studied spectrophotometrically (Figure 10). After a lag phase of 15 to 20 minutes, an absorption band appeared at approximately 310 mμ. This increased with time. Since the extent of the absorbancy at 310 mu corresponded to the intensity of the browning observed visually, it was assumed that this resulted from the presence of the coloured intermediate. After ca. 30 minutes the colour faded from the cuvette, with a subsequent decrease in the absorbancy measured at 310 mμ.

In this system the lag period prior to the occurrance of the peak at 310 mμ was longer than the lag period found when the same system was studied manometrically. The colour intensity seen in the respirometer flasks was much greater than that seen in the cuvettes. Also, the length of time that the brown colour remained was shorter when the cuvettes were used. These features could be explained by the fact that the cuvettes expose only a small portion of the reaction mixture (1 cm^2) to the air. Since oxygen is absolutely essential for the reaction (see Anaerobic), the limited exposure to the air

afforded by the cuvettes, presumably, was responsible for the slight discrepancies mentioned.

Experiments were performed in which the colour formation was examined in the presence and absence of the various cofactors. The band at 310 mμ was absent if only the enzyme and OAA, or if only the enzyme and Mn were present (Figure 10). If, however, enzyme, OAA and Mn were present together only a slight peak appeared at 310 mμ (Figure 12). The degree of absorption suggests that the amount of intermediate formed was quite small. Although this quantity was measurable with the spectrophotometer, no colour was noticed visually. In similar experiments (i.e., minus DCP) using manometers, it was found that slow oxygen uptake would occur without the brown colour (see section on DCP). Figure 12 illustrates that the brown intermediate was being formed, but in amounts too small to be detected visually. This implies that an intermediate, responsible for oxygen consumption, can be formed by the enzyme, OAA and Mn reacting together in the absence of DCP.

If enzyme, OAA, and Mn were allowed to incubate for 10 minutes, the slight peak which occurred at 310 mu intensified immediately upon the addition of DCP (Figure 13). The time required for this increased absorbancy was brief. The data for Figure 11, where DCP was present from time zero, show a lag of 20 - 25 min. before the 310 mμ peak appeared.

Figure 12. Spectrum of enzyme-OAA mixture in the absence and presence of Mn.

SYSTEM: enzyme, 0.5 mg protein; acetate buffer, 100 µmoles, pH 5.3; OAA, 20 µmoles, pH 3.4; $MnCl_2$, 3.0 µmoles, added after ScanA. Total volume 3.0 ml with water replacing enzyme in the blank.

A. scan of the enzyme-OAA mixture.
B. scan of the enzyme-OAA mixture after the addition of Mn. This was designated as time zero.
C. scan at time 10 min.
D. scan at time 15 min.

Figure 13. Spectrum of the enzyme-OAA-Mn mixture in the absence and presence of DCP.

SYSTEM: enzyme, 0.5 mg protein; $MnCl_2$, 3.0 µmoles; acetate buffer, 100 µmoles, pH 5.3; OAA, 20 µmoles, pH 3.4; DCP, 15 µmoles, added after Scan B. Total volume 3.0 ml with water replacing the enzyme in the blank.

A. scan of enzyme-OAA-Mn mixture at time zero.
B. scan of enzyme-OAA-Mn mixture at time 5 min.
C. scan of enzyme-OAA-Mn mixture at time immediately after addition of DCP.

FIGURE 12

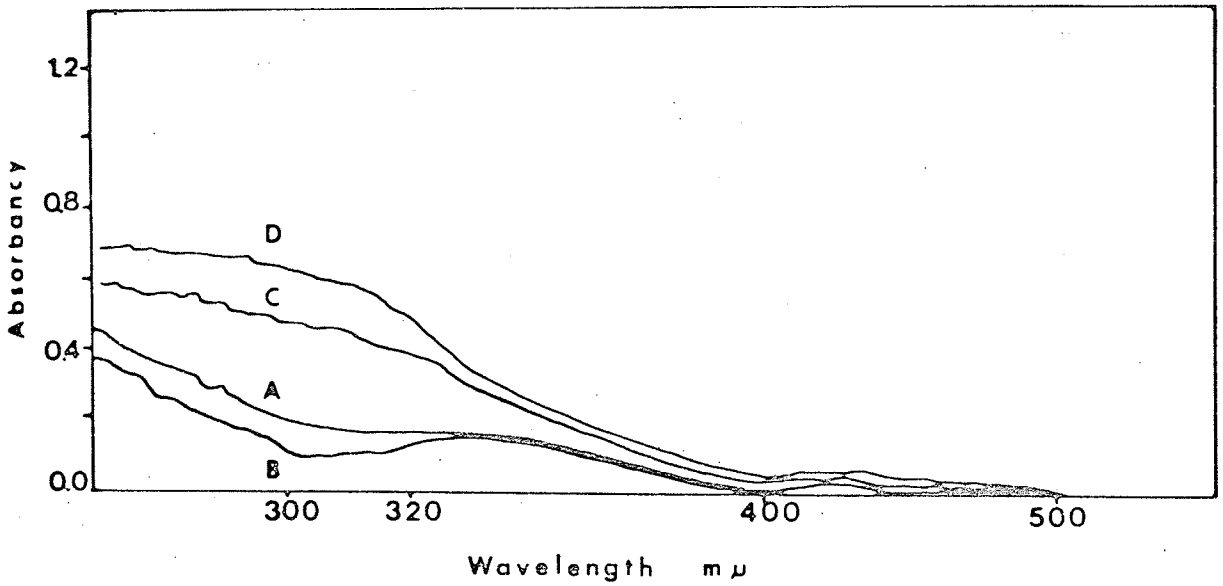
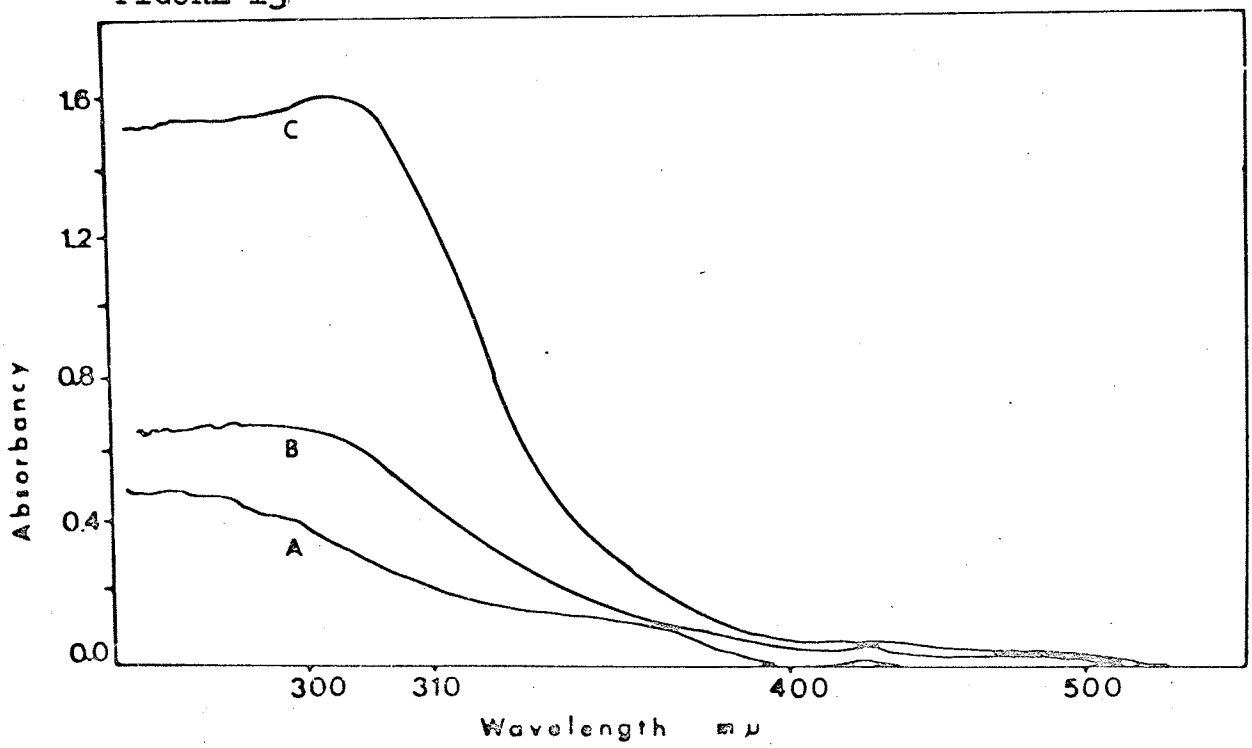


FIGURE 13



This suggests that DCP is required only after enzyme, OAA and Mn have interacted. The complex formed by this interaction is then accentuated by the phenol. This accentuation was expressed by the appearance of the brown colour.

If H_2O_2 was added to those cuvettes containing the complete reaction system, the 310 mu peak formed instantly (Figure 14). The absorbancy was much greater than that found in a complete system devoid of H_2O_2 (i.e., Fig.10). Further experiments, as confirmation of those done manometrically, were performed to observe the effects of H_2O_2 on a reaction mixture from which various cofactors had been omitted. If DCP was omitted, the addition of the peroxide produced only a slight peak at 310 mu (Figure 15a). In the absence of Mn^{++}, the addition of peroxide caused the peak at 310 mu to form immediately (Figure 16). However, the reaction solution in the cell was a cloudy brown (similar to the manometric observations). Although Figure 15b showed a definite peak at 310 mu, the spectrum produced was not the same as that found in a 'standard' system (cf., Fig.10).

The reaction mixture, when OAA was omitted, browned immediately upon the addition of H_2O_2 (Figure 16), and became cloudy. Again, the spectrum produced was not the same as that shown in Figure 11.

The requirements for a phenolic cofactor can be met by resorcinol as well as DCP. In earlier work the resorcinol

FIGURE 14

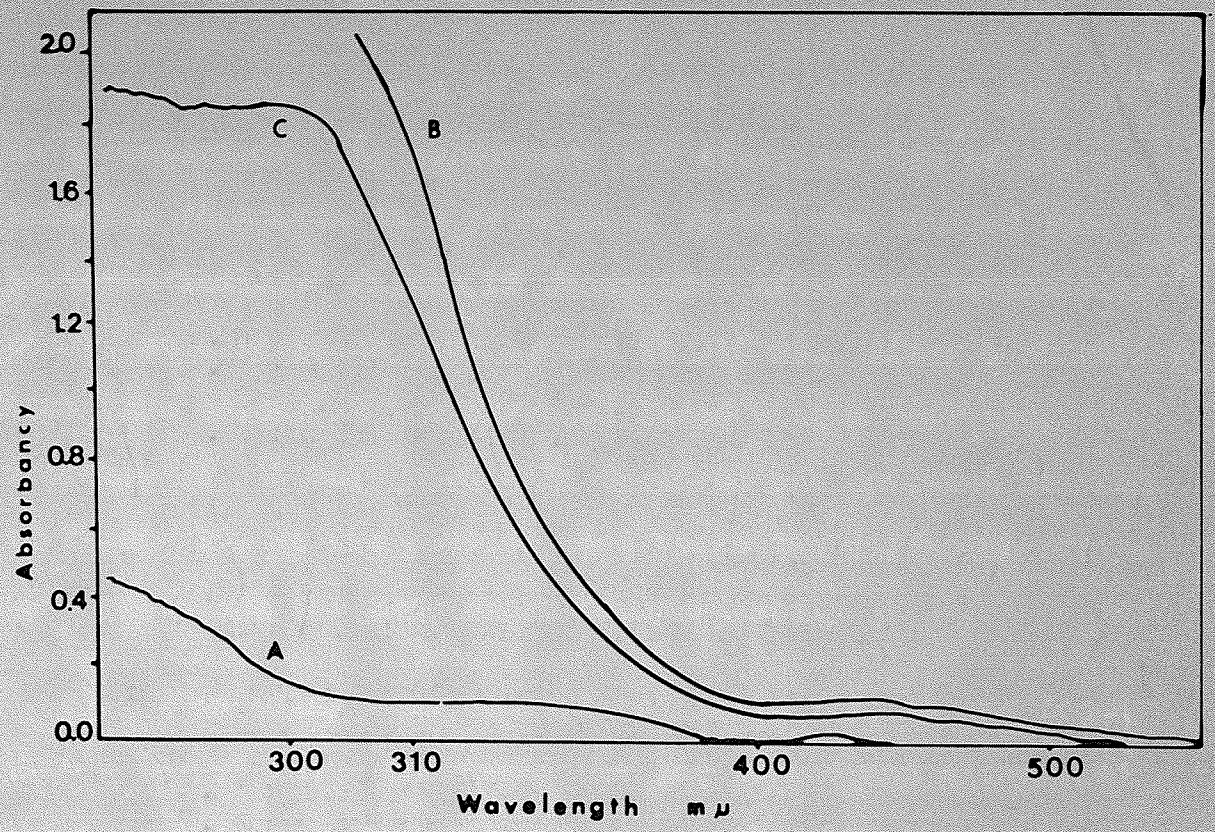
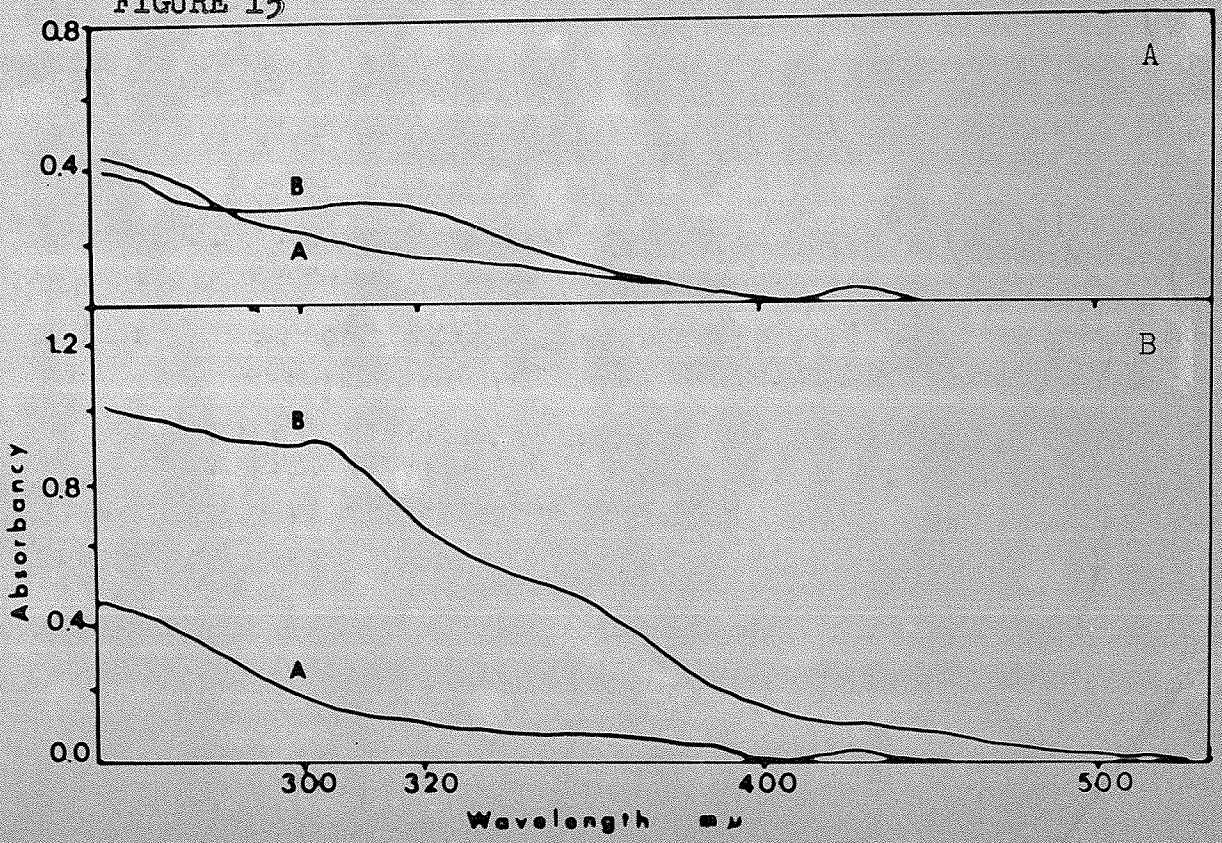


FIGURE 15



system produced a brown colour during the oxidation of OAA. As a comparison, the appearance and disappearance of the browning phenomena in a resorcinol system was studied spectrophotometrically. Figure 17 shows that after the initial lag phase, an absorption peak occurs at 310 mu. After a short time, the colour in the cuvettes faded with a corresponding decrease at 310 mu. When this spectrum was compared to that in Figure 10, it was obvious that these two different systems were producing the same brown intermediate.

9. pH. When using acetate buffers (Table VI), it was found that a low pH value shortened the lag period and eliminated the browning phenomena while a higher pH value caused an increase in the lag phase and allowed the formation of the brown colour. In terms of velocity the optimum pH occurred between pH 4.5 - 5.0 with acetate buffer.

TABLE VI. EFFECT OF pH ON OAA OXIDATION IN THE PRESENCE OF ACETATE BUFFERS.

pH	Time of browning in min.	Time of O_2 uptake in min.	Velocity ul/5 min.
3.5	no browning	5	23
4.0	no browning	5	25
4.5	8	10	32
5.0	11	14	35
5.4	13	16	29
5.6	16	19	26

SYSTEM: enzyme, 0.45 mg protein; dichlorophenol, 15 umoles; $MnCl_2$, 3.0 umoles; acetate buffer, 100 umoles, pH varied; OAA, 20 umoles, pH 3.4, added at time zero.

Figure 16. Spectrum produced when H_2O_2 is added to reaction system with OAA omitted.

SYSTEM: enzyme, 0.5 mg protein; dichlorophenol, 15 umoles; $MnCl_2$, 3.0 umoles; acetate buffer, 100 umoles, pH 5.3; H_2O_2, 0.5 umoles, added after scan A. Total volume, 3.0 ml with water replacing enzyme in blank.

A. scan of reaction system with OAA omitted.
B. scan of reaction system with OAA omitted immediately after addition of H_2O_2.
C. scan of reaction system with OAA omitted 5 min. after addition of H_2O_2.

Figure 17. Spectrum of the appearance and disappearance of the brown colour when resorcinol replaced DCP.

SYSTEM: enzyme, 0.5 mg protein; resorcinol, 15 umoles; $MnCl_2$, 3.0 umoles; acetate buffer, 100 umoles, pH 5.3; OAA, 20 umoles, pH 3.4 added at time zero. Total volume 3.0 ml with water replacing enzyme in blank.

A. scan at time 2 min.
B. scan at time 20 min.
C. scan at time 25 min.
D. scan at time 35 min.

FIGURE 16

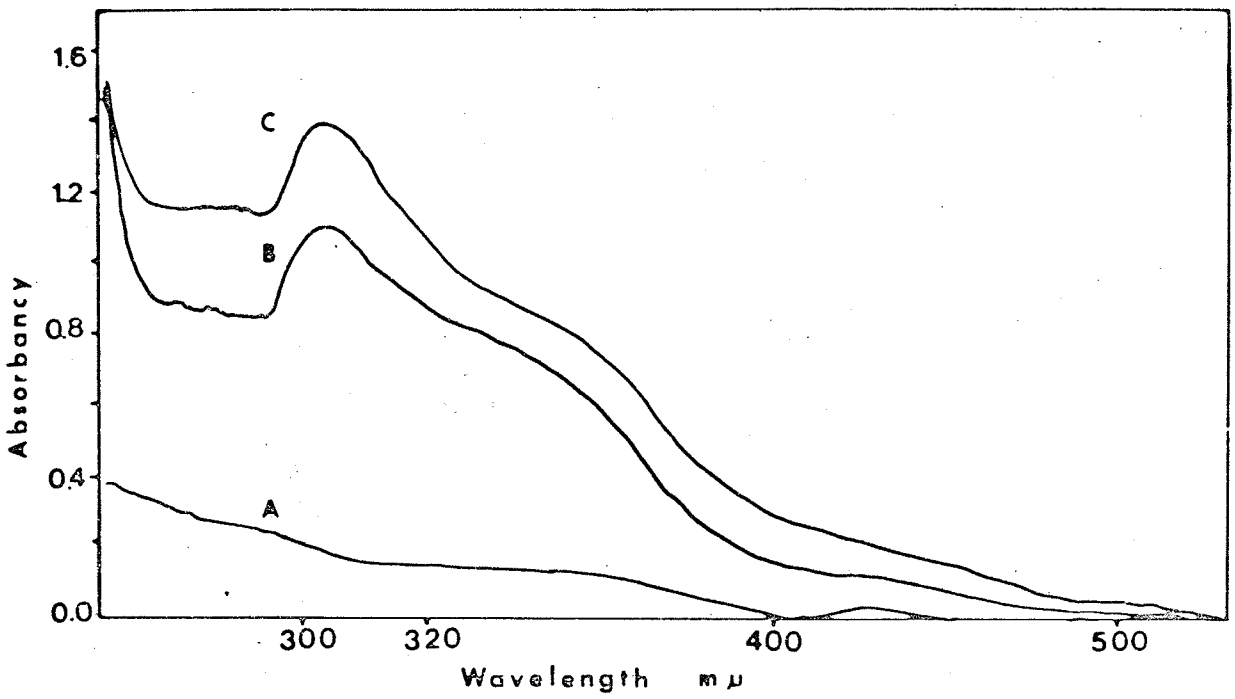
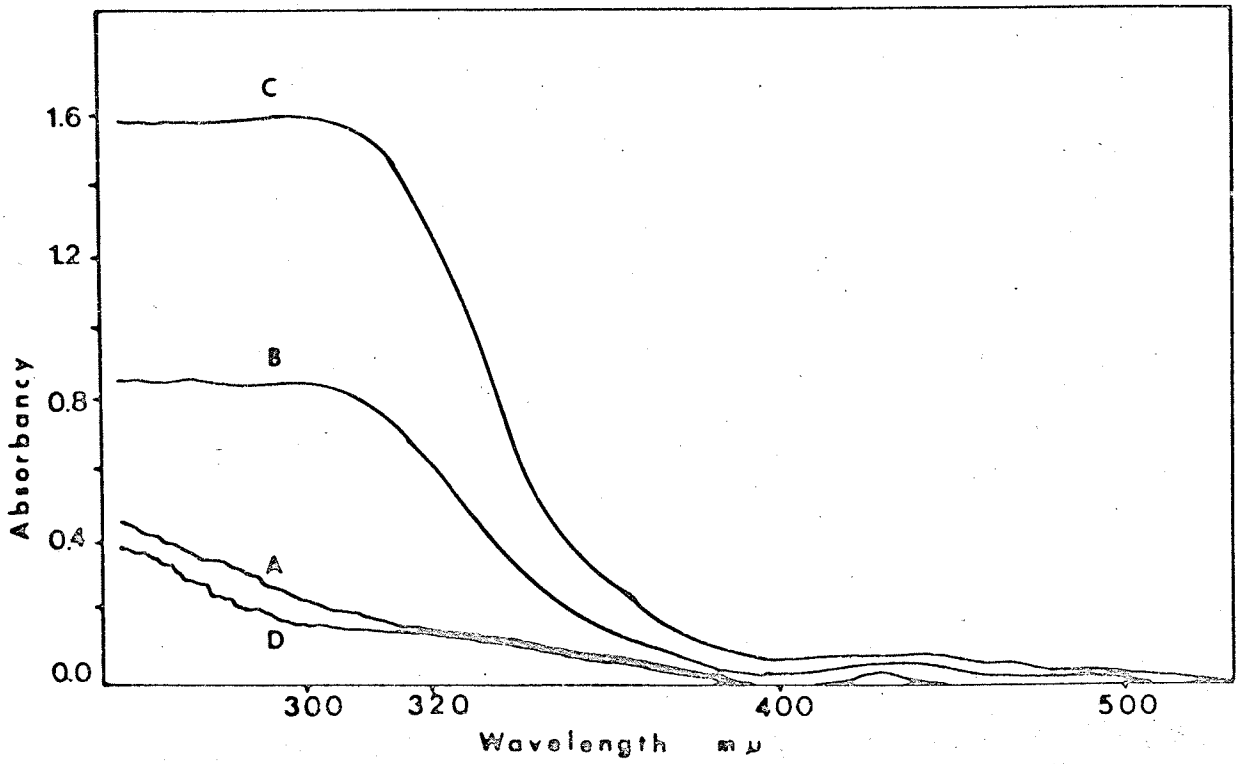


FIGURE 17



The pH optimum shifted to pH 5.5 - 6.0 when an ortho-phosphate buffer was employed (Table VII). The changing of the buffer had a considerable effect on both lag period and colour formation. The ortho-phosphate buffer shortened the lag phase at higher pH values. The browning, in comparison to that observed with the acetate buffer, was of a lower intensity. At pH values of 7.0 and above, a precipitate formed when Mn^{++} was added. This has been reported before and was attibuted to the formation of manganese phosphate (Stutz, 1956).

TABLE VII EFFECT OF pH ON OAA OXIDATION IN THE PRESENCE OF PHOSPHATE BUFFER

pH	Time of browning in min.	Time of O_2 uptake in min.	Velocity µl/5 min.
3.5 A	no browning	5	18
4.5 A	5	8	27
5.4 A	10 (very brown)	12	31
5.5 P	8 (faint)	5	22
6.5 P	8	5	35
7.5 P	precipitate	-	-

SYSTEM: enzyme, 0.45 mg protein; dichlorophenol, 15 µmoles; $MnCl_2$, 3.0 µmoles; acetate buffer (A), or ortho-phosphate (P), pH varied, 100 µmoles; OAA, 20 µmoles, pH 3.4, added at time zero.

10. Chromatographic Analysis Figure 18 shows a chromatogram prepared from modified 'standard' reaction mixtures. Authentic standards of pyruvate, oxalacetate, and malonate were applied to the chromatogram along with the systems for the purpose of

identifying the products. The D-xylose-analine spray caused malonate to appear as a reddish brown spot, pyruvate as a yellow brown spot, and OAA as a dull yellow spot. The Rf values were 24, 62, and 4 respectively.

The complete reaction system showed malonate and pyruvate as the only products. No malonate was detected if the enzyme or if Mn were omitted from the complete reaction system. In these latter two systems, there was an obvious production of pyruvate. Chromatograms of the dinitrophenylhydrazones of the complete reaction system showed that no keto-acids (besides pyruvate) were being formed. Malonate appears to be the sole product of enzymatic oxidative decarboxylation of OAA.

Other chromatographic studies established oxalic acid to be the only product of ketomalonate oxidative decarboxylation.

11. Polyacrylamide Gel Disc Electrophoresis The disc electrophoresis separated the 'dialysed' wheat enzyme into 4 bands (Figure 19). There also appeared to be a non-diffusable group of proteins at the origin which may be attributed to either:

1) denaturated protein in the enzyme solution,
2) non-separation on the 7 1/2% acrylamide gel used at pH 4.5.

Figure 18. The chromatographic separation of the products resulting from OAA degradation.

1. pyruvate
2. oxaloacetate
3. malonate
4. 'standard' system (see Methods and Materials)
5. 'standard' system minus enzyme
6. 'standard' system minus Mn.

Figure 19. Result of disc electrophoresis on the commercial 'Peroxidase' (A) and the dialysed wheat extract (B).

FIGURE 18

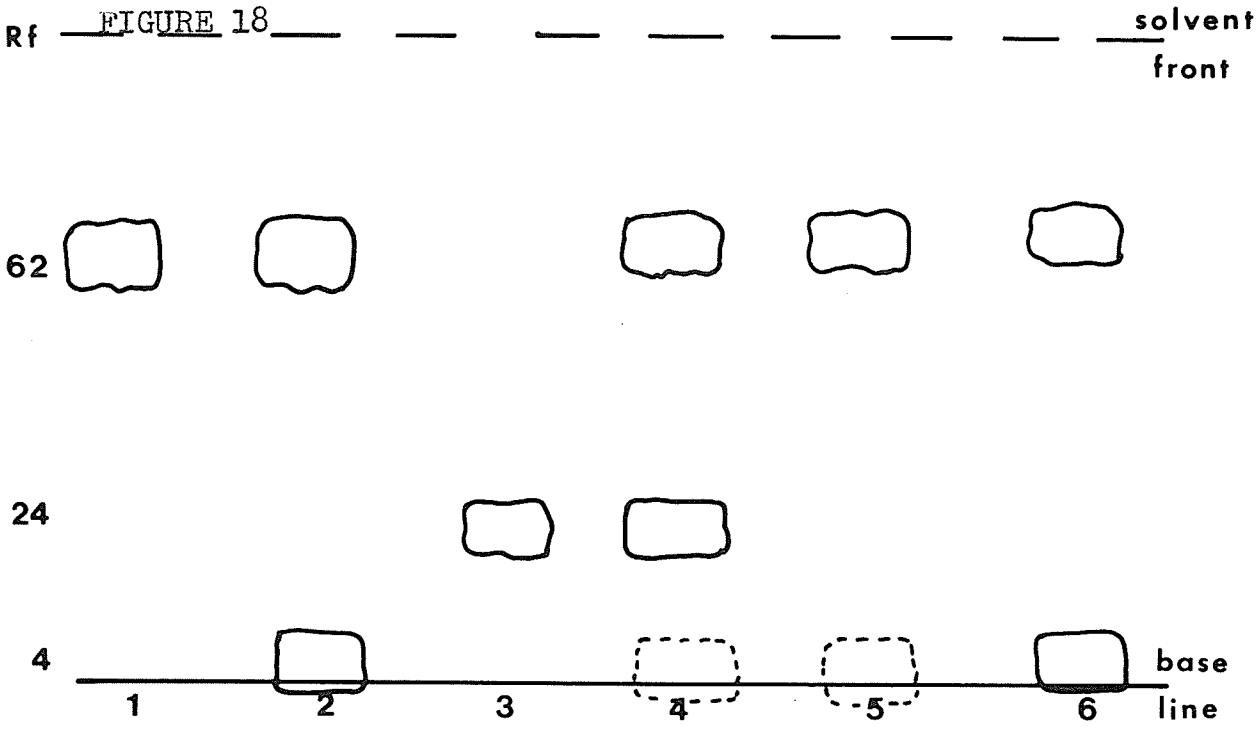
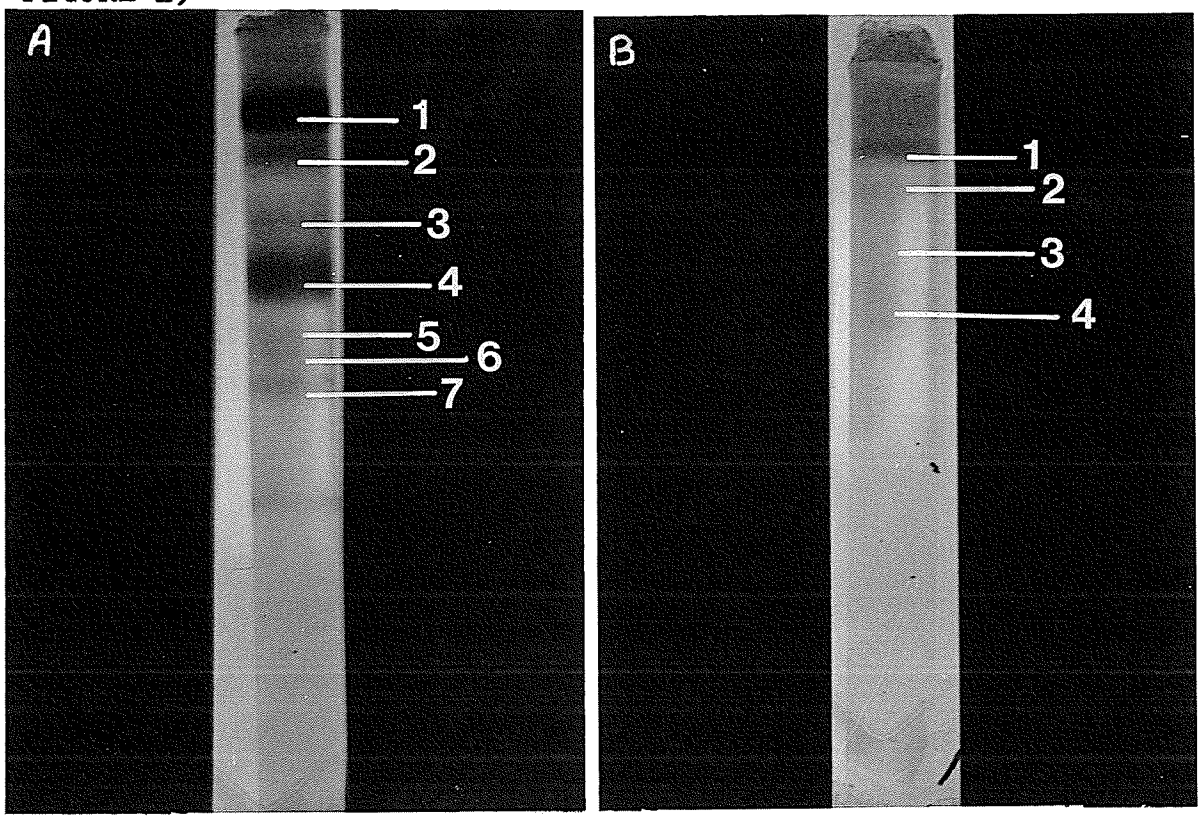


FIGURE 19



The undialysed, commercially prepared 'Peroxidase' (Type II) separated into 7 distinct bands. There appears to be some correlation between the first 4 bands of the two gels. This implies that, at least in some aspects, the dialysed wheat extract is similar to commercial 'Peroxidase.

12. Reaction Sequence The mechanism by which OAA is enzymatically destroyed could be thought to occur in at least two distinct stages. The lag or induction period which takes place prior to oxygen consumption would be the 'initiation' stage. The period of actual oxygen uptake would be the 'oxidation' stage. With this arbitrary separation of the mechanism, studies were done to determine which cofactors were required for each reaction.

It was assumed that those components which were able to shorten the lag phase were involved in the 'initiation' reaction. The concentrations of enzyme, Mn, and OAA when varied, were found to influence the lag period (for details refer to particular section). The DCP concentrations, except when extremely low (below 5×10^{-5}M), did not affect the length of the lag. The lag period appeared to be that time required for the three components to interact. The incubation of any two of these components, together, with the subsequent addition of the third component did not reduce the lag (the lag being measured after the addition of the last

component). This means that all 3 must be present at the same time. It appears that a 'mass action' type of reaction might be taking place during the lag. These results suggest that an 'intermediate' consisting of enzyme, Mn, and OAA is formed prior to oxygen consumption.

The 'oxidation' phase would occur with just the enzyme, Mn, and OAA; but was accelerated by the presence of DCP. When all four components were incubated together, the lag would end after approximately 10 minutes. If the three 'initiation' components (enzyme, Mn and OAA) were incubated for 10 minutes before the DCP was added, the O_2 uptake occurred immediately upon its addition. Therefore, it seems that the presence of DCP has no effect on the 'initiation' reaction, but enhances the 'oxidation' reaction.

13. Sodium Azide This classical inhibitor of heme enzymes was tested using the dialysed wheat extract in the 'standard' reaction system. Sodium azide at 1×10^{-4}M had very little effect on the oxidation of OAA. As the azide concentration was increased, there was a corresponding increase in the lag period and decrease in the rate of oxygen uptake. This result was comparable to that obtained if the enzyme concentration was progressively decreased. The oxidative decarboxylation of OAA was inhibited completely at NaN_3 concentrations of 0.1 M. However, the overall effect on the oxidation rate by any particular concentration of azide could be altered by

the addition of more enzyme.

Inhibition by NaN_3 was studied spectrophotometrically (Figure 20). The 'standard' reaction system was allowed to react until the brown colour with its peak at 310 mu became quite distinct. The addition of NaN_3 (1×10^{-2}M) brought about a rapid disappearance of the 310 mu peak. Since this is a heme enzyme it was not surprising that azide would cause inhibition. But it is worthy of note that the brown colour disappeared upon this addition. This strongly suggests that the browning phenomenon is a direct result of the iron group.

14. Anaerobic In the absence of air, the 'standard' reaction system shows neither gaseous uptake nor the browning phenomenon. If, after 30 minutes under anaerobic conditions, the reaction mixture was exposed to the air, the brown colour appeared immediately and the uptake of oxygen commenced (Figure 21). In order to expose the reaction solution to a large quantity of air in a short time, it was necessary to remove the flask from the manometer and to shake manually for a few seconds. Before the flask could be reattached, the brown colour had appeared and presumably oxygen uptake had started. If the reaction mixture was exposed to the atmosphere merely by opening the stopcocks for a few minutes, the appearance of the brown colour was delayed considerably. But in this latter case, it was found that a small amount (10 µl) of

Figure 20. Spectrum produced when NaN_3 was added to the complete reaction system.

SYSTEM: Concentration of components same as described in Figure 11. Thirty umoles of NaN_3 were added after scan B.

A. scan of complete reaction system taken at time 5 minutes.
B. scan of complete reaction system taken at time 15 minutes.
C. scan taken immediately after the addition of the NaN_3.
D. scan taken 4 minutes after scan 3.

Figure 21. The effect of anaerobic conditions on the oxidation of OAA.

FIGURE 20

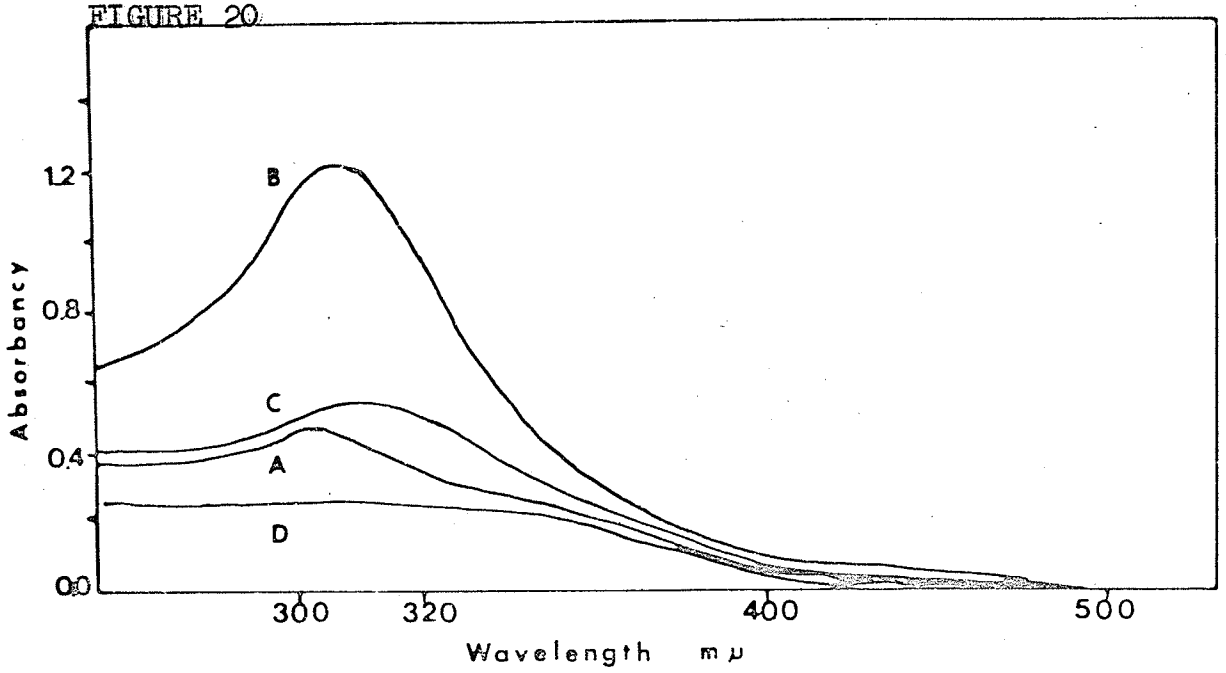
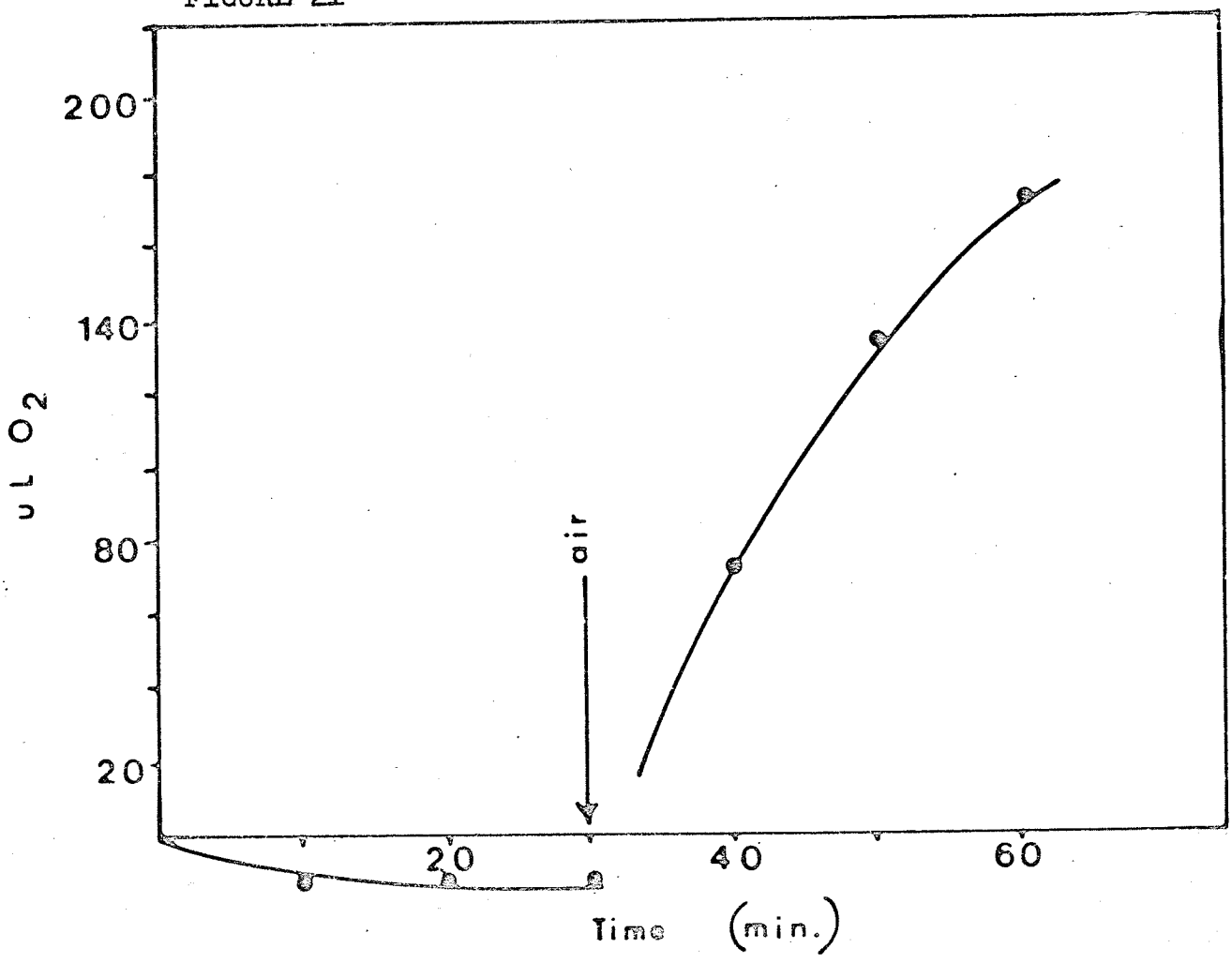


FIGURE 21

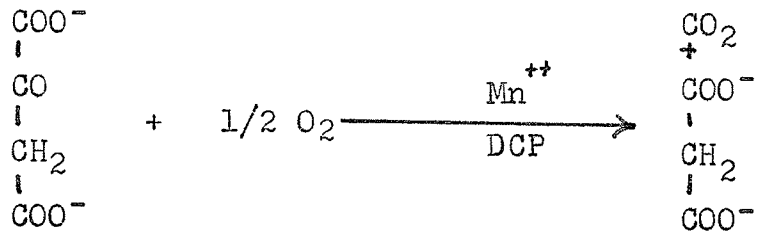


oxygen uptake occurred before the brown colour became visible. These results not only demonstrate the absolute requirement for O_2, but suggest that, since no browning occurs in the absence of oxygen, the colour phenomenon is an oxide of some sort.

Other experiments were performed to detect, if possible, any CO_2 release under nitrogen. This procedure was complicated by the fact that the 'unknown' gas was evolved under anaerobic conditions (Figure 21). However, an extended series of experiments of this nature were attempted. The complementary control for these studies was a complete reaction system minus the enzyme. This control system, under anaerobic conditions, also was found to evolve the unknown gas. In comparison to this control, it was found that a small amount (10-20 μl) of "CO_2" was evolved from the complete reaction system while under anaerobic conditions. This "CO_2" gas, which appeared during the first 10 minutes, occurred consistently through out this series of experiments. Although the results were not conclusive, it does seem that some CO_2 can be enzymatically liberated in the absence of O_2.

V DISCUSSION

The oxidative ∝-decarboxylation of oxaloacetic acid to produce malonate can be presented as follows:



This oxidative ∝-decarboxylation can be visualized as occurring in two distinct stages. These are the 'initiation' and the 'oxidation' phases. As demonstrated in the experimental results, the 'initiation' phase is influenced by the enzyme, OAA, and manganese concentrations. The second or 'oxidation' phase occurs only after the 'initiation' phase has produced some intermediate responsible for oxygen consumption. This 'oxidation' phase is affected greatly by the presence of the phenolic cofactor.

The partitioning of the peroxidase-oxidase reaction sequence is not without precedent. However, there is some controversy as to which cofactors influence the different phases. Indeed, there is still lack of agreement concerning the actual necessity of some cofactors. Sacher (1961) using IAA as the substrate, found that Mn^{++} was not necessary for the 'initiation' phase and suggested that DCP was. However, in accordance with the present findings, he did show that the

initial phase was influenced by the enzyme and substrate concentrations. Stutz (1957), on the other hand, found that without Mn^{++} or DCP no oxygen consumption occurred during IAA degradation. Also, he was able to show that with Mn^{++} present, the oxidation is dependent upon enzyme concentration rather than DCP concentration. The present study (see Fig, 6) is in complete agreement with Stutz (1957).

The experimental results were able to show which factors influenced the 'initiation' phase, but were unable to distinguish their order or sequence of participation. It is doubtful that both the Mn^{++} and the OAA bind simultaneously with the enzyme. Shannon et al. (1964) were able to demonstrate that the Mn^{++} binds to the enzyme prior to the substrate in the oxidative decarboxylation of OAA. There does exist, of course, the alternative whereby the OAA could be the initial component to bind. However, since there is no measurable gas exchange if OAA alone is exposed to the enzyme, it must be assumed that the destruction of the OAA is not accomplished solely by its binding to the enzyme. This leads to the suggestion that Mn^{++} must be concerned in some manner with the decarboxylation, since enzymatic gas evolution occurs only if OAA is present with both the enzyme and Mn^{++}.

With regard to the Mn^{++} promoted β-decarboxylation of OAA to pyruvate, Steinberger and Westheimer (1951) suggest that the essential feature of the enzyme is as a complexing

agent for the metal. To be more precise, they proposed that the protein part of the enzyme has two quite different functions: (a) to impart specificity with respect to substrate to the enzyme system; (b) to complex the metal in such a way as to enhance its activity. In a later paper, Seltzer, Hamilton, and Westheimer (1959) imply that the mechanism of enzymatic OAA decarboxylation to pyruvate is essentially the same as the non-enzymatic. That is: the Mn^{++} while associated with the protein moiety, forms bonds with the keto oxygen and the α-carbonyl of the OAA molecule. Through the resulting bond shifts, the β-carbonyl is liberated as CO_2. If the role of the enzyme protein actually is orientation of the metal with regard to substrate, it would not be difficult to imagine that, in the case of OAA decarboxylation to malonate, the Mn^{++} protects the β-carbonyl while forcing the liberation of the α-carbonyl.

Figure 1 shows that DCP is not absolutely required for the 'oxidation' phase. But, without the phenolic compound, this oxidation is extremely slow. In the section 'Reaction Sequence', it was demonstrated that DCP has no effect on the 'initiation' phase. It is therefore suggested that the phenol, by its presence, enhances the oxidative function of that intermediate formed during the 'initiation' phase. This suggestion is similar to one which Ray (1958) proposed to explain the function of the cofactors in IAA

oxidation. He suggested that the phenol has some promotive effect on the main reaction independent of the presence of Mn^{++}, and that the effect of Mn^{++} may be on the initiation.

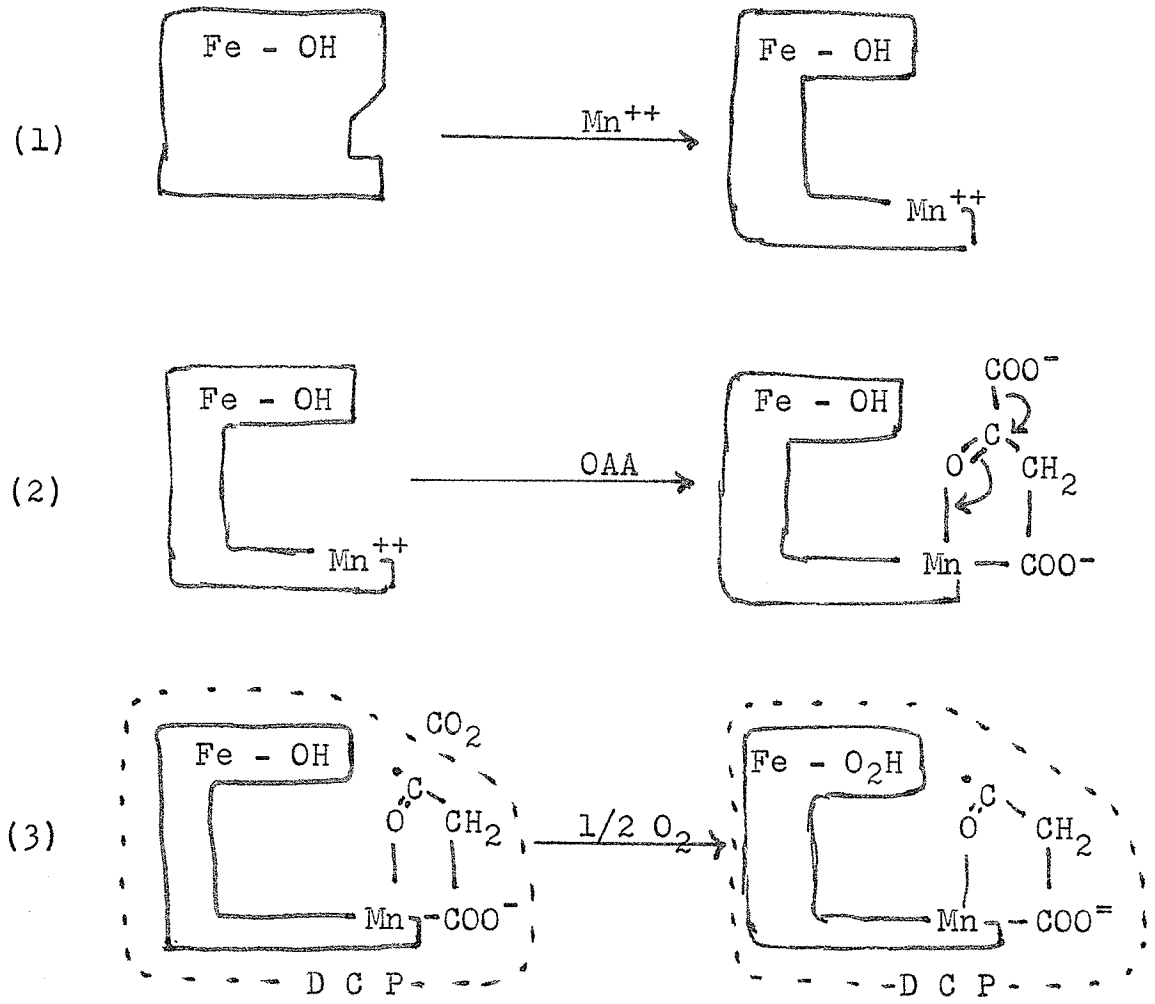
The actual mechanics of the phenolic promotive effect have not been elucidated. However, Yamazaki and Piette (1963) suggest, with regard to ascorbic acid oxidation by HRP-H_2O_2, that the phenol acts as a stabilizer for a free radical intermediate. Also, the fact that in some peroxidase oxidations the phenol can be replaced by pyridoxal-phosphate (Mazelis, 1962) implies a stabilizer role for the phenol rather than that of a hydrogen donor.

There are very few reports of a brown colouration appearing during the oxidation of either OAA or IAA. Vennesland et al. (1946) mention that during the oxidation of OAA by metmyoglobin there appears first a green pigment, then a light brown pigment which has no absorption band in the visible spectrum. Also, Maclachlan (1956) reported the appearance of a brown tint during the oxidation of IAA. In the present study it was found that when IAA was substituted for OAA the browning occurred. Therefore, it is not unlikely that the colour phenomenon mentioned in these previous papers is the same as that reported by this study.

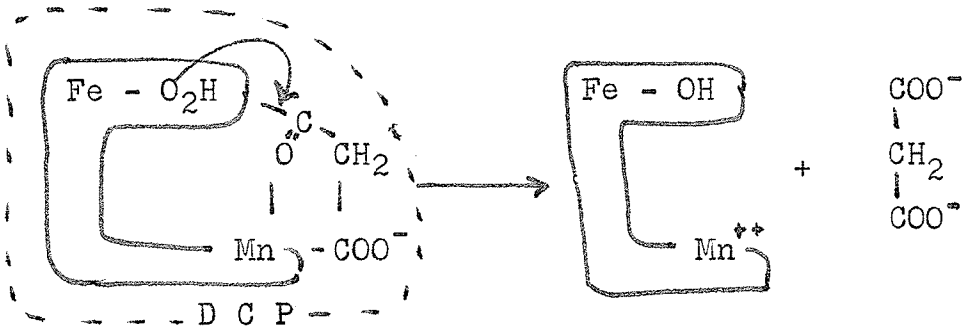
Of considerable interest with regard to the 310 mu absorption band is the work of Evans et al. (1948). In a purely chemical study of a Fe OH -H_2O_2 reaction, they reported

an absorption peak at 310 mμ. This was attributed to the oxidized compound (Fe O_2H^{+2}). If the 310 mμ band reported in this present study is the result of such an oxidized iron compound, then its significance is obvious. It would mean that the heme group of the peroxidase is actively involved in the oxidation of OAA.

In view of the experimental results, and theaforementioned assumptions, the author proposes the following scheme to explain the oxidative decarboxylation of OAA. Steps (1) and (2) represent the 'initiation' phase, while steps (3) and (4) show the 'oxidation' phase.



(4)



The first step is the association of the peroxidase enzyme and the manganese ion. It is depicted diagramatically as effecting a conformational change in the apoenzyme. There is no direct proof of this, but Brill and Weinryb (1967) mention that where a role for the protein has been suggested it has generally been presented as influencing heme group accessibility.

Step two shows the binding of the OAA to the enzyme-metal complex. As previously mentioned, this might occur in a manner not too different from that proposed by Seltzer et al. (1959). According to this scheme, the lag period would be the time required for the formation of the oxygen-consuming intermediate. This apparently is a 'mass action' effect which is influenced by the concentration of the participating factors.

The release of CO_2 is shown to occur before the consumption of the oxygen (Fig. 8). Maclachlan and Waygood (1956b) found the same sequence in the destruction of IAA. Even though the decarboxylation in the Maclachlan-Waygood scheme is shown to occur prior to oxygen consumption, they

maintain that oxygen is required to initiate the reaction. However, although the result can not be called conclusive, the present study demonstrated that a limited amount of enzymatic decarboxylation occurred under anaerobic conditions. As shown in the above scheme, there exists no need for oxygen prior to the decarboxylation.

The free radical which results from this decarboxylation may be responsible for the reduction of the heme group. Nevertheless, the over all effect of step two would be the formation of the intermediate capable of consuming oxygen. The phenol is presented here as stabilizing this intermediate.

The third step is the consumption of one half molecule of oxygen. It is proposed that the oxygen first becomes associated with the reduced iron. This would be similar to the oxygenation of ferroperoxidase (Cmp II) to form oxyferroperoxidase (Cmp III) as described by Ricard and Nari (1967) for IAA oxidation. Presumably this oxygenated iron group is responsible for the browning phenomenon. Supporting this is the fact that a heme group inhibitor such as sodium azide can cause the disappearance of the brown colour (see Fig. 20). The oxygen atom is then transferred in some manner to the ketocarbon of the OAA residue. After the transfer of the oxygen, a re-alignment of the bonds would free the product from the enzyme-manganese complex (step 4).

The assumption that the browning is the result of oxygen-

ated heme is not unreasonable since no colour appears in the absence of oxygen. The previously mentioned findings of Maclachlan (1956) state that the brown tint occurred with the system under high oxygen tension. The reason Maclachlan did not consistently find the colour when under normal atmospheric conditions probably is due to the low IAA concentrations (10 um) which he employed. The present study demonstrated that when substrates concentrations of 10 um or lower are used the colour is not evident. Maclachlan reported the appearance of the brown tint prior to IAA addition. It is difficult to explain this by the above mentioned scheme. It was suggested by Maclachlan that the colour was a result of a quinone condensation. The present study used both DCP (see Fig. 10) and resorcinol (see Fig. 17) in the spectral analysis of the brown colour. The fact that both spectrums are identical would appear to discount the idea of a quinone formation being the cause.

The data obtained from the spectrophotometric analysis of the browning phenomenon can be adequately explained by the proposed mechanism. As would be expected, in the absence of the 'initiation' factors, Mn^{++} (see Fig. 11B) and OAA (see Fig. 11C), no absorption band is formed at 310 mμ. And since oxidation will occur in the absence of the phenol (see Fig. 1), it would be reasonable to expect the formation of a limited amount of an oxygen consuming intermediate in a system devoid of DCP. Figure 12 indicates that such is the case. Further-

more, it would be expected that the 310 mu band should intensify if this intermediate was stabilized by the phenol. The addition of DCP (see Fig. 13) causes an immediate increase in the 310 absorption band. This supports the proposal that the phenolic compound participates in the 'oxidation' rather than in the 'initiation' phase.

The addition of H_2O_2 to a 'standard' reaction system produces the immediate formation of the 310 mu absorption band (see Fig. 14). Apparently the peroxide molecule is able to oxidize the heme group without the formality of a lag period. It was previously suggested that the reason for the lag period was because of a Mn^{++} promoted conformational change of the enzyme. Considering the size of a H_2O_2 molecule as compared to an OAA molecule, it is possible that no conformational change is required in order for the peroxide molecule to reach the heme site. The fact that Mn^{++} is not necessary for the peroxide promoted formation of the 310 mu band (see Fig. 15b) supports this assumption. But it should be noted that the spectra of the peroxide promoted browning with Mn^{++} (Fig. 14) and without Mn^{++} (Fig. 15b) are slightly different. The variation may be due to minor differences in the final protein configuration. It appears that this oxidation can be accomplished without atmospheric oxygen. This is suggested by the findings that less oxygen is consumed if H_2O_2 is present in the system than if it is absent (see Table V). This also would help reduce the lag period.

Once oxidized by the peroxide, it is possible that the heme group becomes more exposed. In other words, the active site becomes more accessible for the OAA molecules. However, in the absence of Mn^{++}, it is difficult to explain how the decarboxylation of OAA would occur.

Without the phenol, the 310 mu absorption band produced by adding H_2O_2 is very slight (see Fig. 15A). This lends further support to the idea that DCP is a stabilizer of the oxygen consuming intermediate.

Both the pH and the type of buffer influence the degree of browning (see Tables VI and VII). Since the colour is believed to be a radical such an effect is not unlikely. It would seem that certain pH values of various buffers have the ability to enhance the stabilization role of the phenol. This susceptibility to ion type and concentration might explain why the lag period is extended when high concentrations of OAA are used (see Table III). Also, it might explain the lack of colour formation when ketomalonate is the substrate.

Whether the oxidative decarboxylation of IAA occurs by the proposed mechanism remains a point of discussion. There are more than a few similarities in the oxidation of OAA as compared to the oxidation of IAA. They both require the same cofactors. Both substrates will produce the 310 mμ absorption band if the proper concentrations are employed. Furthermore, certain data presented by Waygood et al. (1956b) concerning the

promotive effects of Mn^{+3} and MnO_2 are applicable to the present mechanism. The binding of oxidized manganese to the peroxidase enzyme might not only cause a conformational change, but could also reduce the heme group. Waygood et al. (1956b) also suggested that the role of the phenol is to protect such Mn^{+3} radicals. This is similar to the proposal that the phenol protects a reduced iron radical.

However, one of the more noticeable discrepancies is the fact that in the oxidation of IAA the CO_2/O_2 ratio is 1, whereas in the oxidation of OAA this ratio is 2. Neither the Maclachlan-Waygood scheme, nor the present scheme can adequately explain this established fact.

Resolution of the differences may lie in the concept that peroxidases have at least two active sites. Brill and Weinryb (1967) present evidence to support the idea of protein as well as heme participation in the catalytic site of HRP. Supporting this is the fact that Seigel and Galston (1967) were able to remove the heme group from the peroxidase enzyme without affecting its ability to oxidize IAA. Also, Yamazaki and Piette (1963) in their scheme for IAA oxidation imply that more than one pathway is possible.

Therefore, it could be reasoned that any differences in the mechanism of IAA oxidation and OAA oxidation exist as a result of the nature of the peroxidase enzyme. Some enzyme

preparations may contain a peroxidase which can oxidize these substrates without the necessity of the heme group. Other peroxidases may not have such an apoenzyme present and therefore require the heme prosthetic group. Then, of course, there might be preparations in which both sites are active. Any investigation which is able to demonstrate the existence of two sites, and subsequently shows which substrates are oxidized by each, would help untangle the confusion which now exists. Some preliminary work along this line has been done. Kay et al. (1967) has described the catalytic properties of seven homogeneous peroxidase enzymes, and found them to vary with regard to pH, K_m and affinity towards inhibitors.

Polyacrylamide gel disc electrophoresis was used in this study solely to determine the purity of the wheat extract. But, other applications of this technique can be seen. The fact that the 'Commercial' peroxidase contains more bands suggests that other isoenzymes are present in HRP than exist in wheat. It is possible that with these extra bands in the HRP might be able to oxidize substrates which the wheat can not. It would be interesting to know whether or not each band or isoenzyme can function, with regard to oxidizing OAA or IAA as a separate entity.

The present study, while it proposes a new interpretation of OAA oxidation, does not pretend to offer a final solution. The problems which must be overcome in order to describe

the exact mechanism of peroxidase-oxidase enzymes are indeed numerous. But, with the advancements in technology, there is every indication that these eventually will be solved. Polyacrylamide gel disc electrophoresis, for instance, may prove to be useful for peroxidase studies; especially in the separation of the isoenzymes. As mentioned above, the differences in the oxidation of OAA and IAA may be the result of some subtle variations in the protein portion of the enzyme. The approach to the peroxidase problem on the basis, utilizing the more sophisticated techniques available, may contribute substantially to the eventual understanding of this mechanism.

VI SUMMARY

1. The oxidative a-decarboxylation of oxaloacetate to malonate is shown to be catalysed by dialysed wheat leaf extract and also by horseradish peroxidase. The reaction consumes 1/2 mole of oxygen, releases 1 mole of carbon dioxide per mole of OAA

2. There is a lag period prior to the uptake of oxygen. This lag phase can be shortened but not eliminated by increasing the concentrations of enzyme, manganese, and OAA.

3. Manganese appears to be the only essential cofactor requirement for the enzymatic oxidation of OAA.

4. Evidence is presented to show that the phenolic cofactor enhances the oxidation of OAA, but is not essential to the oxidative reaction.

5. The K_m of OAA was found to be between 0.8 and 1.1×10^{-3}. The K_m of ketomalonate was found to be 2.6×10^{-4}.

6. The decarboxylation of both OAA and ketomalonate occurs prior to oxygen consumption. Also, it appears that the decarboxylation can occur under anaerobic conditions.

7. A brown colour appears and disappears in the reaction system during the oxidation of OAA. The colour appears immediately prior to measurable oxygen uptake. It dis-

appears when oxygen has ceased entering the system. A spectral analysis of this colour shows an absorption band at 310 mu.

8. There is no brown colour formation in the absence of oxygen.

9. The 310 mu absorption band will appear when using either the wheat leaf extract or the commercial horseradish peroxidase. Also, the 310 absorption band forms if IAA is substituted for OAA. There is no colour formation when ketomalonate is used as the substrate.

10. The formation of the 310 mu absorption band under atmospheric conditions requires the presence of enzyme, manganese, and OAA. The intensity of the absorption band is increased by the presence of DCP or resorcinol.

11. The addition of H_2O_2 to the complete reaction system decreases the lag period. This addition of peroxide causes the intermediate formation of the brown colour. This H_2O_2-promoted browning can occur in the absence of manganese, but its intensity is very low if DCP is omitted.

12. The pH optimum with acetate buffer is 5.4. The type of buffer and the pH values affect the appearance and intensity of the brown colour.

13. Ketomalonate is oxidatively decarboxylated to oxalic acid. The CO_2/O_2 ratio of this reaction is 2.

14. The oxidation of OAA and the formation of the brown colour is inhibited by NaN_3. This inhibition can be overcome by the addition of more enzyme.

15. Polyacrylamide gel disc electrophoresis of the dialysed wheat extract shows the presence of 4 bands. The horseradish peroxidase shows 7 bands. The bands of the wheat extract have complementary counterparts in the horseradish peroxidase.

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