CHLOROPHYLL A BLEACHING BY A COUPLED OXIDATION WITH LINOLEIC ACID CATALYZED BY LIPOXIDASE

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This is to certify that the

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CHLOROPHYLL A BLEACHING BY A COUPLED OXIDATION WITH LINOLEIC ACID CATALYZED BY LIPOXIDASE

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ABSTRACT

CHLOROPHYLL A BLEACHING BY A COUPLED OXIDATION WITH LINOLEIC ACID CATALYZED BY LIPOXIDASE

by Frank T. Orthoefer

Chlorophyll a was bleached in a model system composed of pigment, linoleic acid, and lipoxidase. The optimum pH for bleaching to occur was 5.7 and the maximum rate was at 25°C. Chlorophyll bleaching was linear for 1.5 to 2 minutes with approximately 30% of the pigment being lost. The maximum bleaching rate occurred at 0.33 mM linoleic acid, corresponding to the critical micelle concentration of the substrate with pigment present. The maximum rate of conjugated diene formation occurred at a higher concentration than the critical micelle concentration because of the effect of chlorophyll on the micelle. The maximum bleaching rate with respect to the pigment occurred at 25mg/l.

Bleaching of chlorophyll was inhibited by the lipid antioxidants butylated hydroxytoluene (BHT) and nordihydroguaiaretic acid (NDGA). The presence of Tween 20 and Triton x-100 also prevented chlorophyll degradation, but the alcohol soluble fraction of soybean lecithin produced a marked increase in the bleaching rate.

The end product of chlorophyll bleaching by lipoxidase was a Molisch phase negative, red fluorescent compound

having an HCl number of 24. The main peaks in the visible spectra occurred at 405 nm and 659 nm with the blue to red peak ratio similar to that of a lactol structure. Infrared spectra showed the presence of the phytol chain and the 9-keto and 10-carbomethoxy groups of the carbocyclic ring. A broad -OH absorption band appeared which could not be removed under rigorous drying conditions.

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heodore
Frank T. Orthoefer

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INTRODUCTION

The color change in leaves and fruits is a familiar sight during autumn or ripening seasons. This color change is usually due to chlorophyll destruction and the resultant unmasking of other pigments present (58). The biochemical pathway of chlorophyll destruction has not been elucidated although positive correlations between leaf protein, pigments, and various other cell constituents have been observed (12, 47, 53, 57). The mechanism of pigment destruction possesses an intrinsic interest to the biochemist and food scientist due to its prime importance in nature and because of its contribution to food color, possibility of color control and the effect of various breakdown products on the total food system. The purpose of this study was to clarify further the mechanism involved in chlorophyll destruction.

Most of the previous studies of pigment destruction have been concerned with chemical degradation or with the investigation of intact plant systems (7, 55, 60). Hoyt (34) has shown that the degradation is enzymatically controlled but has not separated the particular enzymes responsible. Various oxidized chlorophylls have been isolated from degreening plant parts and their structures determined, but their significance to the degradative pathway has not yet been assessed. The attempts by previous investigators (7, 55) to locate compounds from degreening plants which were breakdown products obviously related to chlorophyll often were unsuccessful. The failure of such investigations was ascribed by Egle (13) to the extreme rapidity of reactions converting chlorophyll to compounds which are common metabolites.

This study was performed using components found in degreening plant tissues which are known to have a profound effect on plant pigments. Strain (59) originally described chlorophyll destruction with these components and Holden (26) further elucidated the mechanism. However, in both previous studies crude enzyme preparations were used which limited the description of the parameters involved. It was determined in this study that the active enzyme in the crude preparations of the above investigators was lipoxidase (E.C. 1.13.1.13 linoleate: oxygen oxidoreductase) and that the mechanism involved was a coupled oxidation between the pigment and an oxidizing fatty acid (linoleic acid). The importance of chlorophyll destruction by this means is exemplified in the work of Wagenknecht et al. (79) on color loss in frozen peas and Walker's (80) investigation on frozen French beans.

The system was not specific, however, to the chlorophyll pigments. Studies on the coupled oxidation of carotene have elucidated the critical parameters involved for such a mechanism. While it was not specific to chlorophyll, its importance to the destruction of the pigment in stored foods was apparent. Pigment destruction during senescence or ripening also may occur by the same mechanism. Spencer (58) noted that endogenous lipids were involved in the ripening processes. Lee (43) found that an increase in peroxide value occurred soon after treatment of peas with the ripening hormone, ethylene, and a definite increase in the lipoxidase enzyme prior to degreening of apple tissues has been shown to occur (82). Heath and Packer (24) showed that chlorophyll was bleached when isolated chloroplasts were illuminated and that the degradation could be correlated with lipid oxidation. Strain (60) has separated oxidized chlorophylls from various leaves subjected to treatments which favor enzymatic oxidations. The cause of pigment oxidation was attributed later to the enzymes present in the system outlined by Holden (26).

Since chlorophyll destruction was known to be affected by the lipoxidase enzyme in frozen vegetables and the possibility existed that a similar mechanism was active during the biological degradation of the pigment, a model system composed of lipoxidase, linoleic acid, and chlorophyll was developed to study pigment destruction. A knowledge of chlorophyll destruction by this means would aid in the control of color loss in frozen peas and beans as observed by Wagenknecht et al. (79) and Walker (80). The model system approach eliminated the complicating factors involved in studying the biological degradation and aided in clarifying the mechanism involved and in isolating the end products of chlorophyll destruction.

LITERATURE REVIEW

Co (7) has presented a comprehensive review of the biodegradation of chlorophyll in natural systems. Because the following study was concerned with destruction using purified components, the literature dealing with each of the components will be reviewed only insofar as it deals with the active mechanism involved. The review of the coupled oxidation of carotene was included because of the similarity with the chlorophyll bleaching system. The following literature review will be limited to 1) the role of lipoxidase, 2) the coupled oxidation of carotene, and 3) the oxidative degradation of chlorophyll. The latter is divided further into enzyme catalyzed oxidation and oxidation by various non enzymatic reactions.

Role of Lipoxidase

Theorell et al. (73) were the first to prepare crystalline soybean lipoxidase (E.C. 1.13.1.13 linoleate: oxygen oxidoreductase). Balls et al. (3) noted that the oxidative enzyme had a specificity for fatty acids containing a cis, cis-1,4-pentadiene group. The typical substrates of lipoxidase were linoleic, linolenic and arachidonic acids (29). It had been noted that a peptide activator was apparently required for enzyme activity (3). Holman (27) found no activator requirement for homogeneous systems.

Koch (40) has recently shown that, after prolonged dialysis of crystalline lipoxidase, Ca^{+2} was essential for oxidizing activity and that Mg^{+2} could not be substituted for the Ca^{+2}. Tappel (66) utilized the fact that lipoxidase was active in both colloidal and homogeneous systems (65) to distinguish lipoxidase from heme catalyzed lipid oxidation, which is active only in the heterogeneous system. The activity of the enzyme in the heterogeneous or colloidal system was found to be decreased.

The optimum pH of lipoxidase activity has been found to be above pH 7 for fatty acids (27). The decreased rate of oxidation of fatty acids below pH 7.0 was not believed to be an effect of acid on the enzyme. Tappel (68) theorized that the pH effect was due to substrate solubility since the optimum pH for linoleate oxidation occurred at pH 9.0 (27) and for methyl linoleate at pH 6.5 (46).

Kinetic studies are difficult because of the substrate solubility effect on enzyme activity. A linear relationship between enzyme concentration and initial reaction velocity occurred at intermediate enzyme levels but decreased at greater concentrations (41), while the presence of an induction period was found to be dependent on substrate concentration (69). The induction period did not occur at catalytic levels of substrate, but at higher levels it was found to be present. Hanning and Axelrod (22) noted that induction periods were abolished by the presence of enzymatically oxidized or autoxidized substrates.

The K_m value for the oxygen requirement was found to be dependent on substrate concentration using sodium linoleate as substrate and indicated a competition between the oxygen and substrate for binding sites on the enzyme (68).

The mechanism of lipoxidase action as proposed by Holman (27) consisted of hydrogen abstraction at the methylenic carbon atom of the cis,cis-1,4-pentadiene system, with electron shifts giving hydroperoxides at either end of the pentadiene chain. Because of the competition between substrate and oxygen binding to the enzyme, Tappel (68) theorized that the lipoxidase bound oxygen abstracts the methylenic hydrogen resulting in a free radical which undergoes an electron shift to conjugated double bonds. The electron shift in the newly created conjugated system results in a change from the cis to trans form. The transformation could be the result of steric effects on the resulting hydroperoxide which would be less strained in the cis-trans configuration. Free radical intermediates are produced by lipoxidase catalysis as shown by the work of Fridovich and Handler (16). Hamberg and Samuelsson (21) have noted a disproportionation between hydroperoxides produced. Dolev et al. (10) have shown that lipoxidase action produced only hydroperoxides at carbon atom 13 of linoleic acid. The oxygen involved in the hydroperoxide formation was gaseous oxygen only and not from the aqueous phase (11).

Amino acid analysis showed lipoxidase to be abundant in valine, leucine, and isoleucine and low in sulfur containing acids (30). A hydrophobic region on the surface of the protein was apparent since each of the abundant amino acids possessed non-polar side chains (48). The hydrophobic areas would have an affinity for non-polar moieties of the substrate. Holman (27), in agreement with the above observation, suggested that saturated moieties of the fatty acids are primarily responsible for their affinity to the enzyme.

Inhibitors of lipoxidase activity are the lipid antioxidants such as α-tocopherol, nordihydroguaiaretic acid (NDGA), and propyl gallate (68). Cyanide, azide, and diethyldithiocarbamate showed no lipoxidase inhibition because the enzyme does not require a metal for activity. Common -SH inhibitors showed no essential sulfhydryl groups present.

Various methods have been used to assay for lipoxidase activity. Blain et al. (4) used the coupled oxidation of carotene and Theorell et al. (72) developed a spectrophotometric assay utilizing the absorbance at 234 nm for conjugated diene production. The rate of oxygen uptake has also been used as an assay by various investigators.

Coupled Oxidation of Carotene

Haas and Bohn (18) obtained a patent for the bleaching of yellow pigments from bread doughs by the addition of soybean preparations. Frey et al. (15) prepared a bleaching system containing a soybean preparation, lipid, and carotene to study destruction of the pigment. Sumner and Dounce (62) attributed the bleaching to carotene oxidase. Small amounts of lipid were found to be required for bleaching to occur (63). Excessive quantities of lipid caused a decreased bleaching rate and, with previously oxidized oils, bleaching did not occur indicating a coupled oxidation of the pigment with the lipid component. Tauber (70) showed that the enzyme bringing about the pigment bleaching was not a carotene oxidase but a fat oxidase, lipoxidase.

The carotene pigment itself can act as a proxidant in lipid oxidation (74). Monaghan and Schnitt (49) noted that only oxidized carotene was pro-oxidative, whereas the non-oxidized pigment acted as an antioxidant. Thompson and Steenbock (74) found no differences in the oxidative activity of oxidized or non-oxidized carotene, both being pro-oxidative. Part of the pro-oxidant activity was a result of shortening the induction period of fat oxidation. Kunkel and Nelson (42) showed that the oxidative ability of carotene was concentration dependent; lower concentrations being proxidative and higher concentrations antioxidative. Tookey et al. (77) found only slight differences in oxidative capacity of carotene over a wide concentration range.

The system of Blain et al. (4) for carotene oxidation consisted of carotene and lipid dispersed in an aqueous buffer followed by addition of purified lipoxidase. It was noted that since the coupled oxidation occurs in a two phase system, the rate would be a reflection of the degree of dispersion of the reactants. The optimum pH for bleaching occurred at 6.0, while for lipid oxidation the optimum occurred at pH 9.0. The fatty acid was soluble as the salt at the higher pH, whereas at the lower value the fatty acid was insoluble resulting in a maximum interaction between carotene and lipid constituents.

Sumner and Sumner (63) reported that the maximum rate of pigment bleaching occurred at low levels of linoleate. Balls et al. (3) theorized that an activator of lipoxidase was responsible, but Tookey et al. (77) showed that this maximum rate occurred at the critical micelle concentration of the linoleate. It was also noted that at this same concentration the maximum rate of conjugated diene formation occurred. The rate of bleaching was also dependent on carotene concentration with higher levels increasing the bleaching rate. The rate of oxygen uptake and conjugated diene formation was inhibited during the coupled oxidation although the effect was slight. The greatest inhibition occurred above or below 20 mM carotene.

The inhibitors of lipoxidase activity or of fatty acid oxidation prevented carotene bleaching. Shearer and Blain (56) found nordihydroguaiaretic acid to be more effective than Santoquin while other antioxidants were somewhat less effective in preventing bleaching.

The oxidized products formed from the coupled oxidation of carotene were varied. Sumner and Dounce (62) noted that lipoxidase added gaseous oxygen to the double bonds of the carotenoids. Holman (28) noted an increase in absorbance at 337.5 nm indicating cis bond formation in the early phases of bleaching. The absorption peak later decreased, implying the formation of a transitory compound. Kunkel and Nelson (42) proposed a mechanism whereby the carotene reacts either by supplying a hydrogen to a free radical peroxide formed during lipid oxidation or by adding to the free radicals produced within the system. Tappel (67) noted that the most recent evidence showed that free radical intermediates of the lipoxidase catalyzed linoleate oxidation react in non specific hydrogen abstraction from carotene. The mechanism would result in the formation of unstable compounds which would serve to perpetuate or initiate the reaction (42).

Hunter and Krakenberger (35) found that β -carotene epoxide resulted from an oxidation of the terminal double bond of the pigment and that the epoxide was converted to products of various stages of oxidation. The epoxides which could be isolated were mutachrome, aurochrome, and luteochrome. Friend (17) isolated β-carotene mono epoxide, β- apo-13-carotenone, and polyene ketones.

When crude soybean extracts were used as the source of lipoxidase in the bleaching system the reaction was more complicated (52). Blain and Styles (5) found that the bleaching reaction was slow at pH 4.5 with fresh linoleate but was rapid using oxidized linoleate. This difference indicated that a peroxidase could also function in the bleaching reaction using linoleate hydroperoxide as the oxygen donor.

The Oxidative Degradation of Chlorophyll

Enzyme catalyzed oxidation

Strain (59) observed that chlorophyll was oxidized by a system containing an aqueous extract of soybeans, a fat, and oxygen, Mapson and Moustafa (46) noted that chlorophyll was bleached during the oxidation of glutathione in a system containing a pea extract and linoleic acid. Glutathione oxidation was attributed to lipoxidase catalyzed oxidation of linoleic acid.

Wagenknecht et al. (78) noted that chlorophyll was degraded in frozen raw peas whereas blanched peas showed no pigment loss. When preparations of various enzymes were added to enzymatically inactive peas, the greatest loss of chlorophyll (35.8%) occurred when both lipase and lipoxidase were added (79). Lipase alone caused 27.7% chlorophyll loss and lipoxidase brought about 12.3% loss. It was not shown if the chlorophyll had been changed merely to pheophytin, the metal free chlorophyll derivative.

Walker (80) related fat peroxidation to chlorophyll loss in frozen green beans. When an extract of French beans was incubated with a crude chlorophyll preparation and linoleic acid, the chlorophyll was first degraded to pheophytin under both aerobic and anaerobic conditions. After a lag period, the chlorophyll was bleached aerobically but not anaerobically. With purified chlorophyll there was neither conversion to pheophytin nor a lag period before the color started to disappear. It was stated, without giving experimental details, that purified lipoxidase gave similar results as the bean extract.

Holden (26) investigated the chlorophyll bleaching activity of several legume seed extracts and their response on addition of several different fatty acids. Calvary clover (*Medicago echinus*) and soybeans (*Glycine max*) had the greatest bleaching activity when linoleic acid was added. Linoleic and linolenic acids showed the greatest bleaching rate in the presence of the soy extract. Chlorophyll *a* was bleached more rapidly than chlorophyll *b* and loss of the pigment was inhibited by various antioxidants, butylated hydroxytoluene (BHT) being the most effective with soy extracts. When purified lipoxidase was added to the seed extracts, the extent of bleaching was increased; however, neither peroxidized linoleic acid nor lipoxidase plus lipid bleached chlorophyll. It was postulated that a co-oxidation factor was required between chlorophyll and linoleic acid since lipoxidase plus lipid did not bleach the pigment as rapidly as the crude soybean extract.

Non-enzymatic chlorophyll oxidation

Chlorophyll, upon illumination in a lipid environment, has been assumed to have an effect similar to lipoxidase on linoleic acid (36).√ Hall and Mackintosh (20) showed that an increased peroxide value of pork sausage resulted when sage was added. Replacing the sage with a chlorophyll preparation produced a similar increase. If chlorophyll-lipid systems were kept in the dark the chlorophyll served as an antioxidant (71). Cobern et al. (8) showed that chlorophyll catalyzed linoleic acid oxidation was different from the lipoxidase catalyzed oxidation in that the hydroperoxides occurred at carbon atoms 9, 10, 12, and 13 of linoleic acid.

Isolated chloroplasts, suspended in an isotonic salt solution, swelled and the chlorophyll was bleached upon illumination (50). The extent of bleaching increased with increasing light intensity and was inhibited by ascorbate. Heath and Packer (24, 25) showed that a cyclic peroxidation occurred, and malondialdehyde was produced. The peroxidation was inhibited when the antioxidant BHT was used.

Lu (45) has shown that chlorophyll photosensitized the decomposition of hydroperoxides when exposed to red light, resulting in oxygen evolution and bleaching of the pigment. An unstable oxychlorophyll intermediate could be shown which would undergo further oxidation to an unknown pigment. The photobleaching of chlorophyll has been shown to undergo a similar sequence (44).

Chlorophyll, when illuminated in the presence of certain oxidants, transferred an electron to the oxidant (39). Oxygen had to be excluded or an interaction with the excited state of chlorophyll occurred with resultant loss of the pigment. Tollin and Green (76) noted that the oxidation occurred in the dark only when very strong oxidizing agents were used.

In the dark, chlorophyll in an absolute methanol solution reacted almost instantaneously when ferric chloride was added forming a yellow solution (8). The chlorophyll could be regenerated by the addition of ascorbic acid. Tolbert and Burris (75) found that chlorophyll was converted to a similar product when glycolic acid was added to barley extracts. The glycolic acid was converted to glyoxylic acid through a postulated organic peroxide intermediate.

The allomerization of chlorophyll occurs when methanolic solutions are exposed to air (37). Fischer and Pfeiffer (14) postulated that the initial product is a hydroperoxide which labilizes the cyclopentanone ring. A transient free carboxyl occurs at the C_6 carbon, subsequently leading to a reaction with the solvent, lactonization and production of a lactone ether. Holt (31) purified purpurin 7-lactone ether diester, among others, produced by allomerization of chlorophyll a, agreeing with the postulated product of Fischer and Pfeiffer (14). Pennington et al. (5) showed that a C-10 hydroxychlorophyll a was also produced.

Strain (60) has separated various oxidized chlorophylls from killed leaves subjected to various treatments. The most abundant oxidized pigment was identical to an allomerized chlorophyll a. Pennington et al. (51) has shown this oxidized pigment to be C-10 hydroxychlorophyll. It was postulated that the pigment was produced by enzymatic catalysis similar to that of the Holden bleaching system (26).

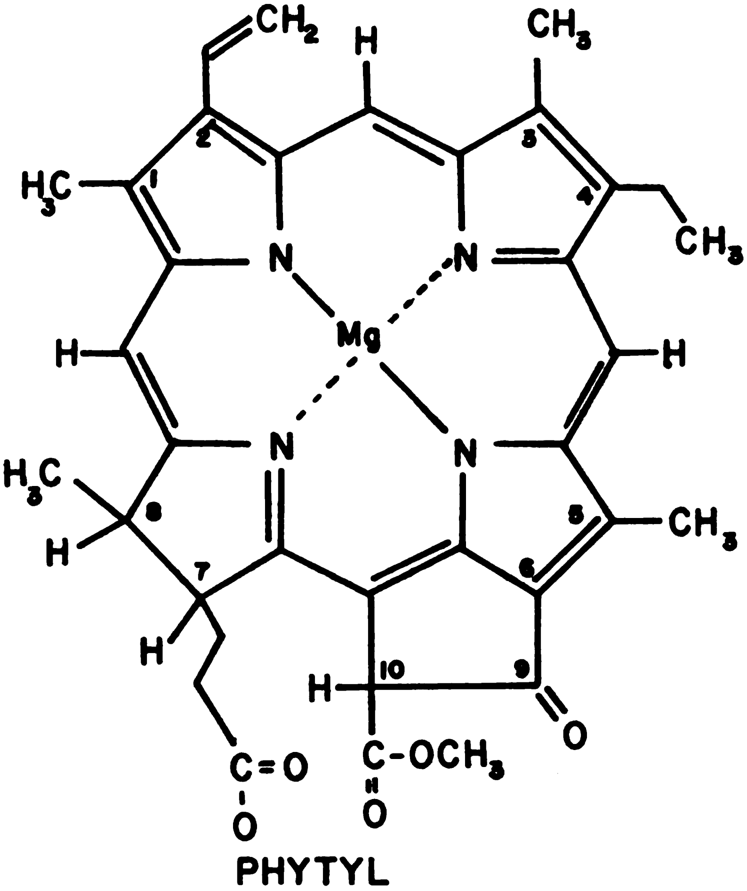


Figure 1. Structure of chlorophyll _a_

MATERIALS AND METHODS

Assay System for Chlorophyll Degradation

Certified Harasoy soybeans were used in the evaluation of chlorophyll bleaching as described by Holden (26). The fatty acids and purified soybean lipoxidase were obtained from the Sigma Chemical Company. Chlorophyll a was purified from fresh spinach using the method described by Strain and Svec (6).

The crude soybean extracts were prepared by soaking the seeds overnight in distilled water, removal of the testas, and grinding 5 grams of the cotyledons with sand in a mortar in 15 ml. pH 5.9 acetate buffer (0.2M acetate). The suspension was centrifuged at 5000 x g for 10 minutes and the supernatant used as the enzyme preparation.

The oil free soybean extracts were prepared by grinding soybean seeds in a mechanical mortar (Torsion Balance Co., Clifton, New Jersey) using both acetone and hexane to remove the lipid fraction. The ground seeds were re-extracted with acetone and hexane in a suction funnel and then dried under vacuum. Five grams of the dried seed meal were stirred into 15 ml. pH 5.9 acetate buffer, centrifuged at 5000 x g for 10 minutes, and the supernatant used as the enzyme source. The purified lipoxidase obtained from The Sigma Chemical Company had 1600 units/mg activity using

Surrey's method (64) to determine lipoxidase activity. This was used as obtained. The required amount was weighed and dissolved in 0.2M acetate buffer (1mg/ml.) just prior to use.

The bleaching system was prepared as described by Holden (26) for both crude extracts and purified lipoxidase. The system consisted of the addition of a 5% linoleic acid solution in methanol (W/V) to the chlorophyll in acetone solution followed by rapid addition of pH 5.7 acetate buffer (0.01M acetate). The total reaction volume was 20 ml. Final concentrations of the reaction system were: 0.75% methanol, 2% acetone, 1.3 mM linoleic acid and 25mg/liter chlorophyll *a*. The various enzyme sources containing 972 units of lipoxidase were then added to the above preparations and the reaction carried out under dim lighting with continuous agitation. The rate of chlorophyll bleaching was proportional to enzyme concentration in this system.

One ml aliquots of the bleaching system were used to determine chlorophyll loss. Acetone (one ml.) was added to stop the reaction and the chlorophyll was then converted to pheophytin using oxalic acid (61). Acetone was added after the conversion to a final volume of five ml. The final pigment concentration was determined from the red absorbance maximum (666 nm) of pheophytin. Pheophytinization as a function of linoleic acid concentration was determined using the method of Dietrich (9). Absorbancies were determined on a Beckman DU spectrophotometer equipped with a Gilford readout.

The lipoxidase activity of the enzyme preparations was determined using the method of Surrey (64). Conjugated diene formation was determined by diluting 1.0 ml aliquots of the bleaching system with 2 ml ethanol. Ethanol (60%) was then added to a total volume of 10 ml and the absorbance determined at 234 nm. Conjugated dienes were found to be caused only by the linoleic acid component by determining the absorbance of samples not containing the substrate. The rate of chlorophyll bleaching was calculated from the linear portion of the degradation curve and the critical micelle concentration was measured with a du Noüy ring tensiometer as described by Alexander and Johnson (1).

End Product Analysis

Extracts of control samples with and without lipoxidase were compared chromatographically to extracts taken after a five minute reaction time to determine the products formed from chlorophyll in the reaction system. The reaction was stopped by diluting with methanol to a 50% concentration and the total system was extracted immediately with petroleum ether using concentrated NaCl solution to aid in the extraction of the pigments. The petroleum ether extracts were evaporated under vacuum to a volume of approximately three ml, then diluted to 10 ml with benzene and placed on a powdered sucrose column. The column was developed using 0 to 10% (V/V) n-propanol in benzene (31,60). The remaining chlorophyll and pheophytin was eluted from the column with 0.5% n-propanol. A single band which was eluted with 4% n-propanol in benzene was found only in the reacted system. To insure purity of the isolate it was rechromatographed a minimum of three times with the above solvent system and with solvents composed of ethanol in benzene, and n-propanol in petroleum ether. Redistilled solvents were used for the isolation of the end product.

Thin layer chromatography was performed using the method of Hagar and Bertenrath (19) by modification of the developing solvent. The modified solvent consisted of heptane; isopropanol; 1 M. acetic acid (100:12:0.25).

The Molisch phase test for allomerization of the end product and determination of the hydrochloric acid number was performed as described by Seely (54). Visible spectra were obtained with a Beckman DK-2 Spectrophotometer. Infrared analysis was performed using a Beckman IR-12 Infrared Spectrophotometer. The solid state infrared spectrum was obtained by drying a concentrated carbon tetrachloride solution of the isolate onto potassium bromide plates under a stream of nitrogen. Spectral grade carbon tetrachloride was used for the liquid spectra.

RESULTS

The results are presented in two divisions. The first deals with the parameters of the chlorophyll bleaching system and the second will be concerned with analysis of the end product of the bleaching reaction.

Kinetics of the Chlorophyll Bleaching Reaction

Chlorophyll bleaching by lipoxidase

Chlorophyll degradation by purified lipoxidase exhibited an optimum pH of 5.7 (Figure 2), while the optimum temperature of bleaching occurred at 25°C (Figure 3). Pigment loss was linear with time up to approximately 30% destruction of the chlorophyll (Figure 4). No induction periods were observed either for chlorophyll loss or diene conjugation. The slope of the linear portion of the chlorophyll bleaching curve was used as a measure of the reaction rate.

To insure that the soybeans used in this study possessed the same bleaching activity as those used by Holden (26), a reaction was carried out as outlined with 64% degradation occurring after two minutes. These soybeans were used throughout this study since the amount of bleaching was similar to that reported.

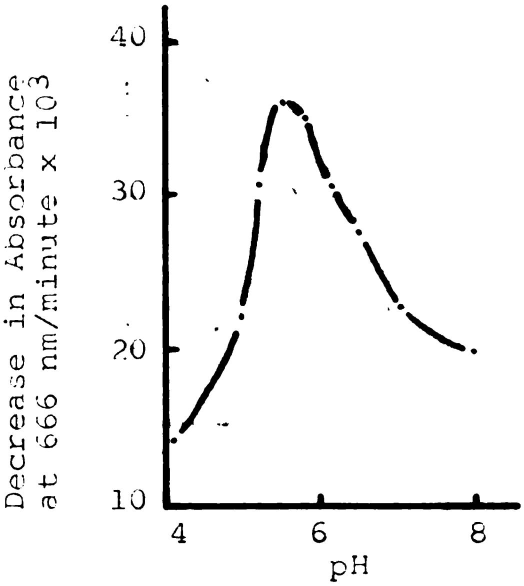


Figure 2. Effect of pH on the rate of chlorophyll bleaching by purified lipoxidase

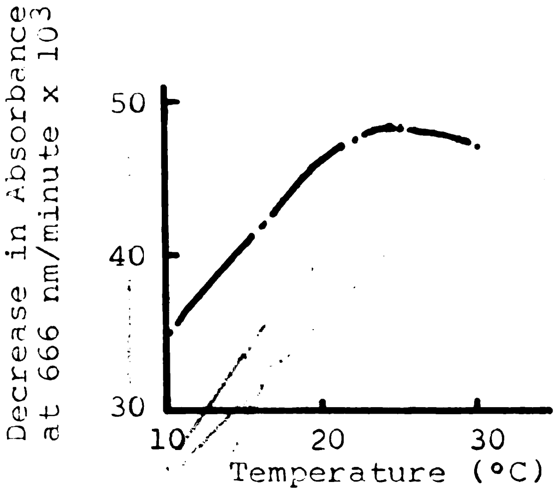
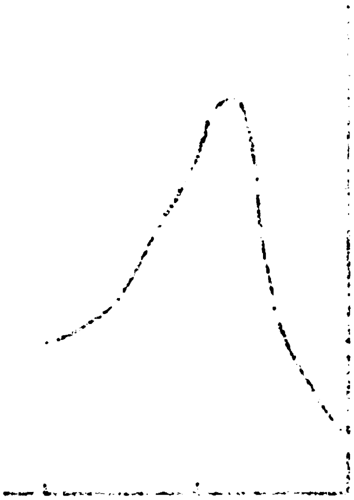
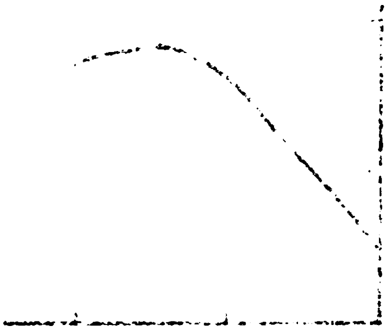


Figure 3. Effect of temperature on the rate of chlorophyll bleaching by purified lipoxidase





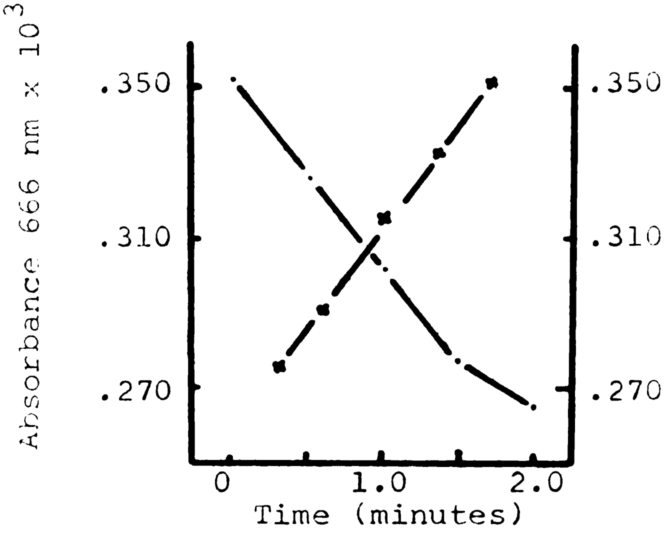


Figure 4. Changes in absorbancy of the chlorophyll bleaching system with time showing chlorophyll destruction (·—) at 666 nm and conjugated diene formation (×) at 234 nm.

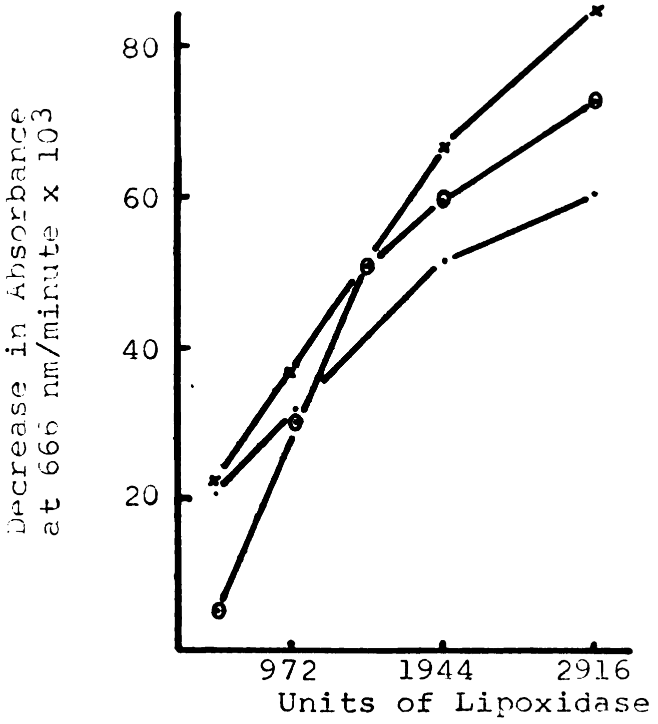
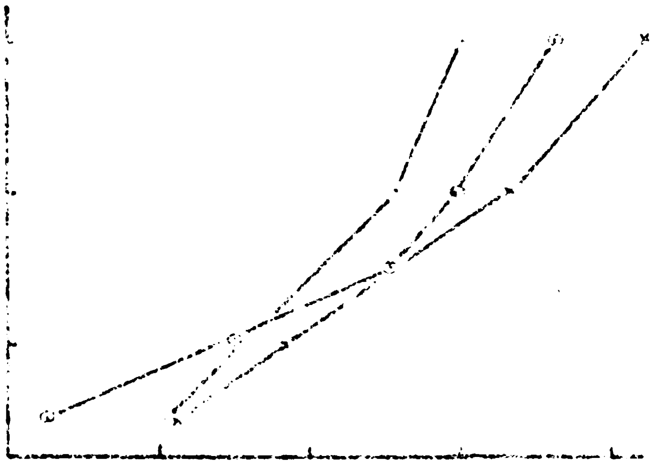
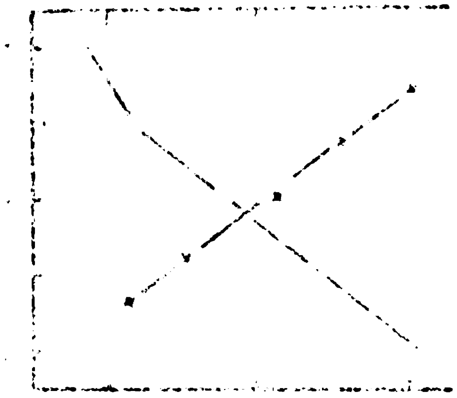


Figure 5. Chlorophyll bleaching rate with respect to enzyme source and concentration: lipoxidase ⊖ , lipid extracted soybeans ·—, and crude soybean extract × .





Chlorophyll bleaching by the various enzyme preparations

Bleaching rates using the various enzyme preparations at equivalent lipoxidase activity are given in Figure 5. The rate of bleaching was found to be dependent on enzyme concentration. Both of the crude soybean extracts exhibited a linear relationship from 486 to 1944 units of lipoxidase, whereas bleaching with the purified lipoxidase was linear from 486 to 1458 units. The rate of bleaching decreased markedly at higher enzyme concentrations.

The crude soybean preparations and purified lipoxidase exhibited a maximum rate of bleaching as a function of chlorophyll concentration (Figure 6). The maximum occurred at 25mg/liter chlorophyll concentration for the purified lipoxidase and the lipid extracted soybean preparation, whereas the maximum occurred at 12.5 mg/liter for the crude soy preparation.

Chlorophyll bleaching rate as a function of linoleic acid concentration showed a maximum at low levels of the fatty acid above which a decreased rate occurred (Figure 7). The maximum occurred at 0.34 mM linoleic acid regardless of the enzyme source, although the crude enzyme extract exhibited a more pronounced effect. The maximum rate of bleaching corresponded to the critical micelle concentration of linoleic acid (0.34 mM) with chlorophyll present (Figure 8). Without chlorophyll in the reaction mixture, the critical micelle concentration was 0.10 mM linoleic acid.

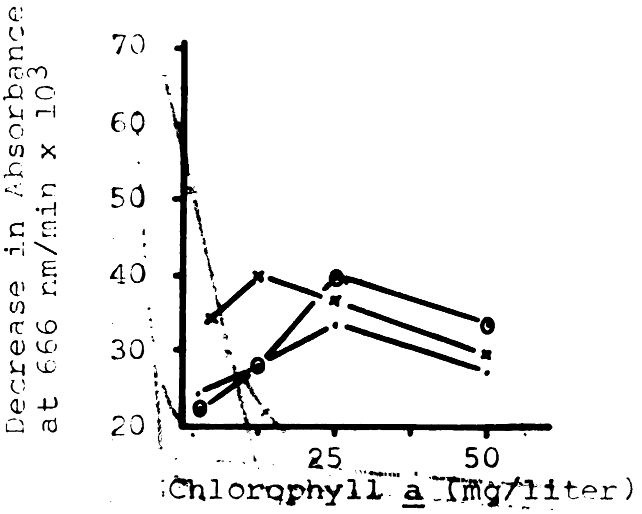


Figure 6. Effect of chlorophyll concentration on the bleaching rate by the various enzyme sources: lipoxidase ⊖, lipid extracted soybeans •—, and crude soybean extract ×.

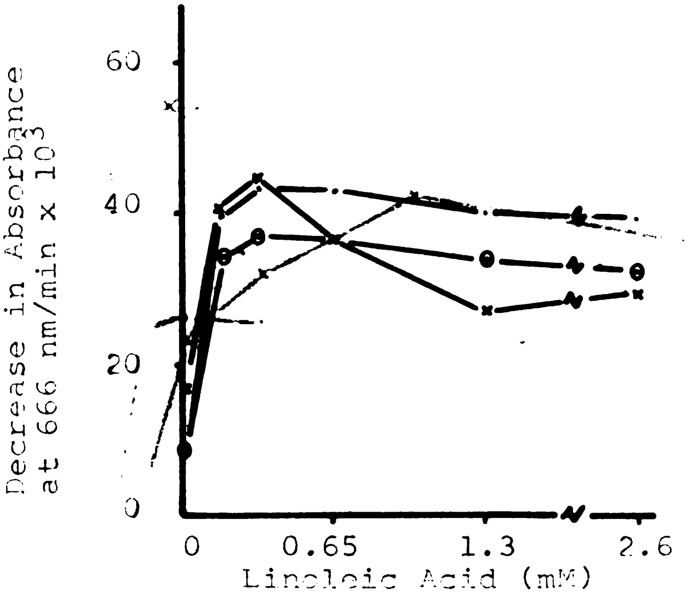
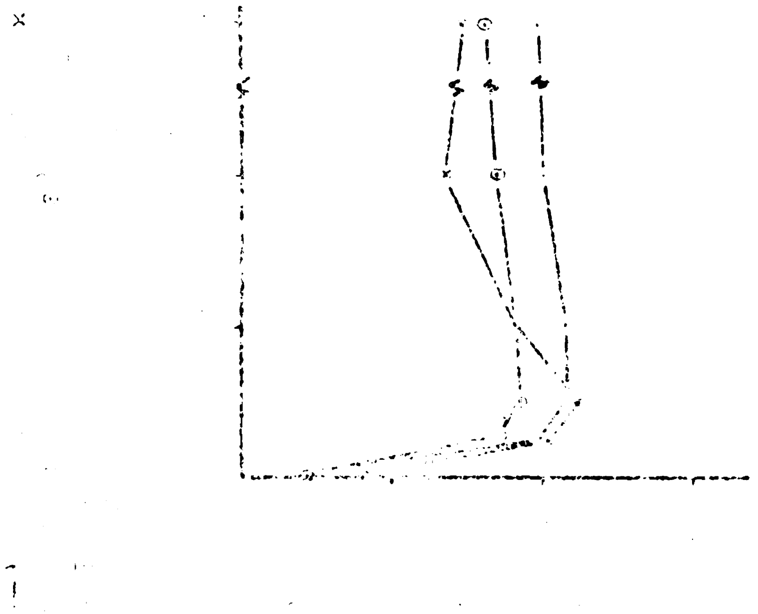
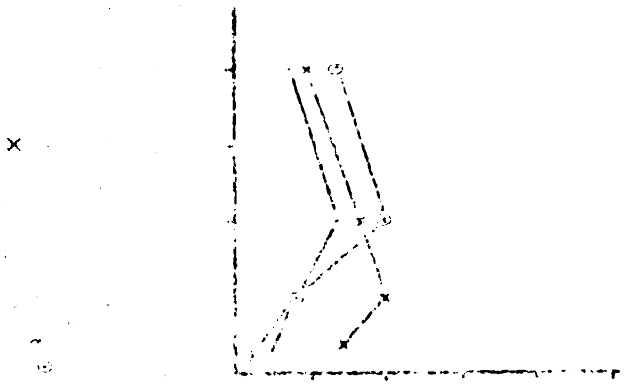


Figure 7. Effect of linoleic acid concentration on the chlorophyll bleaching rate by the various enzyme sources: lipoxidase ⊖, lipid extracted soybeans •—, and crude soybean extract ×.





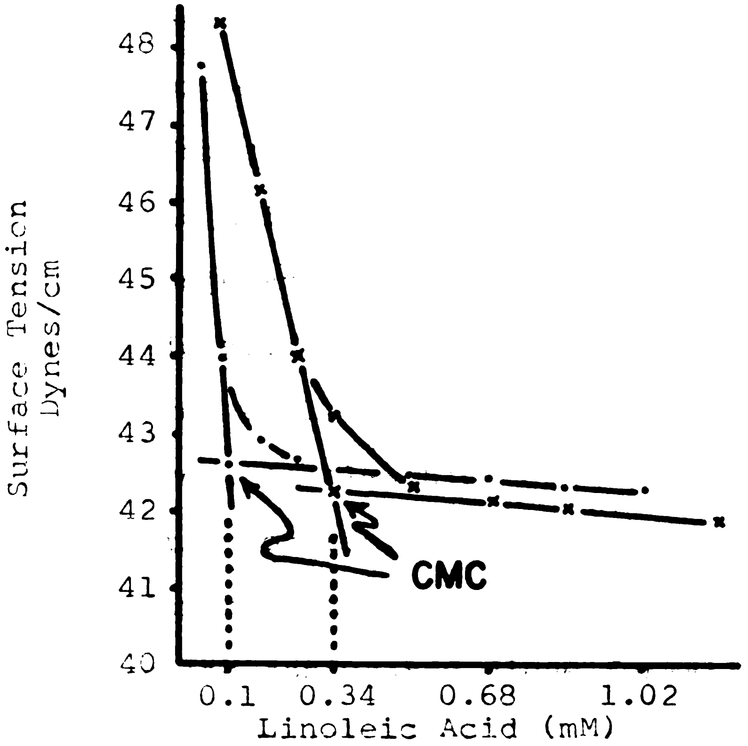


Figure 8. Critical micelle concentration of the linoleic acid: without chlorophyll •—, and with chlorophyll x.

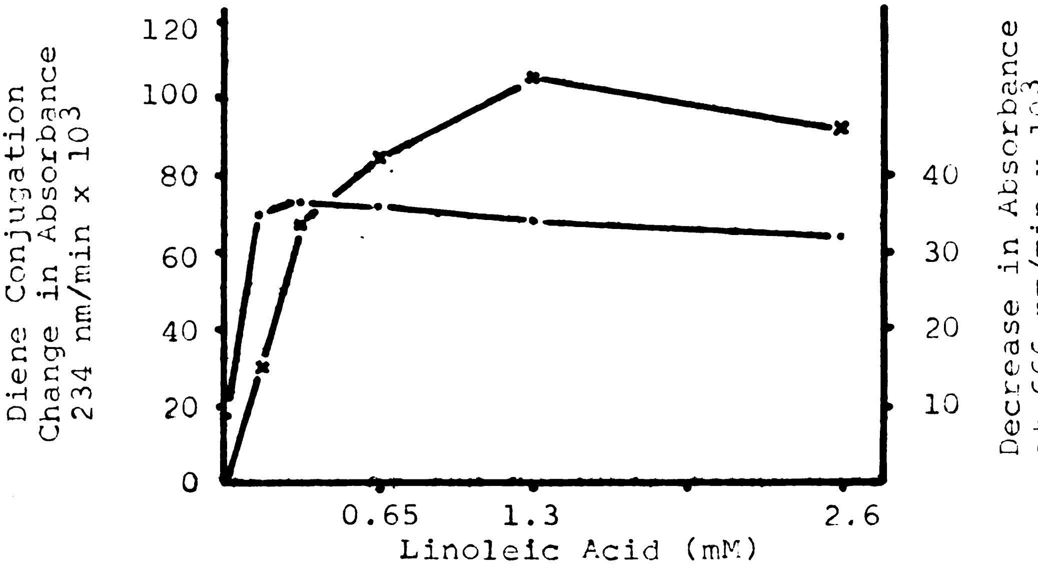
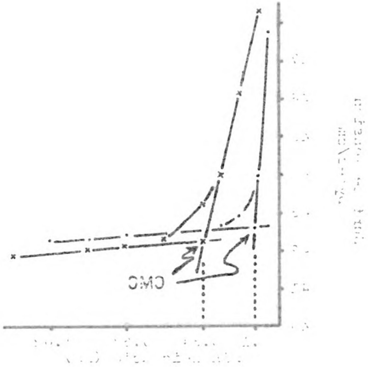
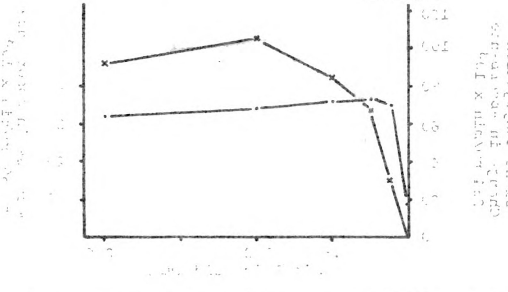


Figure 9. Effect of linoleic acid concentration on chlorophyll bleaching •— and diene conjugation x by purified lipoxidase.


CMC



Conjugated diene formation and chlorophyll bleaching

The rate of conjugated diene formation as a result of linoleic acid oxidation and pigment degradation was followed as a function of linoleic acid concentration (Figure 9). The rate of pigment bleaching paralleled the rate of conjugated diene formation to the critical micelle concentration of linoleic acid. Both the rate of chlorophyll degradation and conjugated diene formation decreased at increasing linoleic acid levels. The maximum rate of diene conjugation occurred at 1.3 mM linoleic acid while the maximum rate of chlorophyll bleaching occurred at the critical micelle concentration of the substrate.

No relationship was found between the rate of chlorophyll degradation and conjugated diene formation as a function of chlorophyll concentration (Figure 10). The maximum rate of conjugated diene formation was found to occur at 6.3 mg/liter chlorophyll _a_, with higher concentrations decreasing the rate.

Conversion of chlorophyll to pheophytin

Chlorophyll _a_ was converted to pheophytin upon suspension in the buffer system when linoleic acid was present. The rate of conversion was independent of linoleic acid concentration over a wide range with the rate being affected only at the extremes of the concentrations used (Figure 11). Neither the buffer alone nor methyl linoleate brought about the conversion. Oleic and stearic acids caused a similar conversion.

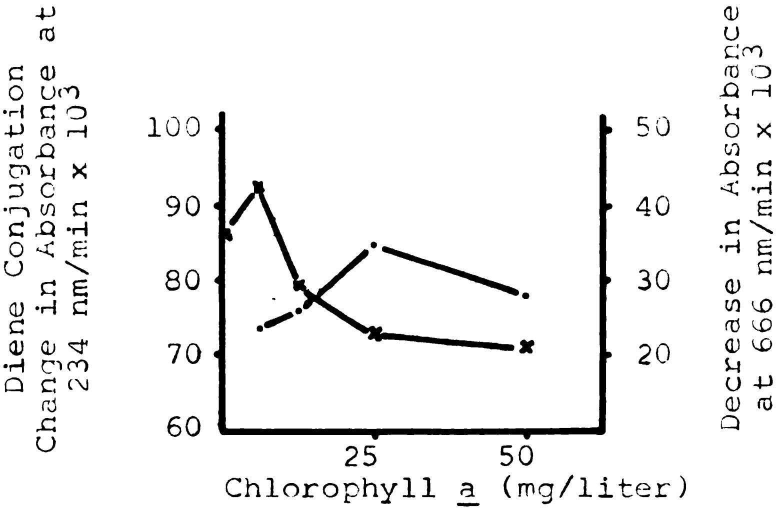


Figure 10. Effect of chlorophyll concentration on diene conjugation ×, and chlorophyll bleaching•—, by lipoxidase.

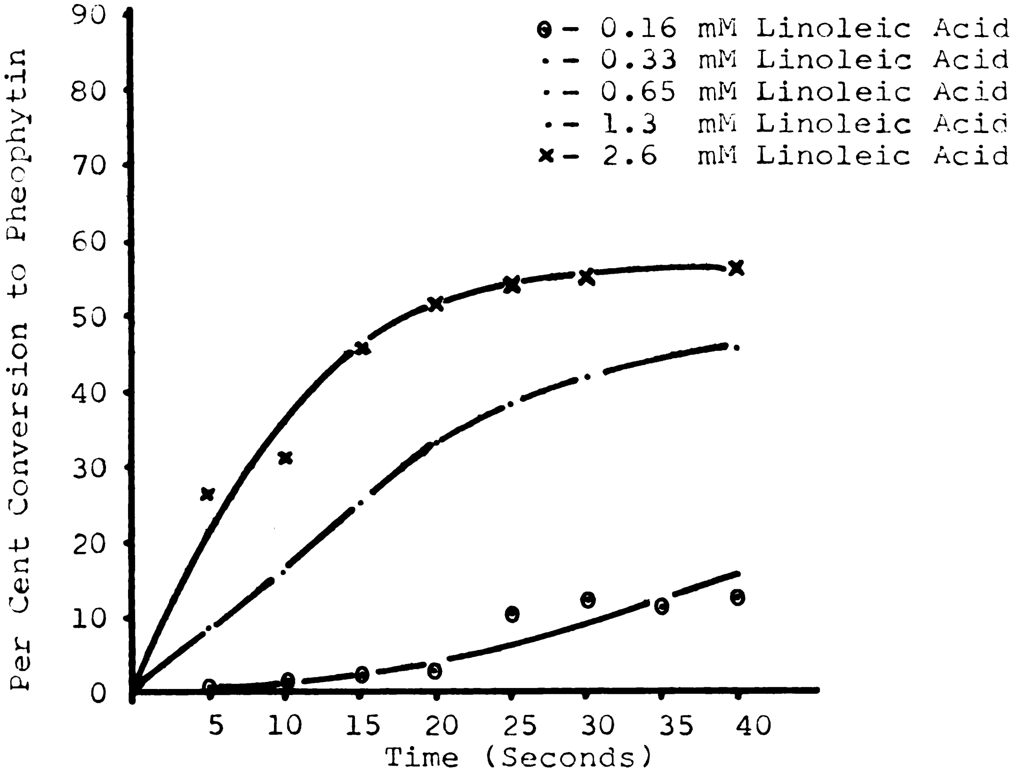
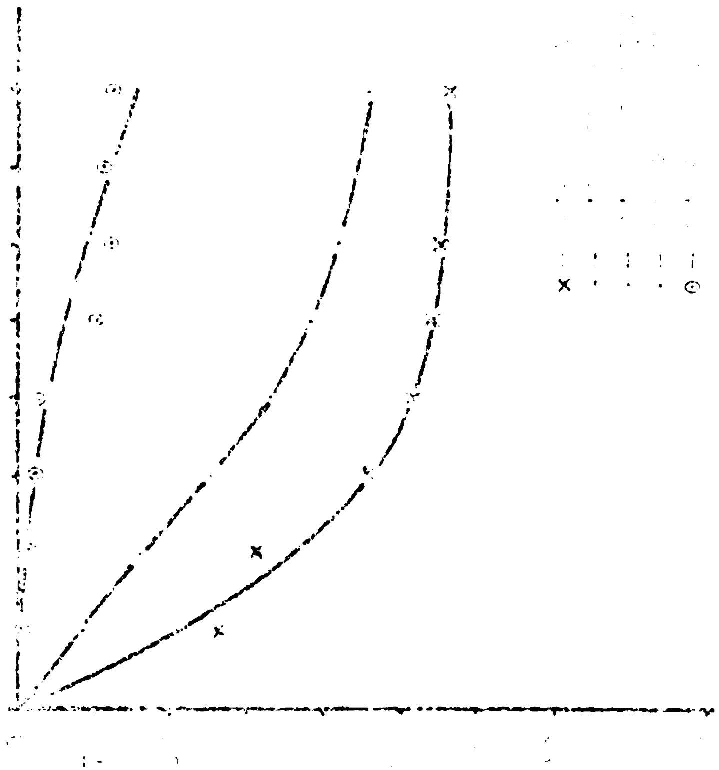
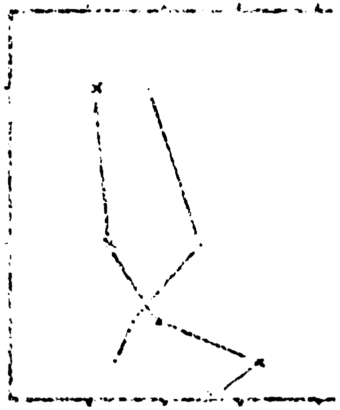


Figure 11. Effect of linoleic acid concentration on conversion of chlorophyll to pheophytin.





Inhibition of chlorophyll bleaching

The effect on the bleaching system of substituting for linoleic acid three fatty acids which are not substrates for lipoxidase is listed in Table 1. Also listed is the effect of two lipid antioxidants. Linoelaidic and stearic acids produced 100% inhibition while the oleic acid, containing 13% linoleic, caused 93% inhibition. Methyl linoleate, which is a substrate of lipoxidase, did not bring about pigment bleaching.

Table 1. Fatty acid and antioxidant inhibition of chlorophyll bleaching

Fatty Acid or Antioxidant	Concentration	Per Cent Inhibition
Stearic Acid	1.3 mM	100
Oleic Acid	1.3 mM	93
Linoelaidic Acid	1.3 mM	100
Methyl linoleate	1.3 mM	100
Butylated hydroxytoluene	10mg/liter	61
Nordihydroguaiaretic Acid	90mg/liter	100

The antioxidants, BHT and NDGA, caused a marked decrease in the bleaching rate. The BHT was found to show a maximum of 62% inhibition at 10mg/liter whereas NDGA caused total inhibition at a concentration of 90mg/liter (Table 1).

Spectral analysis of the reaction system

The visible spectra at various reaction times are presented in Figure 12. A decrease in absorbance occurred in all regions with a marked increase in the ratio of blue to red absorbancy from 2.5 to 3.0 occurring with increasing reaction time (Table 2). While no distinctly new absorbance peaks appeared, an increase in the shoulder of the 409 nm peak appeared relative to the 409 nm peak itself.

Table 2. The 409nm/666nm peak ratio of the visible absorption spectra of the reaction system at various reaction times

Reaction Time (minutes)	Peak Ratio 409nm/666nm
0	2.5
1.5	2.6
5.0	2.8
10.0	3.0

End Product Analysis

Chromatography

The end product of the chlorophyll bleaching reaction was purified by sucrose column chromatography using 4% n-propanol in benzene (V/V) as the developing solvent. The unreacted chlorophyll and pheophytin was removed at lower n-propanol concentrations. The red fluorescent band, the chlorophyll oxidation product, moving with 4% n-propanol in benzene was rechromatographed with ethanol in benzene and n-propanol in petroleum ether moving at a 3% alcohol

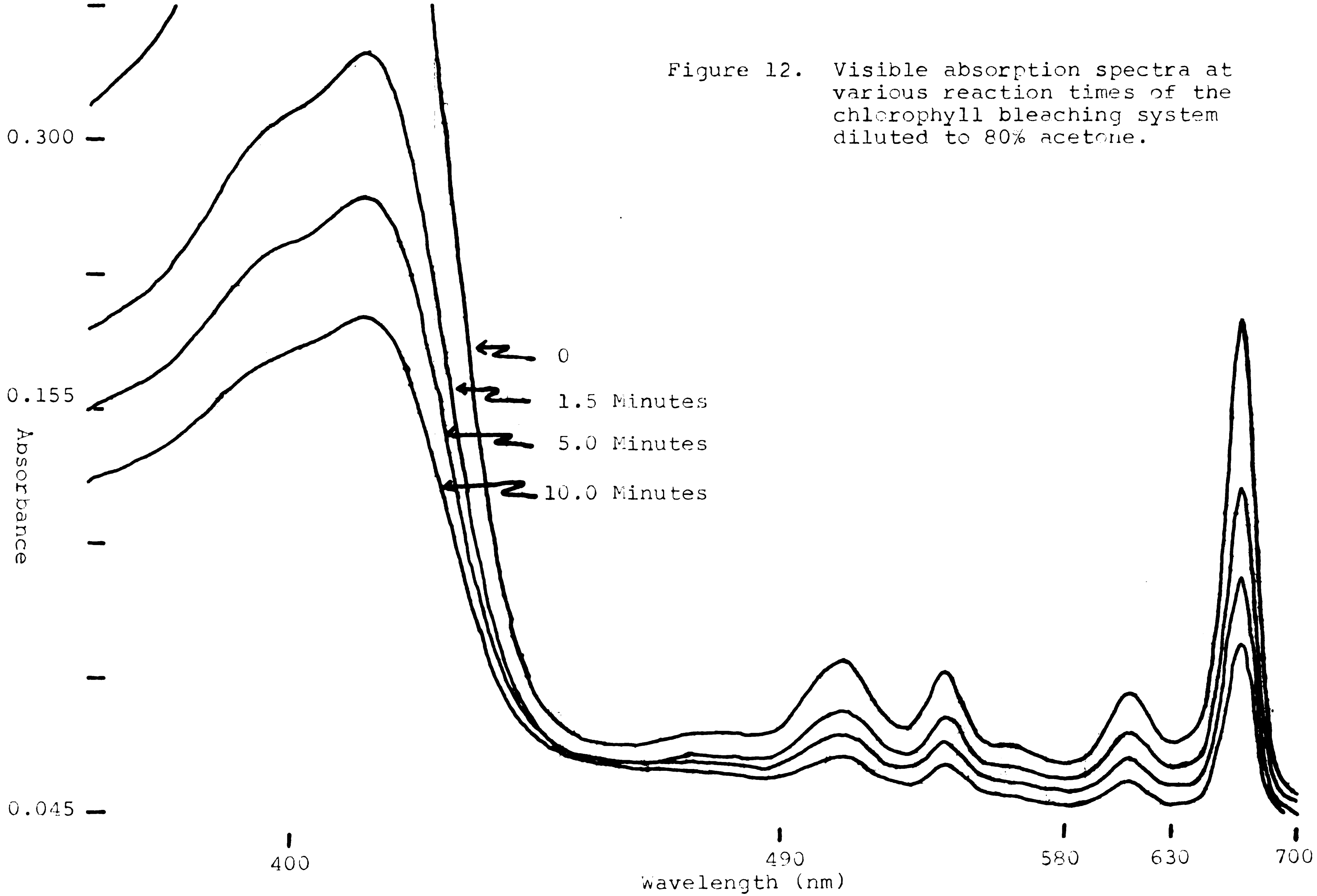
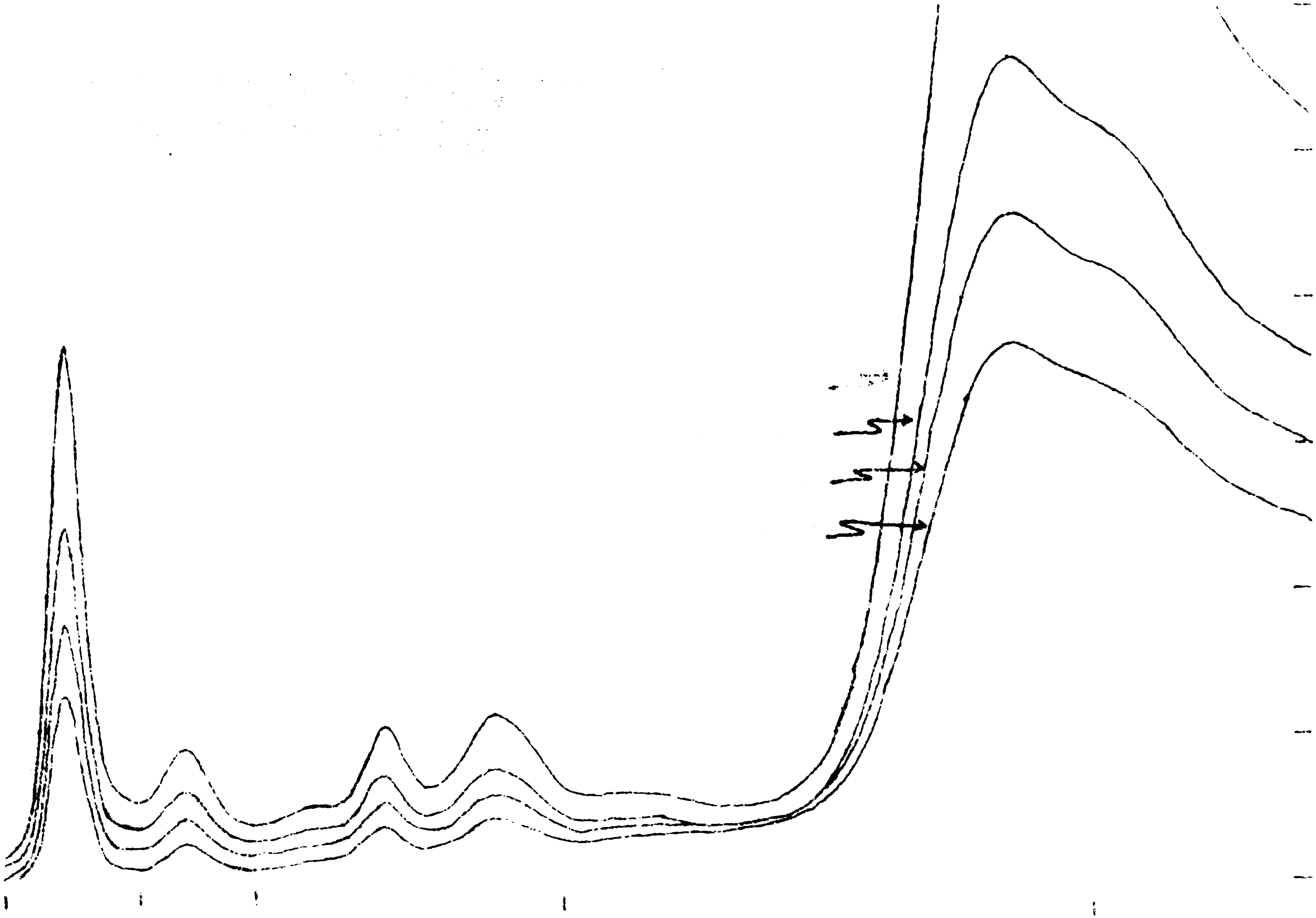


Figure 12. Visible absorption spectra at various reaction times of the chlorophyll bleaching system diluted to 80% acetone.



concentration in both solvent systems. This band was chromatographed as a single band using the Hagar and Bertenrath (19) method with the modified developing solvent. The oxidized chlorophyll moved with an $R_f = 0.28$.

Characteristics of the chlorophyll oxidation product

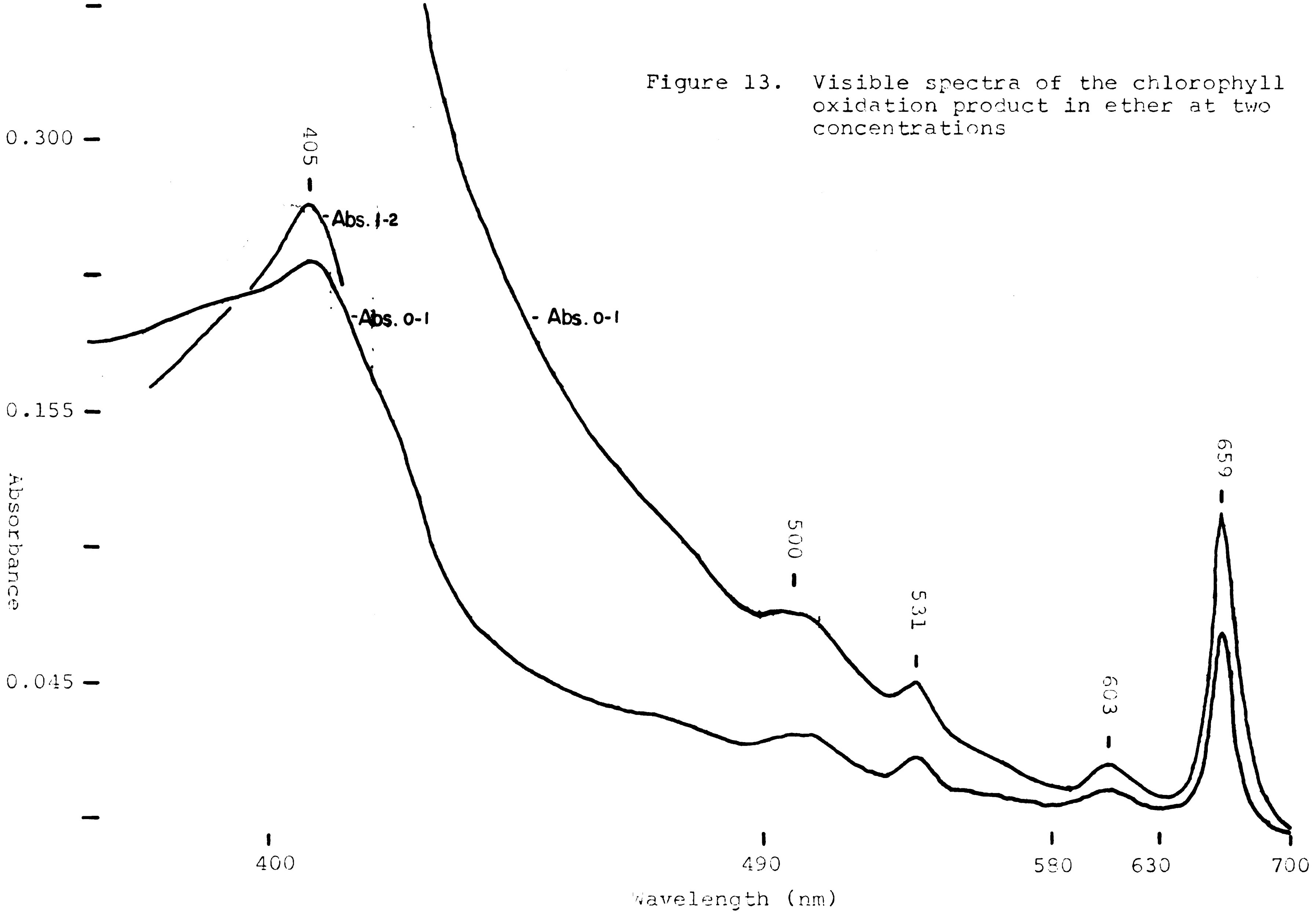
The visible spectrum of the red fluorescent oxidized chlorophyll is presented in Figure 13. The principal absorption peaks appeared at 405 nm and 659 nm with minor peaks at 500 nm, 531 nm, and 603 nm. Spectra were also obtained after treatment of the oxidized chlorophyll with HCl and oxalic acid to insure that it was free of magnesium. No band shifts were apparent in the resulting spectra which were indicative of pheophytinization.

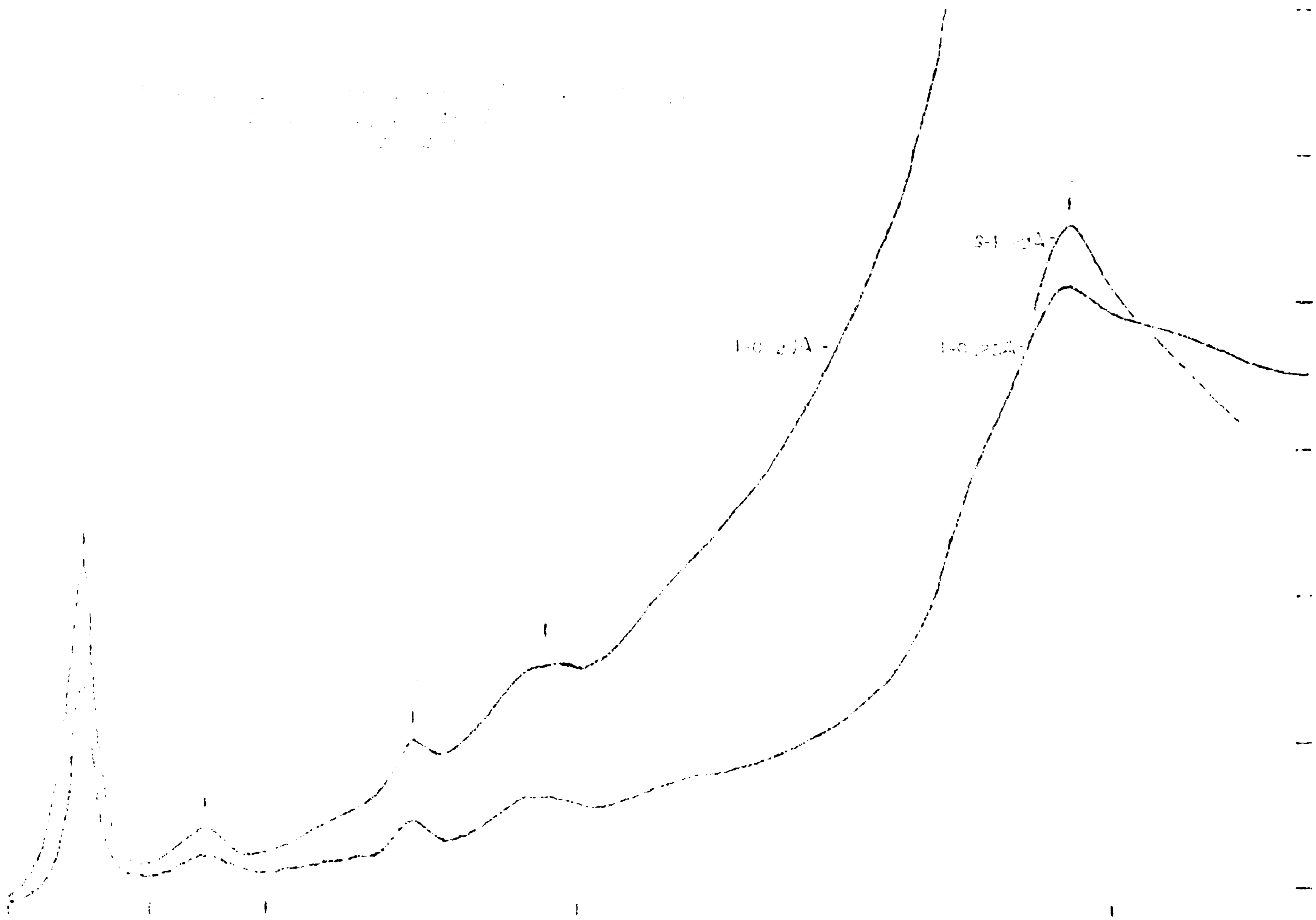
The oxidized pigment gave a negative Molisch phase test for allomerization and the hydrochloric acid number was found to be 24. It could not be extracted into 0.01N. aqueous potassium hydroxide or into 5% aqueous sodium carbonate from an ether solution.

Infrared spectra of the oxidized chlorophyll

The IR spectrum of the oxidized chlorophyll is presented in both the solid state and carbon tetrachloride (Figures 14 and 15). The spectra are divided into the four regions described by Katz (38): (a) 4000 to 2700 cm^{-1}, (b) 1750 to 1600 cm^{-1}, (c) 1600 to 1300 cm^{-1}, and (d) 1300 to 600 cm^{-1}.

Figure 13. Visible spectra of the chlorophyll oxidation product in ether at two concentrations





1·5 μA
1·0 μA
1·0 μA

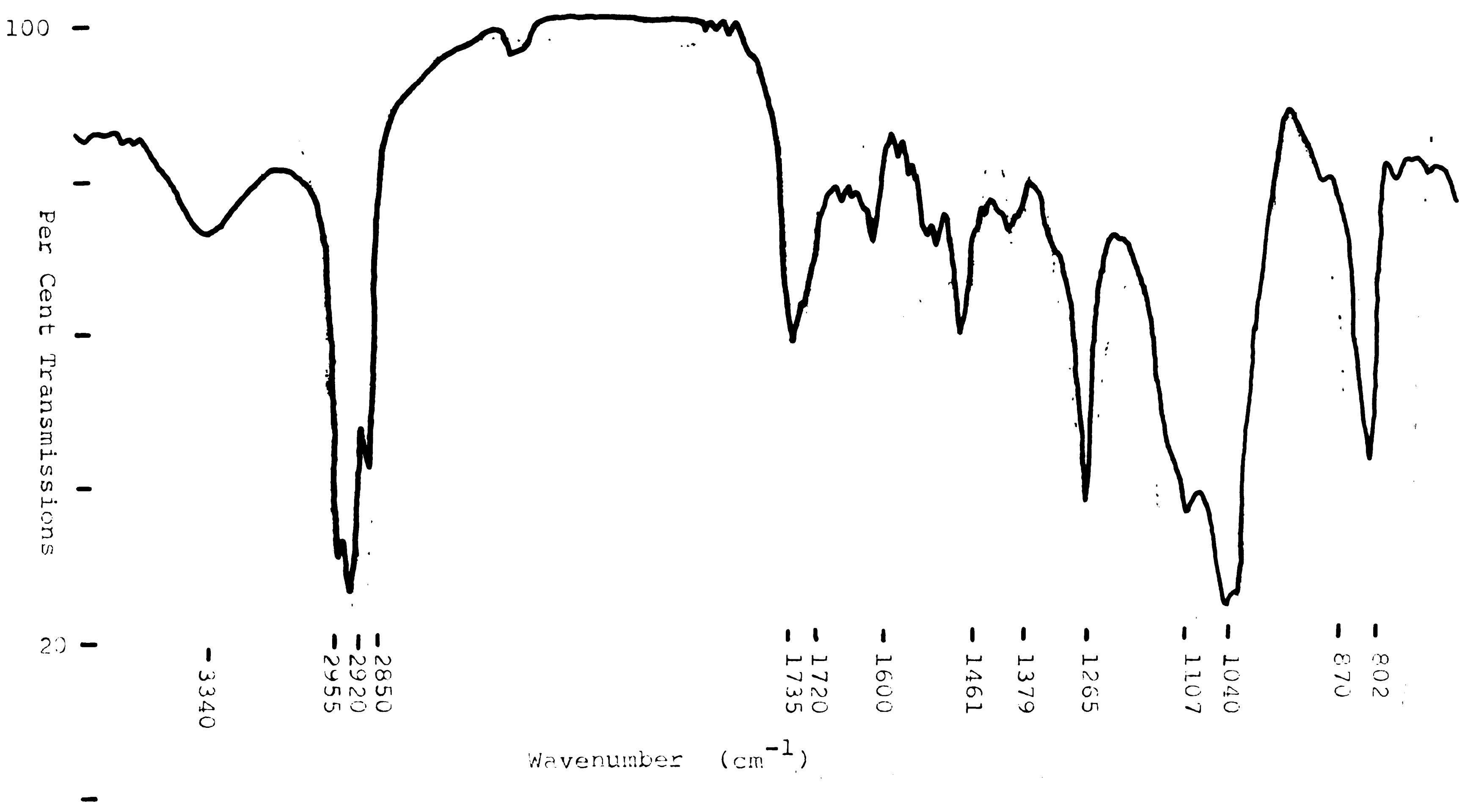
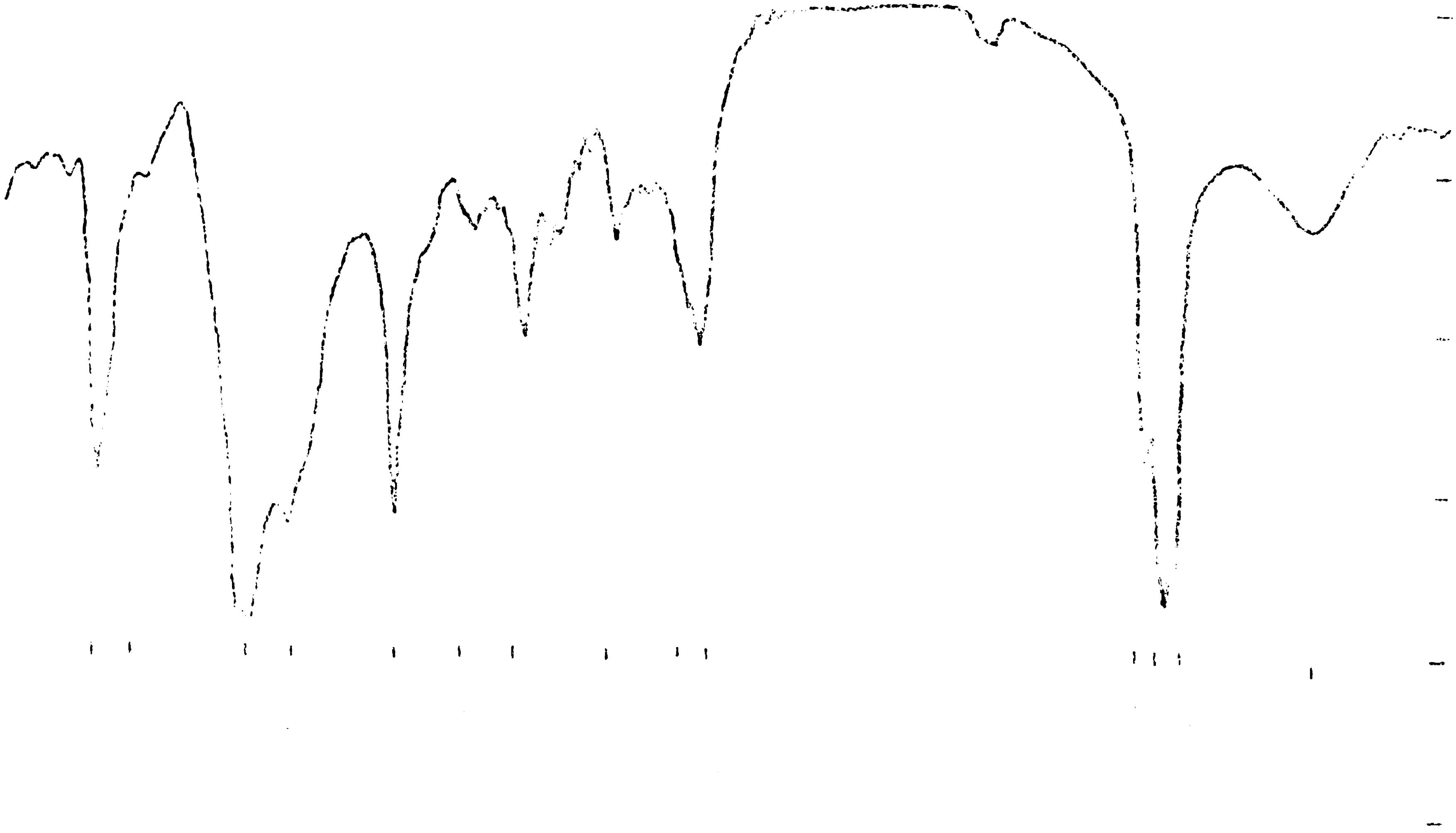


Figure 14. Infrared absorption spectrum of the chlorophyll oxidation product in the solid state



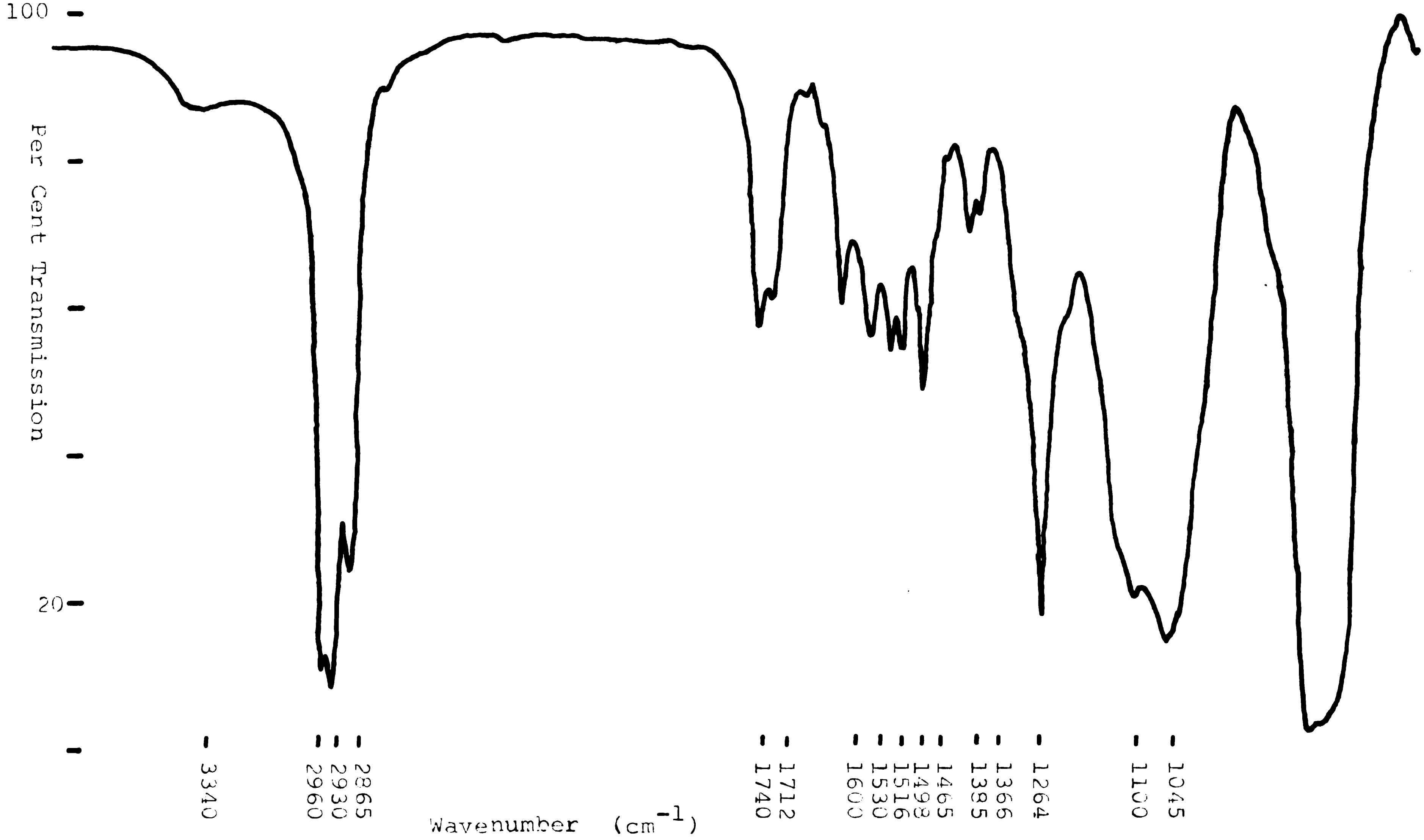
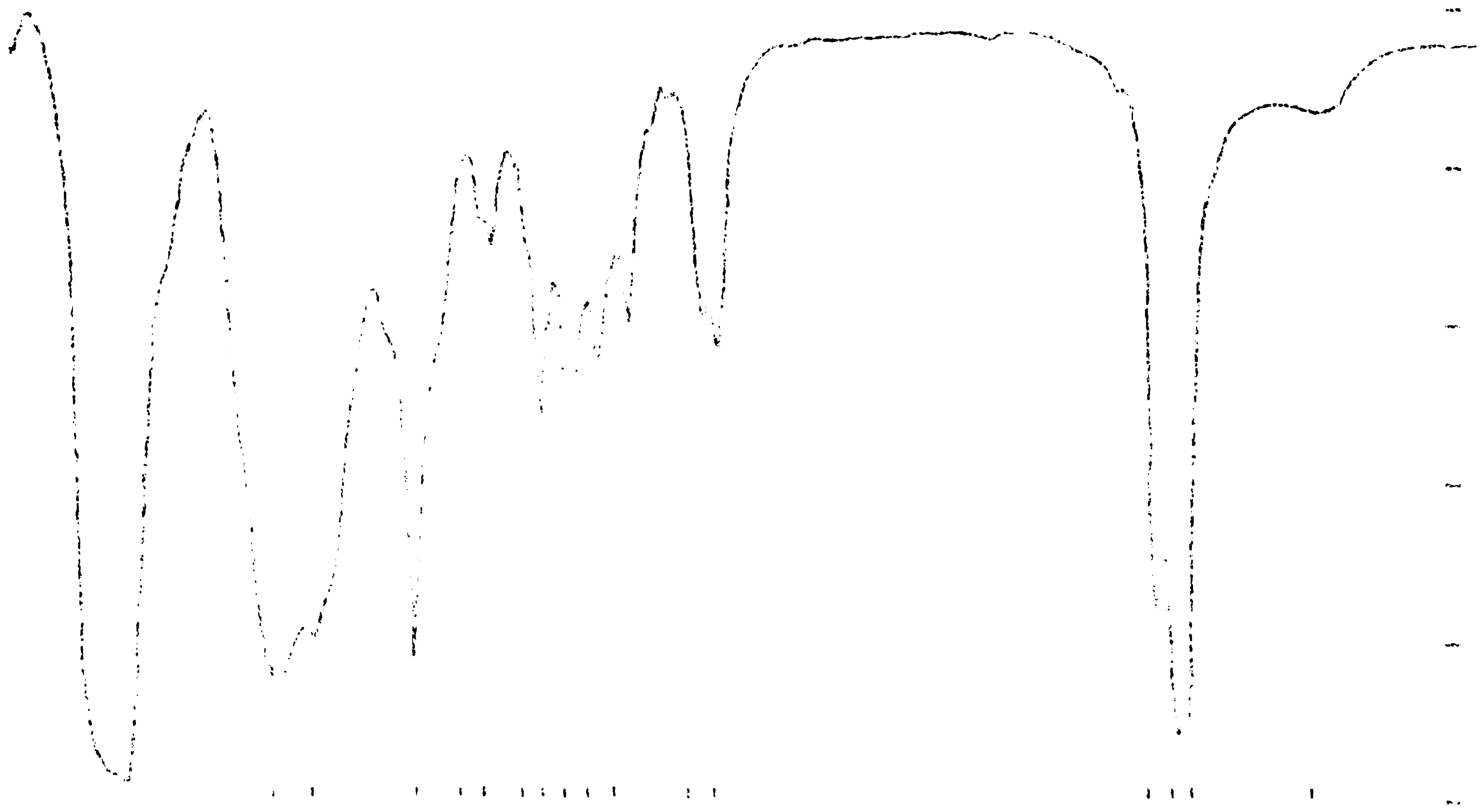


Figure 15. Infrared absorption spectrum of the chlorophyll oxidation product in carbon tetrachloride



(a). The 4000 to 2700 cm^{-1} region:

The spectra in both the solid state and carbon tetrachloride had a broad band centered at 3400 cm^{-1}. This was more pronounced in the solid state spectrum and is attributed to N-H stretching and O-H stretching of water. After extended drying of the oxidized chlorophyll in the solid state, the spectra still possessed the -OH band.

The absorption at 2965 - 2955 cm^{-1} is assigned to C-H stretch of $-CH_3$, at 2940 - 2925 cm^{-1} to antisymmetric C-H stretching of CH_2, and that at 2880 - 2865 cm^{-1} to overlapping of symetric $-CH_3$ and $-CH_2$ stretching modes.

(b). The 1750 to 1600 cm^{-1} region:

The C-7 and C-10 esters and ketone carbonyl group have bands in this region. A broad band in the solid state spectrum occurred at 1735 cm^{-1} with a shoulder at 1720 cm^{-1}. The spectrum in CCl_4 had distinct bands at 1740 cm^{-1} and 1712 cm^{-1}. The first band is assigned to the C-7 and C-10 carbonyl absorptions and the second strong absorption to the stretching mode of the C-9 ketone carbonyl group. The band at 1600 cm^{-1} in both spectra is present in pheophytin at 1616 cm^{-1} to 1619 cm^{-1}. The band is assigned to a general skeletal vibration which is characteristic of dihydroporphyrin ring systems.

(c). The 1600 to 1300 cm^{-1} region:

Absorptions in this region arise from skeletal vibrations of the tetrapyrrole macrocycle and the carbon-hydrogen bending modes of the alkyl substituents of the chlorin ring. Pheophytin has bands at 1560 - 1550 cm^{-1}, 1540 - 1520 cm^{-1}, 1500 - 1490 cm^{-1} and 1355 - 1345 cm^{-1} assigned to C=C and C=N skeletal vibrations of the chlorin ring. The oxidized chlorophyll did not have the 1355 - 1345 cm^{-1} band and had an additional band at 1465 cm^{-1}. The phytol gives a doublet at 1385 - 1375 cm^{-1}.

(d). The 1300 to 600 cm^{-1} region : finger print region:

The absorptions in this region are associated with molecular motion involving in and out of plane bending and breathing vibrations of ring structures. The spectra are complex and only tentative assignments have been made. An exceptionally intense band occurs in the oxidized pigment at 1264 cm^{-1} in CCl_4 and 1265 cm^{-1} in the solid state and is not present in pheophytin.

DISCUSSION

The oxidative enzyme, lipoxidase, had been implicated by various investigators (26, 59, 79) to cause chlorophyll bleaching but the parameters involved had not been reported for lipoxidase catalyzed bleaching nor had a model system been developed using the purified enzyme. When the crude soybean preparations described by Holden (26) were partially purified in this laboratory according to the method of Theorell et al. (73) for lipoxidase crystallization, the active component was found in the fraction containing lipoxidase. The rate of chlorophyll bleaching with purified soybean lipoxidase nearly equaled that by the crude soybean preparations. A chlorophyll bleaching system was then constructed using the purified enzyme. Therefore, the following discussion is concerned first with chlorophyll bleaching by lipoxidase and the mechanism involved. A comparison is then drawn between bleaching by the pure enzyme and the crude soybean extracts. Finally, an analysis and characterization of the end product found in the reaction system is discussed.

Chlorophyll Bleaching by Lipoxidase

Optimum pH

Holden (26) noted that another factor besides lipoxidase must have been responsible for bleaching activity because the optimum pH for chlorophyll degradation by crude soybean extracts was much lower than for peroxidation of linoleic acid by seed extracts. Tappel (68) noted that the pH effect on lipoxidase activity was due to substrate solubility. The optimum with sodium linoleate occurred at pH 9.0 where it is soluble but at a low pH, where the substrate was insoluble, the activity of the enzyme was depressed. The optimum pH with methyl linoleate as substrate occurred at pH 6.5 indicating that the decreased activity at the low pH with sodium linoleate was not due to the effect of acid on the enzyme. The optimum pH for lipoxidase catalyzed chlorophyll bleaching was found to be 5.7 in this study and was close to that reported for bleaching by the crude soybean preparation (pH 5.9). A similar value was also obtained for the coupled oxidation of carotene catalyzed by lipoxidase and was shown by spectrophotometric methods to reflect an interaction between pigment and substrate (4). The interaction occurred because of insolubility of the linoleic acid at the lower pH while at pH values above 7.0 the substrate formed soluble soaps. Attempts to show a similar correlation with the chlorophyll bleaching system used here were not successful because of the nature of the chlorophyll in suspension, that is, turbid solutions were formed. However, both the carotene and chlorophylls are lipid soluble and, in a linoleic acid suspension formed as the result of a low pH, the pigments would be dissolved in the lipid globules or micelles. The pH optimum indicated, then, that micelles were also required for chlorophyll bleaching as they are for carotene bleaching in a similar system and suggested an interaction between chlorophyll and linoleic acid.

Course of the bleaching reaction

The formation of conjugated dienes was clearly related to the loss of chlorophyll and was similar to that observed for the coupled oxidation of carotene except that an induction period up to 0.5 minute duration usually occurred with the carotene system (77). Tappel (69) noted that induction periods for lipoxidase catalyzed linoleate oxidation were dependent upon the reaction conditions. Induction periods were not observed at any time during this study. The chlorophyll bleaching system differed also from the carotene oxidation system in the extent of pigment loss. Tookey et al. (77) noted that in the coupled oxidation of carotene the degradation was linear to 50% destruction of pigment over a four minute time interval, but in this study only 30% of the chlorophyll was degraded in the 1.5 minute linear portion of the degradation curve indicating either a difference between the two pigments in susceptibility to the reaction or a difference in the type of interaction between lipid and pigment. The carotenes, being highly unsaturated linear molecules would be more subject to oxidation than the nearly planar porphyrin ring of chlorophyll. The chlorophyll molecule itself, as opposed to carotene, would present a steric hindrance to alignment with linoleic acid.

The optimum temperature of chlorophyll bleaching occurred at 25°C (Figure 3) corresponding to that for the coupled oxidation of carotene (4). The degradation curve was linear for only one minute at 25°C while at 10°C degradation was linear for 1.5 to 2 minutes. No difference in the extent of pigment loss occurred. Therefore, the remaining kinetic studies were carried out at 10°C because of the rapidity of the reaction at the optimum temperature.

Kinetics of the bleaching reaction

The rate of chlorophyll bleaching increased linearly with increasing lipoxidase concentrations from 486 to 1458 units with decreasing rates at greater enzyme concentrations (Figure 5). The same effect occurred in the carotene oxidation system (6) and in lipoxidase catalyzed linoleic acid oxidation (41). The decreased rate at the higher enzyme concentrations would be expected from normal enzyme kinetics.

The rate of bleaching as a function of chlorophyll concentration was found to go through an optimum concentration (Figure 6). This effect of chlorophyll on the reaction was a reflection of the type of the lipid-chlorophyll micelle formed upon suspension in the buffer system since at low chlorophyll concentrations the micelle would be made up primarily of lipid which would act as a protective layer about the pigment. The micelle would consist primarily of pigment at higher chlorophyll concentrations which would prevent enzymatic oxidation of the lipid. The maximum bleaching rate as a function of chlorophyll concentration occurred, then, at a particular ratio of pigment to lipid. Tookey et al. (77) found in the oxidation of carotene by lipoxidase that the pigment was destroyed at an increasing rate with higher carotene concentrations. This system, however, was limited by the amount of carotene that could be dispersed, otherwise a decrease in the rate at higher pigment concentrations may also have been observed. Carotene also showed only a slight decrease in the rate of diene conjugation whereas chlorophyll caused a marked decrease (Figure 10). The effect may have resulted from chlorophyll sterically protecting the linoleic acid from lipoxidase action.

The maximum rate of chlorophyll destruction occurred at low levels of linoleic acid (Figure 7) similar to that found for carotene destruction (63). Tookey et al. (77) found that it corresponded to the critical micelle concentration of the linoleate added to the carotene oxidation system with or without added pigment. The maximum rate of chlorophyll destruction also was found to correspond to the critical micelle concentration of linoleic acid, but only when pigment was present. The critical micelle concentration without pigment present was approximately one-third that with chlorophyll. This difference would be caused by the pigment since the chlorophyll, being in suspension itself, would act to distribute the linoleic acid and prevent the formation of large lipid micelles.

The effect of linoleic acid concentration on the bleaching system was similar to that of pH. The maximum rate of pigment loss occurred in both instances when micelles were formed. The concentration effect was also a reflection of a particular ratio of pigment to linoleic acid as previously noted for the effect of chlorophyll concentration on the bleaching rate.

Effect of chlorophyll on lipoxidase activity

Tookey et al. (77) noted that the maximum rate of diene conjugation occurred at the critical micelle concentration of linoleate in the carotene bleaching system. The maximum rate of diene conjugation in the chlorophyll bleaching system occurred at a considerably higher linoleic acid concentration than the critical micelle concentration with pigment present. The linoleic acid could have been sterically protected by the pigment in the micelle causing the decreased rate of diene conjugation at the critical micelle concentration.

The rate of diene conjugation at pH 9.0 using sodium linoleate with and without chlorophyll present was compared to determine the active mechanism involved in the inhibition of linoleic acid oxidation. If lipoxidase activity was decreased, then chlorophyll would be acting as an inhibitor since lipid micelles would not be formed. However, lipoxidase was not found to be inhibited because nearly equal rates of diene conjugation were obtained with or without chlorophyll added (Table 3). The effect of the chlorophyll would then be derived from its steric effect on the lipid micelle, protecting the substrate from lipoxidase action.

Table 3. Effect of chlorophyll on the rate of diene conjugation at pH 9.0

Bleaching System	Diene Conjugation. Change in Abs. 234nm/minute
Without Chlorophyll _a_	
Trial 1	.448
Trial 2	.432
With Chlorophyll _a_	
Trial 1	.442
Trial 2	.431

The effect of solubilizing agents on chlorophyll bleaching

Surrey (64) used 0.25% Tween 20 to enhance catalysis in the determination of lipoxidase activity. Loss of the pigment did not occur when Tween 20 was used over a wide concentration range in the chlorophyll bleaching system. Triton X-100 was also found to be inhibitory. These detergents would have interfered either with micelle formation or type of micelle formed between the chlorophyll and linoleic acid by solubilizing the pigment or lipid constituents.

The rate of chlorophyll bleaching by purified lipoxidase increased when the alcohol soluble fraction of soybean lecithin was added to the bleaching system (Figure 16). The lecithin did not affect diene conjugation nor did bleaching occur without linoleic acid present. The critical micelle concentration was found to be decreased to the same value as for linoleic acid in buffer alone (Figure 17).

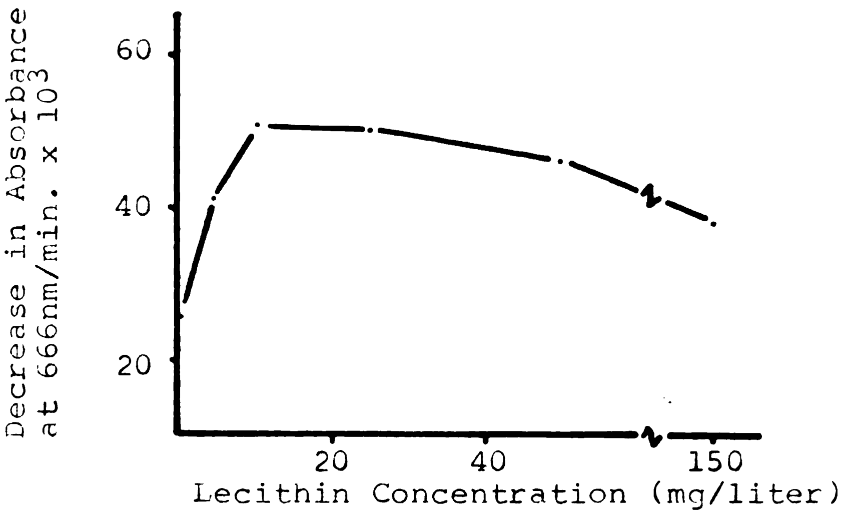


Figure 16. Effect of lecithin on chlorophyll bleaching with purified lipoxidase

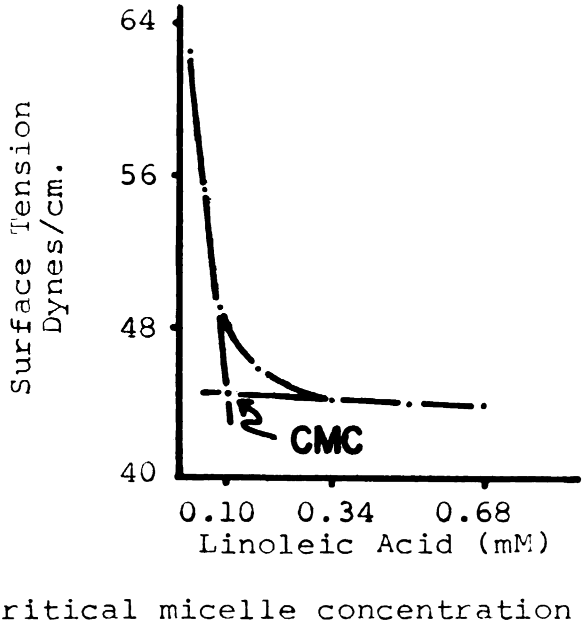
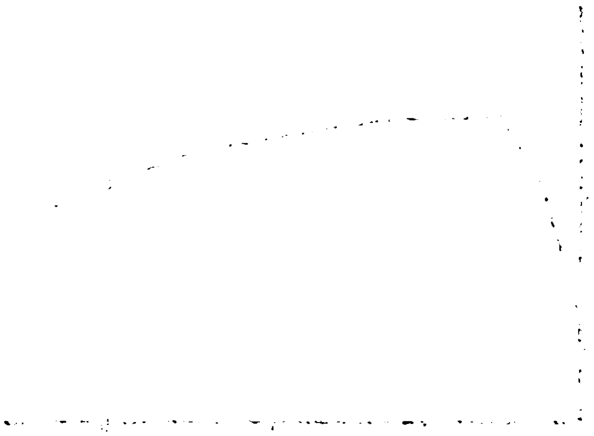
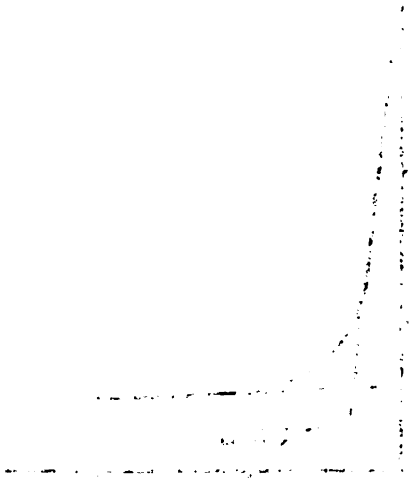


Figure 17. Critical micelle concentration of linoleic acid with chlorophyll and soybean lecithin (10mg/liter)





The effect of this constituent, then, would have been on the production of a greater number of micelles within the suspension since micelles formed at a lower linoleic acid concentration, thereby creating more sites for enzymatic action.

Mechanism of lipoxidase catalyzed chlorophyll bleaching

Carotene destruction in a model system similar to that used for chlorophyll bleaching was found to occur by a coupled oxidation with the lipid component. The following evidence was obtained which showed that chlorophyll bleaching by lipoxidase also occurred by a coupled oxidation with linoleic acid.

Chlorophyll was not destroyed when antioxidants were included in the system or when the linoleic acid was replaced by fatty acids which were not substrates for lipoxidase (Table 1). The pigment was not bleached by linoleic acid which was previously oxidized by the enzyme or by a suspension of chlorophyll and linoleic acid in buffer over a 24 hour period. Lipoxidase did not affect chlorophyll suspensions without the presence of linoleic acid and the parameters for the degradation of chlorophyll were similar to those of the coupled oxidation of carotene (4, 77). Chlorophyll also is known to be lost when hydroperoxides are formed. Tolbert and Burris (75) found that chlorophyll was bleached during the conversion of glycolic acid to glyoxylic acid in plant extracts. The conversion occurred through a hydroperoxide intermediate. Heath and Packer (25) presented a cyclic peroxidation theory for chlorophyll bleaching in isolated chloroplasts in which the chlorophyll initiated lipid oxidation. Chlorophyll bleaching which occurred during the oxidation was inhibited by the addition of lipid antioxidants. Bleaching of chlorophyll by lipoxidase could have occurred in a similar manner because hydroperoxides would be formed during lipoxidase catalyzed linoleic acid oxidation. It was not shown in the above investigations how bleaching occurred, but in the lipoxidase catalyzed bleaching pigment loss resulted from a coupled oxidation since chlorophyll was not bleached by preformed hydroperoxides of linoleic acid. The free radical mechanism postulated for the coupled oxidation of carotene could apply for chlorophyll bleaching since lipid antioxidants which function by preventing free radical formation inhibited the bleaching reaction. Tappel's theory (67) for the coupled oxidation of carotene involved a hydrogen abstraction from the pigment by free radical intermediates of the lipoxidase catalyzed linoleate oxidation. Holt (31) noted that a labile hydrogen occurs at the C-10 position of chlorophyll and was easily abstracted by oxygen when chlorophyll was in a methanol solution. It could be this hydrogen that would be abstracted by free radical intermediates of linoleic acid oxidation. Chlorophyll bleaching by lipoxidase, then, could have been similar to allomerization as outlined by Fischer and Pfeiffer (14) since the same carbon atom is involved.

The chlorophyll bleaching system exhibited a requirement for an association between pigment and substrate since bleaching did not occur without micelle formation. Blain et al. (4) showed that the coupled oxidation of carotene required the same association for pigment bleaching to occur. The formation of micelles in the chlorophyll bleaching system was dependent both on linoleic acid concentration and the pH. The rate of bleaching was affected by the amount of chlorophyll contained in the micelle.

Lipoxidase Versus Crude Soybean Extracts

Differences in assay method

Holden (26) noted a rapid increase in hydroperoxides when chlorophyll was bleached by the crude soybean extract and that bleaching by purified lipoxidase occurred at a decreased rate compared to the crude extracts even when large quantities were added to the reaction mixture. Nearly equal rates of bleaching occurred with either of the enzyme sources in the results presented here. The disparity resulted either from the method of preparation of the system or in the chlorophyll assay. The differences noted between the methods used by Holden (26) and those used in this laboratory were as follows: The quantity of purified lipoxidase added to the chlorophyll bleaching system was equal to the lipoxidase activity of the crude enzyme extracts. Secondly, prior to suspension of the chlorophyll in the buffer, the linoleic acid was added to the chlorophyll in acetone solution. A rapid conversion of chlorophyll to pheophytin occurred when the buffer was added but conversion to pheophytin did not occur when the chlorophyll was suspended in the buffer prior to the addition of the linoleic acid. Erratic results were obtained using the latter method to prepare the bleaching system. The suspension was unstable since a surface layer of linoleic acid could be noted soon after its addition. Lastly, the method of determining the amount of pigment degraded was different. Holden (26) stopped the bleaching with crude soybean extracts by diluting the total system to a 50% acetone concentration. The mixture was then filtered and the chlorophyll determined in the filtrate. It was noticed, when repeated in this laboratory, that a 50% acetone solution did not solubilize all the chlorophyll present. The total chlorophyll added was solubilized when diluted to an 80% acetone concentration. Throughout this study 80% acetone dilutions were used for quantitation of the pigment.

The active component of the soybean extracts

The rate of chlorophyll bleaching using purified lipoxidase was similar to that of the soybean extracts (Figure 5, 6, 7). The differences noted between the crude extract and the purified enzyme were decreased by the use of lipid extracted beans for the enzyme source and indicated the possible removal during lipid extraction of an essential constituent of the reaction or of additional substrate for the lipoxidase enzyme. The bleaching activity was not affected when the lipid extracts were added back to the reaction system when either the purified lipoxidase or the lipid extracted soybeans were used, indicating that it would have been additional lipoxidase substrate since increasing the linoleic acid concentration had no effect upon the bleaching rate (Figure 7). Both of the crude enzyme extracts would have contained lecithin and its effect upon the bleaching system have been previously discussed. The active constituent of the crude extracts then would be the enzyme, lipoxidase, since the soybean extracts and purified lipoxidase exhibited nearly the same kinetic relationship to each constituent of the reaction system. The minor differences would have been caused by various constituents of the crude extracts, principally the lecithin.

End Product Analysis

Chromatography

The high alcohol concentration needed to elute the bleached pigment was similar to that required for chromatography of the chlorophyllides. The oxidized chlorophylls found by Strain (60) also were more absorbed than the chlorophyllides, yet they possessed the phytol group. Chromatographic mobility could not be compared because nearly all the previous investigations were with oxidized chlorophylls rather than the magnesium free derivative. The magnesium free compound was used here because suspension of the chlorophyll with the linoleic acid resulted in removal of the metal from the porphyrin.

Thin layer chromatography was performed using the method of Hagar and Bertenrath (19). The end product streaked the plate when the normal solvent system was used whereas the modified solvent system caused a single band to migrate. Various other thin layer chromatographic procedures were attempted without success. The more polar chlorophyll derivatives are generally more difficult to separate on thin layer chromatography because of their increased polarity and their being strongly attached to the adsorbent. It would be expected that the end product, being more polar than chlorophyllide, would be similarly difficult to chromatograph.

Visible spectra

The spectral shift from 666 nm of pheophytin to 659 nm of the end product of the bleaching reaction was similar to the allomerized chlorophyll of Strain (60) and the 10-hydroxychlorophyll of Pennington et al. (51). The position of the blue peak (405nm) did not correspond to these oxidized pigments, while the red peak (659nm) was the same as the oxidized chlorophylls isolated by Strain (60). The minor absorbance bands were not presented. The peak ratio of blue to red was much greater for the methoxy lactones and was similar to the end product found here. The 10-hydroxychlorophyll had a peak ratio similar to that of the parent chlorophyll.

Phase test and HCl number

A positive Molisch phase test is indicative of the presence of the 9 ketone carbonyl, the 10 hydrogen, and the

10 carboxymethyl groups of chlorophyll. An alteration at any one of these positions gives a negative test. The end product gave a negative phase test indicating that the isocyclic ring had been altered at one of these positions. The position of the change to the molecule cannot be determined by this technique.

The HCl number was found to be slightly less than that of pheophytin and higher than the phytol free chlorophylls or pheophytins. Therefore, either a shorter carbon chain is esterified at C-7 or an additional functional group is present in the molecule. An alteration of the C-7 ester would not have been responsible for the pronounced blue spectral shift of the unknown because even pheophorbides, phytol free pheophytin, showed nearly indistinguishable spectral changes from pheophytin (32). The high blue to red absorbance ratio of the visible spectra was indicative of changes in the isocyclic ring. Cleavage of the isocyclic ring would not have occurred, however, since a more drastic decrease of the HCl number would have been found. The lactone structure would cause but a slight change in HCl number and could account for the results obtained here.

General characteristics of the end product

The visible spectra showed that the end product was different from pheophytin and the 10-hydroxychlorophylls. The non-extractability into 0.01 N KOH was an indication that the pigment was not the same as those found by Strain (60) and non-extractability into sodium carbonate indicated that the C-7 ester was still intact. The IR spectra showed that the phytol, the C-10 carboxymethyl, and C-9 ketone carbonyl groups were still present. The broad absorption band at 3400 cm^{-1} was also found for 10-hydroxychlorophyll and was used to indicate such an altered pigment. This band, since it could not be removed from the end product after prolonged drying, indicated a hydroxyl in the molecule and not absorbed water. The isocyclic ring was altered since it was phase negative and, because it did not correspond to the 10-hydroxychlorophylls, the product would be oxidized to some point further, to the lactol. The high hydrochloric acid number was evidence that the isocyclic ring was still intact. The high peak ratio of blue to red was further evidence of lactol formation.

SUMMARY

Chlorophyll a was degraded in a model system composed of linoleic acid, lipoxidase and pigment. The optimum pH for degradation was 5.7 and the optimum temperature was 25°C. Approximately 30% of the pigment was bleached in 1.5 minutes. The maximum rate of bleaching occurred at the critical micelle concentration of linoleic acid with pigment present but was three times the critical micelle concentration of linoleic acid dispersed in buffer alone. The maximum rate of diene conjugation occurred at three times the critical micelle concentration of linoleic acid. Chlorophyll did not inhibit lipoxidase at higher pH values where linoleic acid forms a soluble soap.

The rate of bleaching increased on addition of the alcohol soluble fraction of soybean lecithin but was inhibited by the addition of Tween 20 or Triton X-100. Pigment loss was not observed when fatty acids were added which were not substrates for lipoxidase or when the lipid antioxidants, BHT or NDGA, were added to the suspension indicating a coupled oxidation of chlorophyll.

The results of kinetic studies indicated that lipoxidase was the enzyme involved in the bleaching of chlorophyll by crude soybean extracts.

Bleaching of chlorophyll resulted in the formation

of a red fluorescent pigment which was Molisch phase negative with an HCl number of 24. The main visible absorption peaks were at 405 nm and 659 nm. The pigment was not extractable into aqueous potassium hydroxide distinguishing it from the 10-hydroxychlorophylls. The peak ratio of blue to red indicated it was similar to the methoxy lactone structure. The C-7 and C-10 esters and C-9 keto groups were still present as shown by the IR spectra. A broad hydroxyl band could not be removed on prolonged drying indicating an -OH on the molecule.

FUTURE RESEARCH

Since chlorophyll degradation through a coupled oxidation mechanism is known to occur in various food products, an investigation of the effect of the end product would be appropriate. Hashimoto and Tsutsumi (23) have shown that a chlorophyll derivative can cause a photosensitive response and a similar response may occur with the end product isolated in this study.

Because the biodegradation of chlorophyll is unknown, an investigation of degreening plants for the bleached product would lend information as to whether this is active during normal degradation. Addition of the bleached pigment to a slurry of degreening plant parts would indicate if it is more subject to the various enzymes present than the parent chlorophyll.

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