Summary

MUTUAL INTERACTION

IN UNSATURATED-FAT OXIDASE SYSTEMS

by

William R. Bannatyne.

September 1960

SUMMARY

One of the main problems involved in studies of the nature and action of the unsaturated-fat oxidase, lipoxidase, has been the similarity of the lipoxidase reaction to haematin-catalysed oxidation of unsaturated fats. While it would be comparatively easy to distinguish between the action of haematin-free lipoxidase and lipoxidase-free haematin with existing methods, crude plant extracts contain mixtures of the two systems and may give rise to confusion. The characteristics of such mixtures can only be adequately studied if haematin-free lipoxidase is available, and while purified haematins can be fairly readily prepared, the isolation of lipoxidase presents major difficulties.

This thesis reports the examination of a number of methods of purification and describes the subsequent separation of a highly active, haematin-free lipoxidase from soya-beans. The fractionation procedure involves precipitation of the enzyme at its isoelectric point with ammonium sulphate.

The characteristics of the haematin-free lipoxidase system are defined using a method of assay which depends on the spectrophotometric measurement of the diene conjugation developed in sodium linoleate by the enzymic action. By means of a slight modification of the lipoxidase assay, the unsaturated-fat oxidase behaviour of the haematin compounds is also examined.

The work described in this thesis includes a study of lipoxidase-haematin interaction. The effect of haematin on lipoxidase behaviour is examined, using the direct spectrophotometric assay system

with respect to such variables as pH, reaction time, linoleate concentration, haematin concentration and lipoxidase concentration.

Rapid breakdown of the haematin molecule in the lipoxidase reaction system is described and the influence of haematin decomposition on diene conjugation in the adopted system is discussed.

Aided by these studies, attention is given to the unsaturated-fat oxidase behaviour of crude soya extracts. The characteristics of soya extracts and of lipoxidase-haematin mixtures are examined and compared using the diene conjugation method of assay. Arising from the data obtained, it is concluded that in unsaturated-fat oxidations catalysed by crude plant extracts, possible interference from haematins cannot be overlooked.

MUTUAL INTERACTION

IN UNSATURATED-FAT OXIDASE SYSTEMS

by

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PREFACE

PREFACE

Certain enzymic factors present in plant tissues are capable of catalysing the oxidation of unsaturated fats. These unsaturated-fat oxidase systems are known collectively as the lipoxidases.

Compounds containing the haematin grouping have been found to catalyse similar oxidative changes in unsaturated fats and these haematin compounds are ubiquitously distributed in plant tissues. This raises the question as to whether extracts of plant material owe some of their unsaturated-fat oxidase activity to the presence of haematins as well as to lipoxidase.

The possibility of haematin interacting adventitiously with lipoxidase in fat oxidations catalysed by crude plant extracts has received little consideration. Furthermore, many reports of lipoxidase activity in plant tissues have been based on evidence which did not exclude the possibility of haematin catalysis.

This thesis represents an attempt to examine the mutual interaction of the two systems.

GENERAL INTRODUCTION

GENERAL INTRODUCTION

The unsaturated-fat oxidase, lipoxidase, has been the subject of considerable study, and a number of reviews have surveyed the extensive literature on lipoxidase (1, 2, 63, 76).

The enzyme has been shown to oxidise only those fatty acids which contain the pentadiene structure, $-CH = CH - CH_2 - CH = CH-$, with the double bonds in the 9, 10 and 12, 13 positions to the carboxy group.

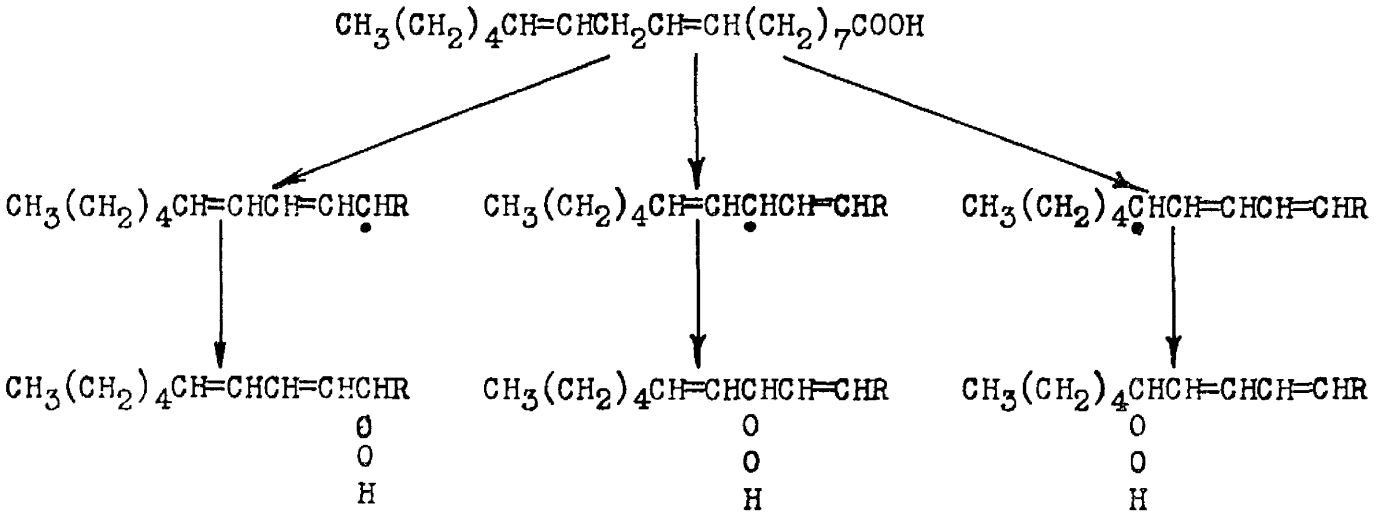
According to a number of earlier investigators (3, 4, 5), only those acids having an unsaturated linkage in the 9, 10 position are attacked by lipoxidase. Balls et al. (6), however, in 1943, found that of a number of unsaturated fatty acids only linoleic, linolenic and arachidonic formed peroxides when oxidised by lipoxidase; work which was later confirmed by Holman and Burr (7). From this evidence it is apparent that the position of the double bond in relation to the carboxy group is not critical, as the first double bond of arachidonic acid is probably in the 8. 9 position.

In 1947, Holman and Elmer (8) showed that only the natural isomers of linoleic and linolenic acids, in which the double bonds are cis, are substrates for soya-bean lipoxidase, whereas the elaidinized or trans isomers are not. The conjugated isomers of linoleic and linolenic acids are also apparently not attacked by lipoxidase (7,9).

It should be pointed out that the fatty acids containing the cis-cis pentadiene grouping are regarded as being nutritionally essential. It is not known, however, whether any physiological significance can be attached to the specificity of lipoxidase for these essential fatty acids.

On oxidation by lipoxidase, linoleic acid gives absorption peaks at 234 mμ which have been shown to be indicative of the presence of conjugated hydroperoxides (7,10,11,12). Infra-red studies have shown these hydroperoxides to consist of cis-trans and trans-trans conjugated isomers (13). If the reaction is allowed to proceed further, carbonyl compounds are formed (14) and breaks occur in the unsaturated fatty acid chain (15).

Finding that the lipoxidase oxidation of linoleic



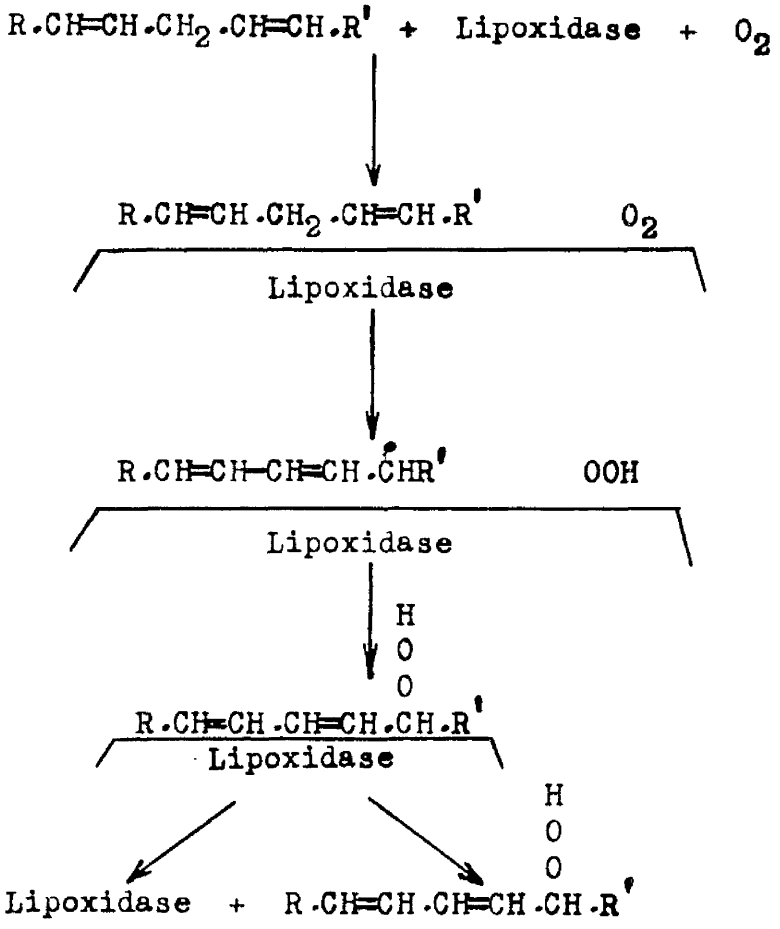
2,4 decadienal 2 octenal hexenal

Holman's Chain Reaction Theory

Fig. 1.

acid leads to the formation of conjugated hydroperoxides which are further oxidised to carbonyl compounds, Holman (16) and Bergstrom (10,17) suggested that a chain reaction mechanism similar to autoxidation of linoleate was taking place. The suggested mechanism is shown in Figure 1. Further evidence of the similarity of the lipoxidase reaction to autoxidation was obtained when antioxidants were shown to strongly inhibit the enzymic oxidation(18).

Tappel, Boyer and Lundberg (19), however, noted several differences in the decomposition of linoleate brought about by autoxidation and by catalysis with lipoxidase respectively. For example, the extinction coefficient at 234 mμ for the peroxides formed in the presence of lipoxidase was higher than that which resulted from the autoxidation of linoleate and corresponded to complete conjugation of the double bonds. This could be explained if the lipoxidase system only adds oxygen at the outside carbon atoms of the unsaturated group of the linoleic acid yielding conjugated products. Moreover, studies of antioxidant inhibition of the reaction showed the antioxidant N.D.G.A. to be rapidly destroyed in the presence of lipoxidase



Tappel's Reaction Mechanism.

Fig. 2.

and linoleate with little or no concomitant oxidation of the linoleate. If the reaction was a chain reaction it would be expected to give conjugated products in the presence of antioxidants.

To replace the assumption that the oxidation of linoleate proceeds as a chain reaction, Tappel and co-workers proposed a mechanism more like that of a simple enzyme system in which they suggested the formation of a biradical from linoleate and oxygen on the enzyme surface. The biradical may accept electrons from the antioxidants, or may react to give the conjugated linoleate hydroperoxide. The mechanism proposed by Tappel et al. (19) is shown in Figure 2.

Holman (11) had previously reported that pure lipoxidase is not inhibited by pyrophosphate, fluoride, cyanide, azide, mercury ions, p-mercuribenzoate or diethyldithiocarbamate, thereby indicating that neither heavy metals nor sulphydryl groups are concerned with the functional activity of the enzyme.

Siddiqi and Tappel (20) recently put forward a reaction mechanism to explain how the enzyme brings about the abstraction of an electron or a hydrogen atom

from the methylene group of the fatty acid without the aid of a prosthetic group or coenzyme. These workers suggest that the lipoxidase protein acts as an electron sink whereby it can momentarily hold an electron from the α-methylene group of the linoleate thus allowing oxidation.

Certain substances have been shown to have an activating effect on the lipoxidase reaction. Balls et al. (6) and Theorell and co-workers (77) believed that lipoxidase is activated by a polypeptide present in soya-beans. A polypeptide which could augment the enzyme activity by as much as 300% was isolated by Kies (78, 79). However, in the single-phase sodium linoleate substrate at pH 9, crystalline lipoxidase has been shown to require no such "activator" (11).

It is likely that the need for an activator demonstrated previously is a property of the emulsion systems used, and that the activator, probably through some surface-active agency, stabilises the system in such a way that the substrate becomes more available to the enzyme.

Lipoxidase oxidation of unsaturated fatty acids may be

accompanied by the concurrent oxidation of substances present other than the primary substrate. Thus, the enzymic oxidation has been shown to induce the oxidative destruction of vitamin A (21, 22, 23), carotene and other carotenoids (3,4,24,25), xanthophylls (4), chlorophylls (4) and certain dyes (4).

The coupled oxidation of the above pigments would appear to occur by the interaction with some intermediate in the enzymic peroxide formation (5,24,26,27).

The lipoxidase-linoleate system has also been reported to oxidise other systems such as amino-acids (29), ascorbic acid (4), haemin (30) and glutathione (31).

Lipoxidase activity has been reported to occur in a variety of plant tissues and lists of plants which have been shown to contain the enzyme are given by several authors (4, 32, 33).

Lipoxidase has been detected in certain vegetable tissues such as potato, potato juice and asparagus (4,34,35,36), but has been found to occur most notably in leguminous seeds (2,37,38,39,40,41). Soya-beans are a particularly active source of the enzyme and the majority of studies have been carried out on extracts of

defatted soya. Only from soya has a pure crystalline lipoxidase been obtained (42,43).

Sumner (44), in 1943, demonstrated the presence of the enzyme in wheat flour, and Miller and Kummerow (45) later compared the activities of wheat mill fractions with that of soya-beans and concluded that the greatest activity is found in the germ fractions. Blain and Todd (46) showed that the scutellum and embryo are the most active centres in the wheat berry.

Histochemical studies of the unsaturated-fat oxidase activity of numerous plants have been made by Van Fleet (47,48). Activity was found to be high in the seedling stage of plants grown in alkaline medium. The oxidase was reported to be most active in regions of the plant that are neutral or alkaline, where water losses occur, below wounded areas or where antioxidants are ineffective. The techniques used in localising these oxidase systems, however, are not of a specific nature and it cannot be stated with certainty that lipoxidases are responsible for the effects observed.

The lipoxidase content has been shown to decrease in germinating soya-beans on the second day after planting,

simultaneously with a reduction in the linoleate and linolenate contents (49) . The actual function of the enzyme in the germination process is not known but Holman (50) has suggested that lipoxidase may act upon the unsaturated acids to initiate their conversion to other metabolites .

Lipoxidase has also been found to take part in the early stages of germination of corn seedlings (51) and of peas (51) .

Much effort has been devoted to the identification of the catalysts in animal tissues responsible for the oxidation of unsaturated fats . Since the only known enzyme which catalyses the direct oxidation of unsaturated fats with oxygen is lipoxidase, some investigators have suggested that lipoxidase may be one of the unsaturated-fat oxidation catalysts present in animal tissues. However, there has been a great deal of conflicting evidence as to whether an animal lipoxidase exists, or whether the oxidizing catalysts are in fact the haematin compounds of animal tissues. (The term haematin is used here in the sense established by

Lemberg and Legge (112) to indicate iron porphyrin compounds in general, irrespective of valency.

The ability of the haematin or iron porphyrin compounds to catalyse the oxidation of unsaturated fats has been well established. Robinson (52), in 1924, first described the accelerating effect of haemoglobin on the rate of oxidation of emulsified linseed oil. In 1938, Barron and Lyman (53) noted the catalytic effect of haemin on the oxygen uptake of emulsified linseed oil. Reporting inhibition of the catalysis by antioxidants, Barron and Lyman suggested that a chain reaction mechanism might be involved . Haurowitz et al. (54), in 1941, found haematin to catalyse the oxidation of oleic acid, although to a lesser extent than that of linoleate or linolenate, and observed, also, that during the catalysis the haematin compound was destroyed. In 1944, Banks (55), in a study of the action of haematin on linoleic acid, pointed out that the catalyst is not haematin itself, but a combination of haematin and linoleate peroxide. In the same year, Simon et al. (56) showed cytochrome C to

catalyse linoleate oxidation.

Meanwhile, attempts had been made to determine whether or not animal lipoxidases as distinct from the haematins of animal tissues, did occur. Banks (57) in 1937, demonstrated a heat-labile system in herring muscle which stimulated rancidity in herring oil. He subsequently found that haemo proteins are involved in this oxidation, and that the properties of this enzyme system are quite unlike those of soya-bean lipoxidase, having optimum activity at low pH values (55). In the same year, Lea (58) found a heat-labile system in pig muscle which accelerated fat rancidity. Watts and Peng (59), however, attributed the fat oxidase activity of pig muscle to the myoglobin and haemoglobin present. Reiser (60), on the other hand, believed the catalytic effect of aqueous extracts of bacon adipose tissue to be due to an enzyme. He based his conclusions on the heat-stability of the catalyst and on the fact that after the removal of the haeme pigments catalysis still occurred. Being unable to repeat Reiser's findings, Tappel (62) suggested that Reiser's method for detecting traces of haemoglobin was not sufficiently sensitive and concluded that pork tissues are free from lipoxidase.

Hove (64) examined extracts of rat gastric mucosa which he found to have unsaturated-fat oxidase activity not due to haemoglobin .

More recent claims of lipoxidase activity in animal tissues have been made by Clegg and McIntyre (65), working with liver extracts, and by Khan (66) who examined fish tissues, but none of these workers produced evidence to show that the observed activity was not in fact due to haematins .

Tappel (67) carried out a study of a wide variety of animal tissues and concluded that the haematin compounds are the predominant catalysts for unsaturated-fat oxidation occurring in animal tissues .

Tappel's method (62,67) of differentiating between haematin oxidation and lipoxidase oxidation in animal tissues was based on the fact that haemoglobin catalyses linoleate oxidation only in heterogeneous systems, whereas soya lipoxidase catalyses oxidation both in heterogeneous and in homogeneous systems . Earlier workers had also reported haematin catalysis to occur in heterogeneous systems (55,105) . A more recent report by Maier and Tappel (106), however, has shown that haemoglobin and haematin catalyse the

oxidation of homogeneous as well as heterogeneous linoleate systems.

While soya lipoxidase and the unsaturated-fat oxidase systems of animal tissues were found capable of catalysing similar oxidative changes in fats, the reaction mechanisms of both types of system appeared to be different, for soya lipoxidase, unlike the haematin compounds, was not found to possess a metallic prosthetic group (43). This led to various workers putting forward criteria of distinction between lipoxidase and haematin, based on differences in reaction kinetics of the two systems.

Thus, Tappel (68,69) confirmed the earlier findings of Barron and Lyman (53) when he showed that oxidation of unsaturated fats in the presence of haematin compounds proceeds via a chain reaction mechanism. Tappel also found that this was not the case with the lipoxidase reaction, and suggested an alternative mechanism (19). It should be pointed out, however, as mentioned previously, that Holman (16) considered pure lipoxidase to initiate a chain reaction mechanism and that although results in support of both theories of the lipoxidase reaction mechanism have been obtained, they have not been conclusive enough to confirm one or the

other.

Long, unexplained induction periods which may be abolished by addition of linoleate peroxide (55,69), have been reported as being a feature of haematin catalysis (62,68,69, 71) but not of the lipoxidase reaction. However, a more recent critical study of soya lipoxidase by Haining and Axelrod (70) has shown that an induction period does occur in the course of the lipoxidase-catalysed oxidation of sodium linoleate and that the induction period can be completely abolished by the addition of traces of linoleate peroxide.

Cyanide inhibition has also been used as a criterion of distinction between the two systems. Boyd and Adams (71) showed that cyanide, in very high concentrations, completely inhibits haematin action and that of animal tissue extracts, but not the action of soya lipoxidase. On the other hand, cyanide has been reported to be inhibitory towards wheat lipoxidase (72).

There are therefore, two classes of unsaturated-fat oxidases, the true lipoxidases which are believed to be without a metallic prosthetic group, and the haematin compounds, which

under the appropriate circumstances are able to catalyse the oxidation of fats in a rather similar fashion to the lipoxidases. On consideration of the available evidence, however, it would seem that the "lipoxidase activity" in animal tissues is due to haematins rather than lipoxidase.

The differentiation of catalysis due to haematin compounds from that due to lipoxidase remains a perplexing problem.

The haematin compounds are known to occur in fairly large amounts in plant tissues (73,74) in the form of cytochromes, catalase and peroxidase, some of which are soluble in aqueous extracts (75). Since most of the earlier studies of the lipoxidase reaction were carried out on crude plant extracts at a time when the significance of haematin catalysis was not fully appreciated, the question is raised as to whether many of the effects attributed to lipoxidase in plants are in fact due to haematins.

Adequate methods of distinguishing between the action of lipoxidase and that of haematin compounds necessitate the use of pure preparations and while pure haematins may be readily obtained, the purification of lipoxidase presents

major difficulties. The successful isolation of lipoxidase, from soya-beans, has only been reported once(42,43); work which has not since been repeated.

While it would be relatively simple to distinguish between the action of lipoxidase-free haematin and haematin-free lipoxidase with existing experimental methods, plant extracts contain mixtures of the two systems and give rise to confusion. The characteristics of such mixtures can only be properly studied if haematin-free lipoxidase is available.

EXPERIMENTAL

PART I : OBSERVATIONS ON THE PURIFICATION OF LIPOXIDASE.

OBSERVATIONS ON THE PURIFICATION OF LIPOXIDASE

1. Previous Attempts at Purification of Lipoxidase

Partial purification of the lipoxidase of soya-beans has been reported by various workers. In 1943, Sullman (80) prepared a 66-fold concentration of the enzyme by fractional precipitation of an aqueous soya extract with acetone. Balls, Axelrod and Kies (6), in the same year, reported the separation of a fraction which had a specific activity 115 times that of a 2·5% water extract of soya. Balls and his co-workers used ammonium sulphate as precipitant at pH 6·8 and found that most of the activity was precipitated between 0·47 and 0·5 saturation.

Cosby and Sumner (81), using a simpler method of ammonium sulphate fractionation, obtained a 60-fold concentration of the enzyme. They extracted the enzyme from the crude soya meal by means of a potassium alum solution, thereby avoiding ballast material. Later, Franke et al. (15(, also using ammonium sulphate fractionation, obtained a 32-fold concentration of the enzyme.

A very simple method, involving precipitation of the

enzyme in the cold with 0·5N disodium phosphate, was used by Sumner and Smith (25) who reported a 50-fold concentration of the enzyme.

Using an improved method of assay, and by means of a method of fractionation based on the original ammonium sulphate method of Balls et al. (6), Theorell, Bergstrom and Akeson (77) obtained a 388-fold concentration of the enzyme on a dry weight basis. The fractionation procedure involved a complex combination of precipitative and electrophoretic methods, and the preparation obtained was identified as a globulin of molecular weight 75,000 - 80,000. On electrophoresis, however, it was discovered that the product was not a homogeneous protein.

In the same laboratory, soya lipoxidase was finally isolated in a crystalline form in 1947, by Theorell, Holman and Akeson(42,43). The scheme of separation used was based on the previous work of Theorell, Bergstrom and Akeson (77,82), which involved the use of ammonium sulphate as precipitating agent. The enzyme was extracted from soya-beans with pH 4·5 acetate buffer, since it had been found that an extract made at pH 4·5 is a purer lipoxidase preparation than

one made at higher pH values (83). The gummy material present in the crude extract was removed by precipitation with lead and barium acetates, and the activity was then precipitated from the solution with ammonium sulphate. The active precipitate was redissolved in a small amount of distilled water and the albumins were removed from solution by heating to 65°C. for 5 minutes. Fractional precipitation of the solution with ammonium sulphate produced an active fraction between 35% and 50% saturation. This precipitate was taken up in phosphate buffer and fractionated with alcohol at 0°C. The active material which precipitated out at 12% alcohol was then further fractionated with ammonium sulphate and the active concentrate subjected to electrophoresis on a preparative Tiselius apparatus. Crystallisation of the enzyme was achieved by dialysing fractions from the Tiselius apparatus against ammonium sulphate solutions of increasing concentration.

The crystalline preparation, which had a specific activity of 850 units per mg., represented a 115-fold concentration from the crude buffer soya extract on a dry matter basis. From studies of electrophoretic mobility, Theorell and his

co-workers (45) calculated the isoelectric point of lipoxidase to be approximately pH 5·4, and from sedimentation and diffusion measurements, the product was judged to be a homogeneous protein of molecular weight 102,400 (45).

The isolation of lipoxidase in a completely pure form has not since been repeated.

It should be pointed out here that three distinctly different and active components were separated electrophoretically during the purification procedure by Theorell et al, and that only one of these components, supposedly lipoxidase, was further concentrated to give a crystalline preparation. Some doubt may therefore be attached to the nature of the material purified.

In their separation of lipoxidase, Theorell and his colleagues (42,45) used alcohol as a precipitating agent, and these workers reported that under suitable conditions alcoholic fractionation gives as narrow a separation as ammonium sulphate, but with less inactivation. This finding led Cameron (84) to devise a simple method for the

purification of soya lipoxidase. The method was based on the alcoholic fractionation procedures developed by Cohn and his colleagues for the separation of plasma proteins (85,86).

Alcoholic fractionation involves the carefully controlled use of organic solvent, pH, salt concentration and temperature. The use of so many controllable variables and the absence of high salt concentrations which tend to mask specific protein properties, permit choice of conditions to give cleaner separations than are obtainable with salts alone.

While isolation of lipoxidase in a homogeneous state was not achieved by this method, Cameron (84) did find that losses from denaturation and overlapping of fractions were less than in the method used previously by Theorell, Holman and Akeson (42,43). From the results of her investigation, Cameron concluded that the use of ethanol as a fractionating agent in the separation of soya lipoxidase offers useful possibilities, provided that strict control is effected over the variables in the system, particularly temperature.

2. Control Methods and Exploratory Experiments

Rationale of the Experiments

In view of Cameron's findings (84), it was considered that alcoholic fractionation might prove successful as a means of separating a haematin-free lipoxidase preparation from soya-beans. Although it was appreciated that at later stages more empirical methods of separation might be required, it was decided that fractionation with alcohol should firstly be examined.

Lipoxidase Assay

To follow the course of isolation of the enzyme a suitable assay system was required. Various methods of assay of lipoxidase, involving its different properties, have been suggested and these methods depend on either of the following:-

1. The measurement of the peroxide values by iodimetry or by the ferric thiocyanate colour reaction (56,57,82,87,88,89).
2. The measurement of the oxygen uptake of the system in a Warburg apparatus (19,26,55,71,77, 80,90,91,92).

3. The measurement of the bleaching of a carotenoid pigment added to the reaction system (6,28,52,61,93, 94,95,96).

4. The spectrophotometric measurement of the diene conjugation developed in pure sodium linoleate by the action of the enzyme (7,17,19,24,42,70,82,84, 96,97).

The unusual feature of the lipoxidase reaction is that it takes place in two phases, the fatty substrate and the coupled reactant being insoluble in the aqueous enzyme phase. Thus, most of the methods employed to assay lipoxidase activity are subject to considerable error because the rate of oxidation is influenced by the degree of dispersion of the substrate. In addition to this disadvantage, those methods based on carotenoid oxidation give proportionality between enzyme concentration and carotenoid destruction over only a narrow range (81).

The spectrophotometric assay developed by Theorell, Bergstrom and Akeson (82) does not have these disadvantages because it uses a more reproducibly homogeneous substrate, and the products of the primary reaction are measured.

Under these conditions, peroxide formation is proportional to time and to enzyme concentration over wide ranges (2).

At the beginning of the present investigation, measurement of carotene-bleaching by the cup - plate technique of Blain (98) was used for estimating lipoxidase activity, as this method has been found to be free from a number of disadvantages of the other carotene-bleaching systems. Although results were satisfactory for some of the preliminary work, it was later discarded because a side reaction rather than the primary phenomenon of hydroperoxide formation was being measured. The direct spectrophotometric method was thereafter adopted for the present work.

The method depends on the fact that during the lipoxidase-catalysed oxidation of linoleate, conjugated hydroperoxides are formed on the site of the original methylene-interrupted double bond system of the linoleate. The degree of conjugation is proportional to the amount of oxidation which has taken place and the process can be readily followed from the ultra-violet absorption band of the conjugated system which shows a maximum in the region 232-235 mμ.

The original method has been modified several times (82,97), but the assay procedure employed in this investigation followed the modification of a previous worker in this laboratory (84). The procedure consisted of adding to 50 ml. of pH 9 ammonia/ammonium chloride buffer, in a conical flask, 1 ml. of sodium linoleate solution and then a suitable quantity of enzyme solution. The enzyme was allowed to react for one minute after which the reaction was stopped by the addition of 1 ml. of a 20% aqueous solution of sodium hydroxide containing 5% potassium cyanide. The test solution was compared on the Uvispek Spectrophotometer at 234 mμ against a control solution prepared as follows. To the buffer solution was added the alkaline stopping reagent. The enzyme solution was then introduced, and, after thorough mixing, the substrate solution was added, the relative levels of enzyme and substrate added being the same as in the case of the reaction solution.

A detailed account of the preparation of reagents and of the assay procedure is given in Appendix 1.

In terms of this system, a unit of lipoxidase activity

is defined as that amount of lipoxidase, which, on acting on sodium linoleate in the assay system described for one minute, increases the optical density of the solution at 234mμ by 0·1 unit.

Criteria of Purity

The specific activity at each successive stage of purification was estimated in relation to protein nitrogen, which was determined by micro-Kjeldahl.

The ultimate criterion of purity in this investigation was complete freedom from haematin compounds. To indicate the presence of haematins in the purified material, the following three methods were used:-

1. The determination of the iron content of the material by Pringle's procedure (99), involving wet oxidation of the enzyme followed by colorimetric estimation of the iron as ferrous-o-phenanthroline.

2. The determination of the catalase activity present by a slight modification of the iodimetric titration method of von Euler and Josephson (101).

3. The determination of the peroxidase activity of

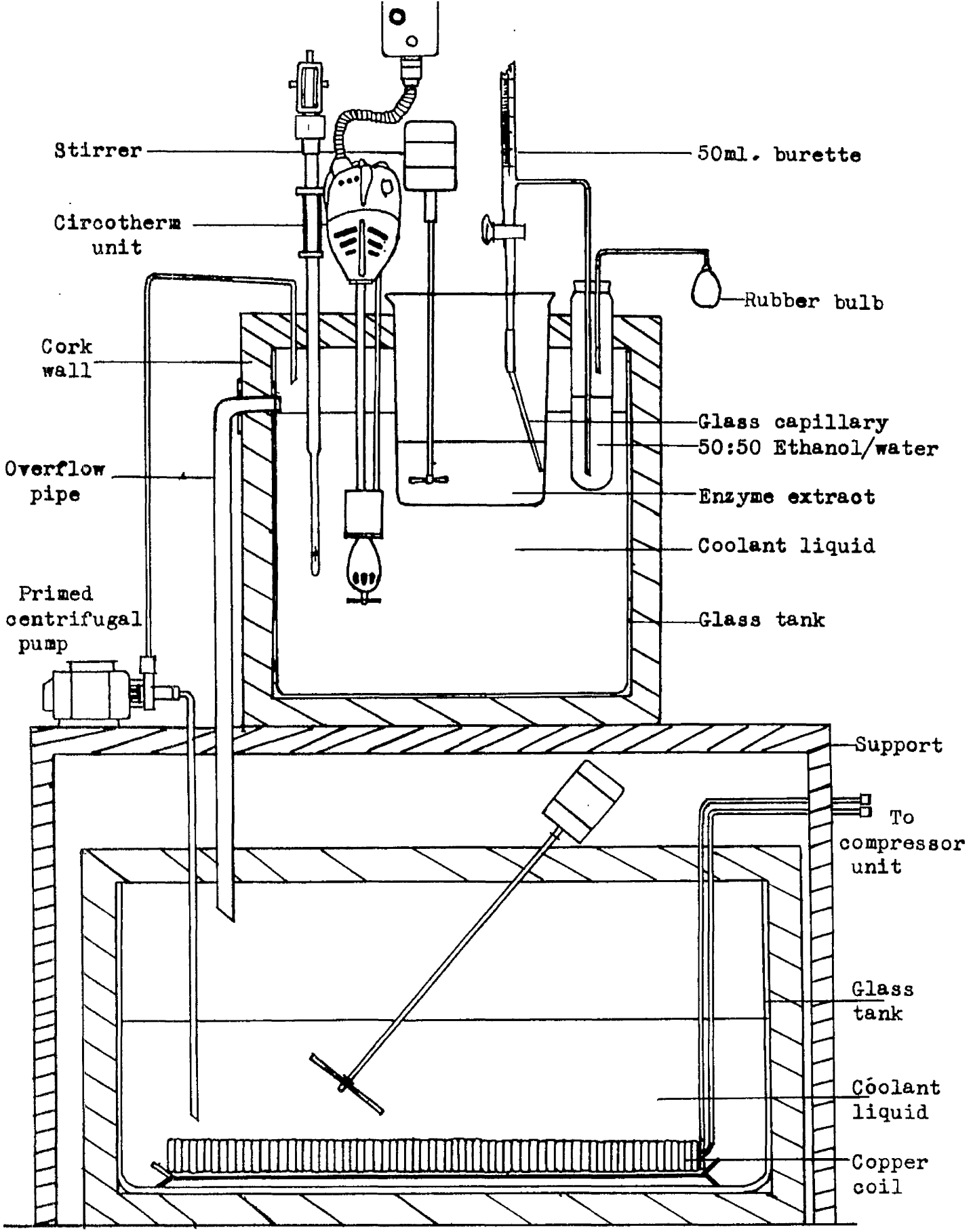


Fig. 3. - Cooling Apparatus for Alcohol Fractionations.

the fraction by the spectrophotometric procedure of George (100), involving oxidation of guaiacol .

The purity criteria were applied at the later stages of the purification where maximum enrichments or specific activity values had been reached .

Alcoholic Fractionation of Crude Soya Extracts .

The first attempts to purify lipoxidase followed the alcoholic fractionation method of Cameron (84) .

Since alcohol, and organic solvents in general, show a marked tendency to denature proteins and inactivate enzymes, the method necessitated working at temperatures between 0° and -5°C . To control the temperature of the fractionations therefore, cooling apparatus was built using a 50% solution of ethylene glycol in water as the cooling medium .

The apparatus, an illustration of which is shown in Figure 3, consisted essentially of two glass tanks, the smaller of the two being mounted above the larger tank . Each of the tanks was surrounded by two-inch thick cork walls . The larger tank, which had a capacity of two cubic

Alcohol Fractionation of Soya-Beans

by Cameron's Method.

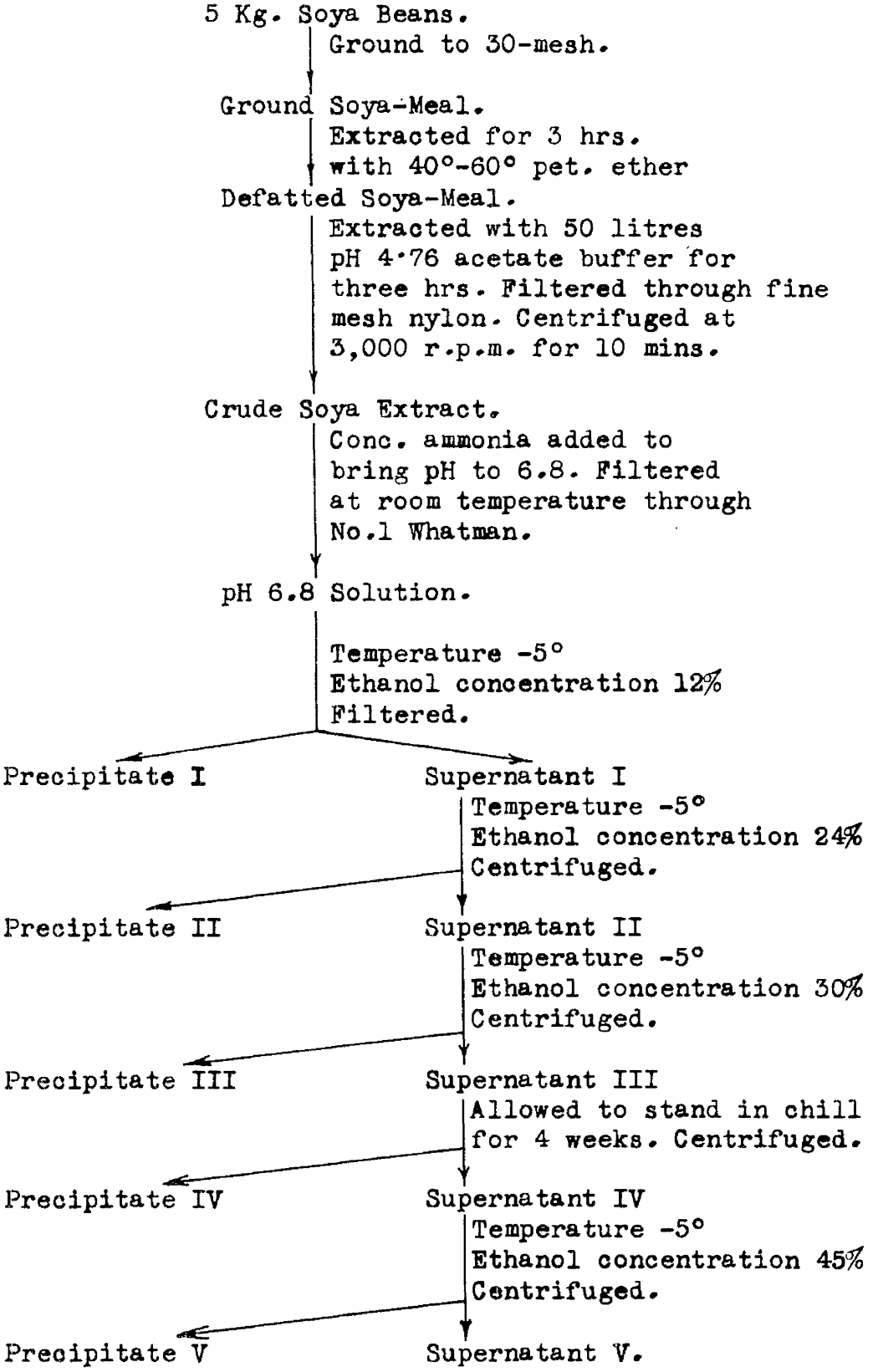


Fig. 4.

feet, was used as a reservoir for the liquid coolant.

A conventional freon refrigerator unit with a copper coil as evaporator, was used to cool the reservoir tank. The smaller glass tank, placed directly above the reservoir tank, which had a capacity of one cubic foot, was used to cool the enzyme extracts during fractionation with ethanol.

The coolant liquid was circulated by means of a centrifugal pump, the upper tank being fitted with an overflow.

The energy used by the compressor was roughly controlled by an energy regulator and the temperature of the coolant mixture in the upper tank was accurately controlled by a Circotherm relay unit.

The conditions for extracting the activity from soya-beans and the method of fractionation followed the procedure used by Cameron (84), which is shown in Figure 4. The fractionation experiments in this case, however, were carried out with 400 ml. volumes of crude soya extract as larger volumes than this could not be handled in the tank.

The method used was as follows:- 400 ml. of a crude 20% acetate buffer extract of defatted soya were brought to pH 6·8 with 0·88 ammonia and filtered through a No. 1 Whatman filter paper. The solution was then placed in a 2 litre

beaker in the cold tank and cooled. The enzyme solution was continually stirred during cooling. When the temperature had reached 0°, the addition of a 50% V/V solution of ethanol in water was started. This was added through a capillary attachment from a 50 ml. burette. The alcohol was pumped into the burette from a storage bottle in the cold tank, so that it had a temperature of -5° on being added to the enzyme extract.

To prevent losses of activity due to ice crystal formation, the temperature at the initial stages of alcohol addition was lowered at such a rate that it was always close to, but never at the freezing-point of the extract. When the temperature had reached -5°, the addition of alcohol was continued until there was a concentration of 10% alcohol on a volume basis in the mixture. The time taken for addition was 30 - 45 minutes.

The solution was then centrifuged at 5,000 r.p.m. for 30 minutes. The centrifuge was cooled with dry ice and the centrifuge bottles and buckets were prechilled. With these precautions, the temperature rise in the solution was kept below 2°. The precipitate obtained at 10% alcohol was taken up in pH 4·5 M/10 acetate buffer and tested for activity by the procedure detailed in Appendix 1. The solution was found

to be inactive and was therefore discarded.

The supernatant was then returned to the cooling tank, brought to -5°, and the addition of alcohol was continued.

The precipitates obtained at alcohol concentrations of 20,30, 40 and 50% were collected by centrifuging, taken up in pH 4·5 M/10 acetate buffer and tested for lipoxidase activity.

The results of the fractionation are shown in Table 1.

TABLE 1

Alcoholic Fractionation of a pH 4·5 buffer extract of Soys

	Units/ml.	Total Activity
Crude Buffer Extract	32.5	13,000
Precipitate between 0-10% alcohol	8·0	160
Precipitate between 10 - 20% Alcohol	15·6	780
Precipitate between 20 -30% Alcohol	21·5	1,065
Supernatant at 30% Alcohol	2·5	920
Precipitate between 30 - 40% Alcohol	3·5	175
Precipitate between 40 -50% Alcohol	0	0

It was found that a great loss of activity had occurred during the precipitations. Most of the recovered activity,

as may be seen from Table 1, was precipitated between 10% and 30% alcohol.

The above experiment was repeated within narrower limits. The most active precipitate, in this case obtained between 12% and 25% alcohol, was separated from the solution by centrifuging at 5,000 r.p.m. for 30 minutes. The precipitate was then taken up in 50 ml. pH 4·5 M/10 acetate buffer, and filtered through a No. 1 Whatman filter paper, the temperature being kept below 0°. The lipoxidase activity and protein nitrogen content of this solution were determined, and the specific activity of the fraction was compared with that of the crude soya extract. The results are shown below.

TABLE II

Fractionation of a Crude Soya Extract with Alcohol

	Units/ml.	Units/mg. Nitrogen	Total Activity
Crude Buffer Extract	34.0	11.2	13,600
Solution of Fraction between 12-25% Alcohol	12,7	14.1	635

This separation was carried out repeatedly with minor variations. The results obtained, however, were essentially similar to those shown.

It is evident that a great deal of activity had been lost during fractionation. Although most of the activity was found to have been removed from the supernatant, the final precipitate contained only 5% of the total activity and the degree of enrichment was negligible.

It was, therefore, decided to attempt separation of the enzyme by means of the more conventional salting-out techniques.

Separation by Salting-out Procedures.

The first salting-out procedure examined followed that of Balls, Axelrod and Kies (6), in which solid ammonium sulphate was used as the precipitant. The method used was as follows:-

50 gm. of finely ground, defatted soya-bean meal were suspended in 1 litre of cold, tap water. The suspension was left overnight in the cold room. Enough M acetic acid was then added to adjust the pH to 5, and the solution was immediately filtered.

The total filtrate was brought to pH 6·8 with M NaOH. Solid ammonium sulphate was added slowly, with stirring, until the saturation level in the solution had reached 50%.

Without delay, M NaOH was added until the pH of the solution was readjusted to pH 6·8. The suspension was then centrifuged at 3000 r.p.m for 30 minutes, the precipitate collected and then dissolved in distilled water.

This solution was dialysed against running water until sulphate-free, and was then refractionated with ammonium sulphate.

The active precipitate, obtained between 30% and 50% saturation, was separated from the solution by centrifuging for 30 minutes at 3000 r.p.m. and then dissolved in 40 ml. distilled water.

To avoid any interference from the nitrogeneous content of the ammonium salt in the protein nitrogen determinations, the enzyme solution was dialysed until sulphate-free. The results of this separation are shown in Table III

TABLE III

Separation by the Method of Balls, Axelrod and Kies

	Units/ml.	Units/mg. Nitrogen	Total Activity
Crude Extract	18·0	6·0	14,940
Solution of Fraction between 30-50% saturation	37·0	35·8	1,480

Although, using this procedure, a 6-fold concentration

of the enzyme was obtained in just a few short steps, only 10% of the total activity had been recovered.

The next salting-out procedure examined was that of Sumner and Smith, (25) in which disodium phosphate was used as precipitating agent. The method employed was as follows:- 200 gm. of finely ground, defatted soya meal were stirred with one litre of ice cold water and the suspension left overnight in the cold room. 20ml 2N acetic acid were stirred in and the mixture was filtered rapidly in the cold room. As fast as the filtrate dripped into the receiving vessel, 0·5N disodium phosphate was added, about 50 ml. for every 250 ml. filtrate being used.

The fine, white precipitate which formed after 2-3 hours was separated from the solution by centrifuging at 3000 r.p.m. for 30 minutes, and dissolved in 210 ml. distilled water.

The results of the fractionation are shown in Table IV

TABLE IV

Separation by the Method of Sumner and Smith

	Units/ml.	Units/mg. Nitrogen	Total Activity
Crude Soya Extract	62·0	6·9	49,600
Solution of Precipitate	25·3	265	5,300
Supernatant	92·5	36·8	85,000

Using this method of separation, it was found that a 38-fold enrichment of the enzyme had been achieved in only one step. The activity per ml. of the lipoxidase concentrate was not apparently very high, however.

The lipoxidase activity of the supernatant was observed to be considerably higher than that of the crude extract. This may possibly have been due to the removal of an inhibitory substance from the crude extract on its treatment with disodium phosphate.

It was decided to attempt further purification of the 38-fold enriched solution using ammonium sulphate as precipitant.

The lipoxidase solution was adjusted to pH 6·8 with 0·88 ammonia and solid ammonium sulphate was added to 40% saturation. The precipitate obtained was removed by centrifuging and dissolved in distilled water. This solution was tested for lipoxidase activity, found to be inactive, and was therefore discarded.

The supernatant was then brought to 60% saturation with sulphate, and the active precipitate so obtained was collected by centrifuging at 3,000 r.p.m. for 30 minutes, and dissolved in 50 ml. distilled water. This solution was dialysed until sulphate-free, and its specific activity duly determined.

TABLE V.

Further Purification of a 38-fold Lipoxidase Concentrate

	Units/ml.	Units/mg. Nitrogen	Total Activity
Crude Soya Extract	62·0	6·9	49,600
Solution of Fraction between 40-60% Saturation	24·8	392	1,440

By this treatment, the degree of enrichment of the lipoxidase extract was found, as shown in Table V, to have increased to 57-fold. The yield, however, had apparently decreased to 2·8%.

Although the enzyme fraction had, by this stage, lost a great deal of its original activity, further purification seemed justified, and for the next step in the purification, precipitation with alcohol was chosen, as it was considered that further fractionation of the enzyme solution with salt might cause overlapping of fractions, thereby inducing increased loss of activity.

Accordingly, the 57-fold concentrate was diluted to 200 ml. with M/15 pH 6·8 phosphate buffer. This solution was placed in a 1 litre beaker in the cooling tank. When the solution had reached a temperature of 0°, the addition of alcohol was started.

The fairly active precipitate obtained between 10% and 25% alcohol was removed from the solution by centrifuging for 30 minutes at 3,000 r.p.m., the centrifuge being kept as cool as possible by means of dry ice.

The precipitate was dissolved in M/15 pH 6·6 phosphate buffer and filtered through a No. 1 Whatman filter paper, the temperature being kept below 0°. The results of the fractionation are shown in Table VI.

TABLE VI.

Further Purification of a 57-fold Lipoxidase Concentrate

	Units/ml.	Units/mg. Nitrogen	Total Activity
Crude Soya Extract	62.0	6.9	49,600
Solution of fraction between 10-25% alcohol	78.4	759	784

The specific activity of the final fraction, separated by a combination of salting-out and alcoholic fractionation procedures, proved to be 110 times that of the original crude soya extract.

Although a high degree of enrichment had apparently been achieved by this method, the lipoxidase preparation was found to be so unstable that its activity decreased very rapidly

on storage.

As mentioned earlier in this section, it was observed during the course of the fractionations that treatment of an acidified soya extract with disodium phosphate resulted in a supernatant solution, the specific activity of which was 5 times that of the original soya extract. It was, therefore, decided to attempt the separation of a more stable lipoxidase concentrate from a phosphated soya extract, by repeated fractionation with ammonium sulphate.

The method used was as follows:-

A crude soya extract, prepared by extracting 1 kgm. defatted soya flour overnight with 5 litres chilled water, was brought to pH 5 with 2N acetic acid and then centrifuged at 2000 r.p.m.for 45 minutes. The solution was filtered rapidly in the cold room.

The filtrate was treated with 0·5N disodium phosphate, 50 ml. being added for every 250 ml. filtrate. The precipitate which formed after 2 - 3 hours was separated from the solution by centrifuging and was then discarded. The supernatant was then dialysed for 48 hours against tap water. An inactive precipitate was at this stage removed from the dialysate by centrifuging and then discarded.

The supernatant was adjusted to pH 6·8 with 0·88 ammonia and brought to 50% saturation with solid ammonium sulphate. The active precipitate so obtained was separated from the solution by centrifuging at 2,000 r.p.m. for 45 minutes, dissolved in 500 ml. distilled water, and was then dialysed until salt-free. An inactive precipitate was removed from the dialysate and discarded.

The supernatant was brought to 30% saturation with ammonium sulphate and the inactive precipitate obtained was removed by centrifuging and discarded. The precipitate at 40% saturation was also discarded. Sulphate addition was continued, and at 60% saturation an active precipitate was obtained. This was separated from the solution by centrifuging at 2,000 r.p.m. for 30 minutes. The precipitate was then dissolved in 100 ml. distilled water and the solution dialysed until salt-free.

The dialysate was refractionated with solid ammonium sulphate and the highly active precipitate obtained between 40% and 55% saturation was collected, dissolved in 50 ml. distilled water and dialysed overnight against tap water.

The results of the fractionation are shown in Table VII.

TABLE VII

Fractionation of a Crude Soya Extract with Ammonium Sulphate

	Units/ml.	Units/mg. Nitrogen	Total Activity
Aq. Soya Extract	69·8	7·6	226,050
Supernatant after addition of Phosphate	103·6	56·3	395,500
Final Fraction separated between 40-55% saturation	710	652	35,500

The highly active fraction obtained by this salting-out method was found to have a specific activity 67 times higher than that of the crude, aqueous soya extract.

In order to avoid loss of activity from the enzyme solution on storage, the lipoxidase concentrate was freeze-dried in 10 ml. ampoules on an Edwards freeze dryer, and the dried material stored at -20° C.

The iron content of the freeze-dried fraction was determined and found to be 0·06%. This figure was apparently almost three times the level found in a 94% pure preparation of lipoxidase by Holman (43). Assuming that the 67-fold enriched lipoxidase had a molecular weight of 100,000, (48) the level of iron present represented the equivalent of almost one atom per molecule of the enzyme. On the basis of this

evidence, the possible presence of traces of haematin compounds in the lipoxidase preparation could not be overlooked.

The catalase activity of the freeze-dried material was therefore determined by the method of von Euler and Josephson (101) and the results are shown in Table VIII.

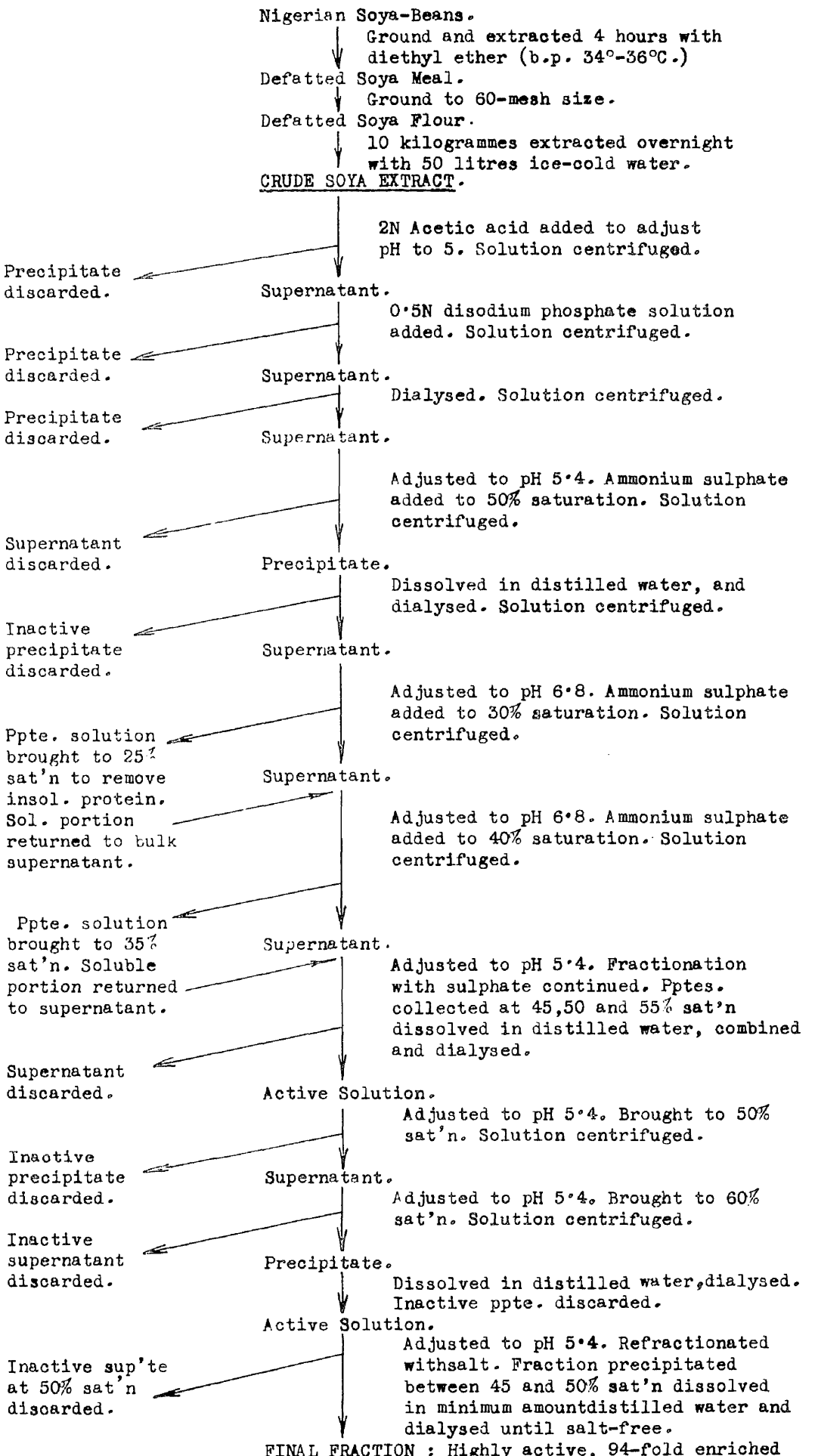
TABLE VIII

Catalase Activities of Defatted Soya Flour and Freeze-Dried Lipoxidase Concentrate.

	Kat f	Equivalent p.p.m. pure catalase
Defatted Soya Flour	0.06	2
Freeze-dried 87-fold Lipoxidase concentrate	0.16	6

The freeze-dried lipoxidase preparation was found to contain the equivalent of 6 p.p.m. pure catalase of Kat f value 30,000. Comparison of the catalase activities of the 87-fold concentrate and of defatted soya flour showed, also, that the catalase present in the crude soya had been concentrated 3-fold during the course of fractionation. However, the method seemed sufficiently promising to justify a further attempt on a larger scale.

Fig. 5- PURIFICATION OF SOYA-BEAN LIPOXIDASE.



3. Purification of Soya Lipoxidase .

The underlying principle of the separation was the precipitation of lipoxidase from the crude soya extract at its isoelectric point , pH 5·4 (43) , using ammonium sulphate.

The results of the previous experiments had shown the enzyme to be precipitated around 50% saturation with ammonium sulphate . The proposed scheme of the final fractionation was to carry out salt additions to saturation levels above 45% at pH 5·4 , in order to favour precipitation of lipoxidase. Removal of inactive protein was to be effected by carrying out the precipitations at pH 6·8 .

The preliminary stages of the separation followed the same pattern as in the preceding experiment , and the procedure of separation is shown in Figure 5 .

10 kgm. defatted Nigerian soya flour were extracted overnight with 50 litres ice-cold water in a large stainless steel tank. The pH of the suspension was then adjusted to pH 5 by addition of 2N acetic acid . The extract was then centrifuged in batches at 1500 r.p.m. for 30 minutes.

The supernatant was returned to the tank in the cold room, and for every 250 mls. enzyme solution , 50 mls. 0·5N disodium phosphate solution was added. The mixture was then left in the cold room and the precipitate which had formed overnight was removed from the solution by centrifuging , and was

then discarded. Dialysis of the supernatant against tap water was carried out for 60 hours and the inactive precipitate obtained was discarded.

The pH of the supernatant was adjusted to pH 5·4 with 0·2M acetate buffer; this treatment did not appear to precipitate the enzyme from solution. Ammonium sulphate was then added to a saturation level of 50%. The addition of salt to the extract was carried out slowly and with stirring in order to avoid local high concentrations. The solution was centrifuged in batches at 1500 r.p.m. for 30 minutes and the active precipitate obtained was dissolved in 5 litres distilled water. The supernatant solution, containing salt at a 50% saturation level, was, at this stage, discarded.

The solution of the active precipitate was dialysed against tap water for 48 hours, after which time an inactive precipitate had formed which was removed from the solution by centrifuging and then discarded.

The pH of the dialysed solution was adjusted to 6·8 with 0·88 ammonia and ammonium sulphate was added until a saturation level of 30% had been reached in the solution. Removal of the precipitate was effected by centrifuging the solution at 2000 r.p.m. for 30 minutes. Further addition of salt to the supernatant produced, at 40% saturation, another precipitate which was also removed by centrifuging. Most of the activity present

in the precipitates obtained at 30% and 40% saturation was then recovered by refractionating the aqueous solution of the precipitates with salt , and returning the soluble protein material so obtained to the 40% saturated supernatant . The solution was adjusted to pH 5·4 and fractionation with ammonium sulphate was continued .

The active precipitates collected between 40-55% saturation were dissolved in distilled water , combined , and the solution then dialysed until sulphate-free . This solution , which was found to have a specific activity 20 times that of the crude extract , was then subjected to further fractionation with salt.

After adjustment of the pH to 5·4 , ammonium sulphate was added to a saturation level of 50%. The relatively inactive precipitate obtained was discarded and the addition of salt was continued . At 60% saturation , an active precipitate was collected and dissolved in the minimum amount of distilled water . The specific activity of the enzyme solution was found to have increased by this stage to a level almost 60 times that of the original soya extract , while the activity had apparently been entirely removed from the supernatant . The active solution was dialysed until salt-free and the dialysate , after removal of an inactive precipitate , was adjusted to pH 5·4 with 0·2M acetate buffer . The solution was then cautiously refractionated with solid ammonium sulphate.

The highly active fraction, precipitated between 45-50% saturation, was collected from the solution by centrifuging, dissolved in the minimum amount of distilled water and then dialysed against running water for 24 hours.

The specific activity of this preparation was found to be 94 times as high as that of the aqueous 20% soya extract. The results of the separation are shown in Table IX.

Table IX

Purification of Soya Lipoxidase.

	Units/ ml.	Units/mg. Nitrogen	Total Activity	Degree of enrichment
Soya Extract	61·5	6·15	2,030,000	1
Supernatant after treatment with phosphate	78·0	24·0	2,784,000	4
Fraction precipitated between 40-55% saturation	250	120	316,000	20
Fraction precipitated between 50-60% saturation	600	356	252,000	60
Final fraction precipitated between 45-50% saturation	1364	565	136,400	94

The highly active lipoxidase concentrate was freeze-dried in 10 ml. ampoules on an Edwards freeze dryer and then stored

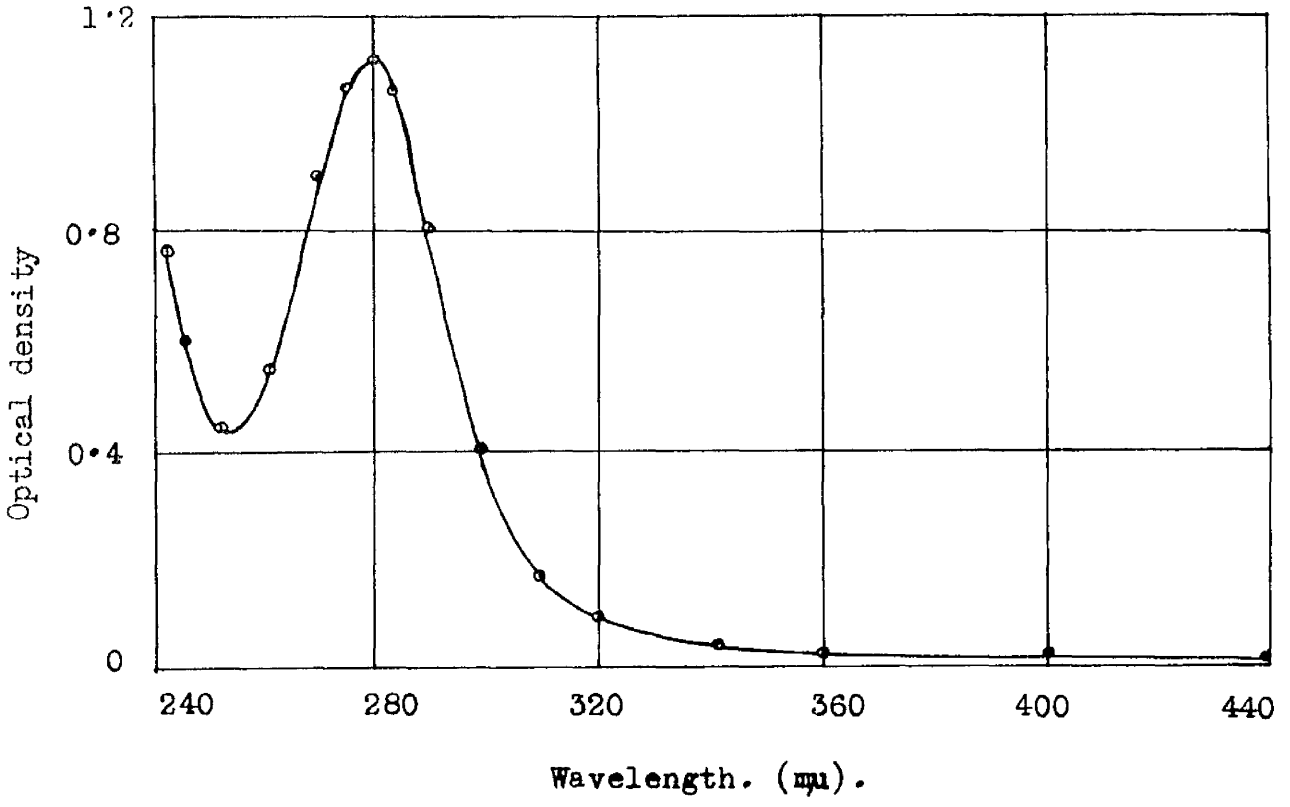


Fig.6.- Absorption spectrum of purified lipoxidase.

under vacuum at -20°C .

The iron content of the purified material was found to be only 0·02%, approximately the same level as that reported by Holman (43) in his purified material , and peroxidase was estimated to be present to the low extent of 0·1 p.p.m. Catalase activity of the freeze-dried material could not be detected by the adopted procedure . However, on the assumption that the catalase activity of the purified fraction was less than the lowest possible level measurable by the adopted method , the catalase content of the preparation was regarded as being less than the equivalent of 0·2 p.p.m. of pure catalase of Kat f value 30,000 .

The absorption spectrum of the purified preparation was similar to that obtained for crystalline soya-bean lipoxidase by Holman (43) . As shown in Figure 6, the absorption spectrum exhibited a fairly high absorption peak at 280 mμ, characteristic of the aromatic amino grouping of proteins. No increased absorption was observed, however, at the higher wavelengths around 405 mμ where haematin (iron porphyrin) absorbs .

On the basis of the above tests, the 94-fold enriched lipoxidase preparation was considered to be substantially free from haematins .

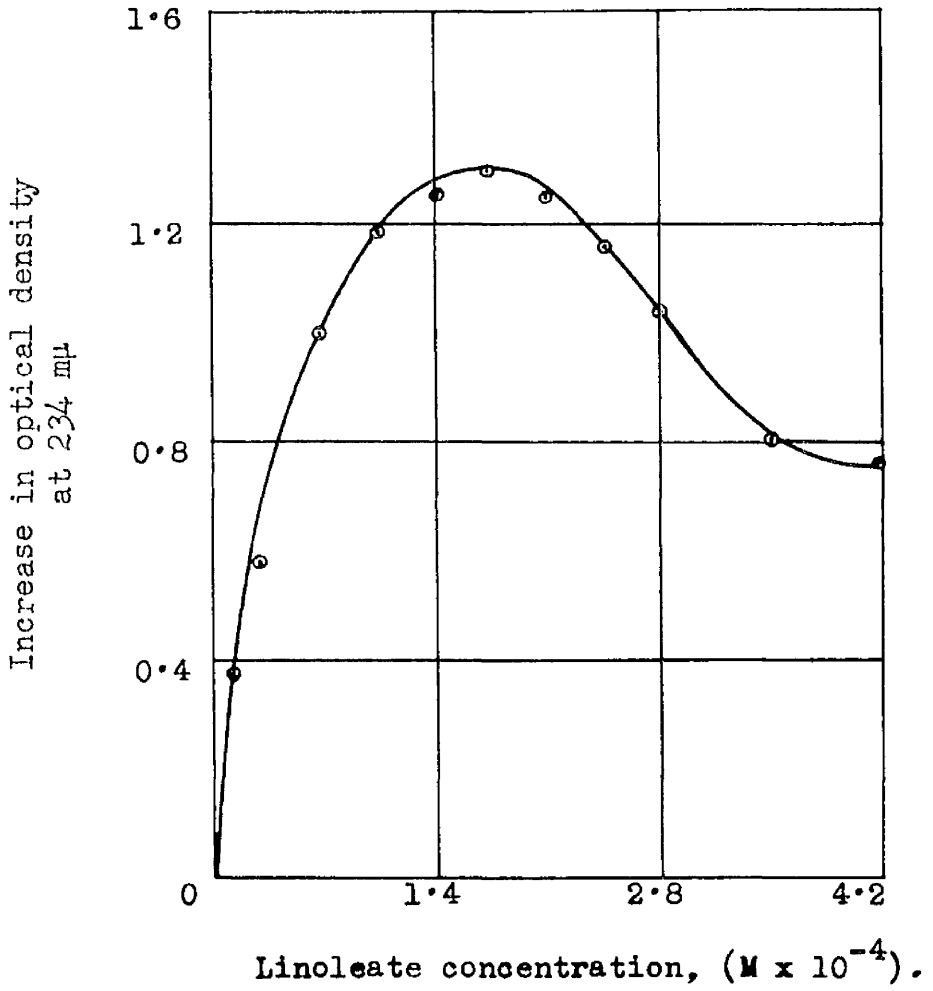


Fig. 7. - Effect of Substrate Concentration on the Initial Reaction Velocity at pH 9.

Lipoxidase level in system : 0·004 mg./ml.

4. Characteristics of the Haematin-free Lipoxidase System.

In studying the following characteristics of the purified lipoxidase system, solutions of the freeze-dried preparation in distilled water were used and were freshly prepared for each experiment. Purified lipoxidase was normally used at a level in the reaction system of 0·004 mg. per ml., unless otherwise stated.

Sodium linoleate was used as substrate and the relative activities were measured, at pH 9, by the spectrophotometric method described in Appendix I, the result being expressed as the increase in optical density at 234 mμ developed after a reaction time of one minute.

Effect of Substrate concentration on the Initial Reaction Velocity.

The effect of varying substrate levels on the lipoxidase system at pH 9 is shown in Figure 7.

The existence of an apparent substrate optimum for soya lipoxidase has been noted by Balls et al. (6), using an assay based on peroxide measurements, and also by Blain et al.(95), who used a system based on carotene-destruction.

Under reaction conditions similar to those used in the present work, Tookey et al. (96), also observed a maximum in lipoxidase activity at rather low levels of sodium linoleate. These workers found this substrate optimum to correspond to

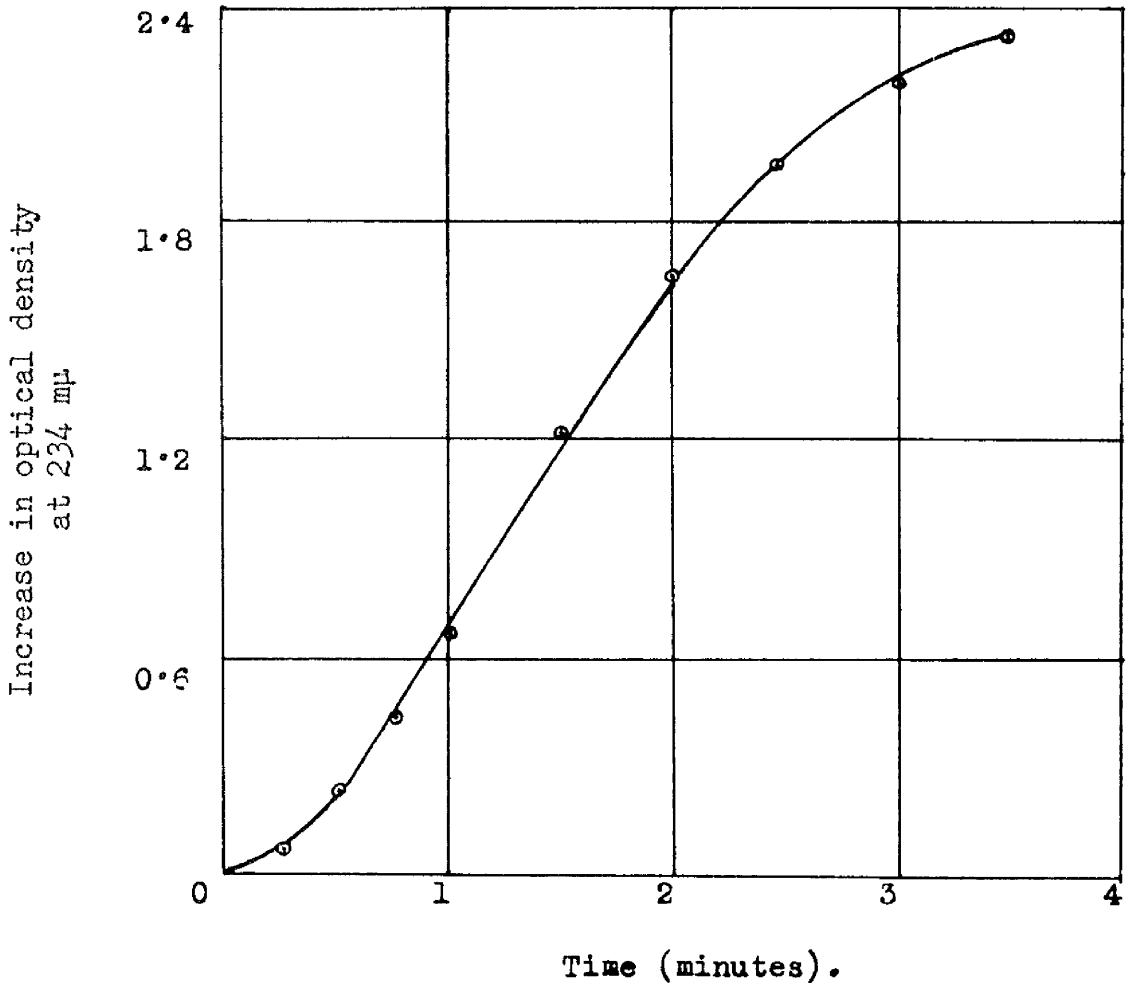


Fig. 8. - Effect of Time on the Lipoxidase Reaction at pH 9.

Lipoxidase level in system : 0·002 mg_4/ml.
Sodium linoleate concentration : 1·4 x 10^{-4} M.

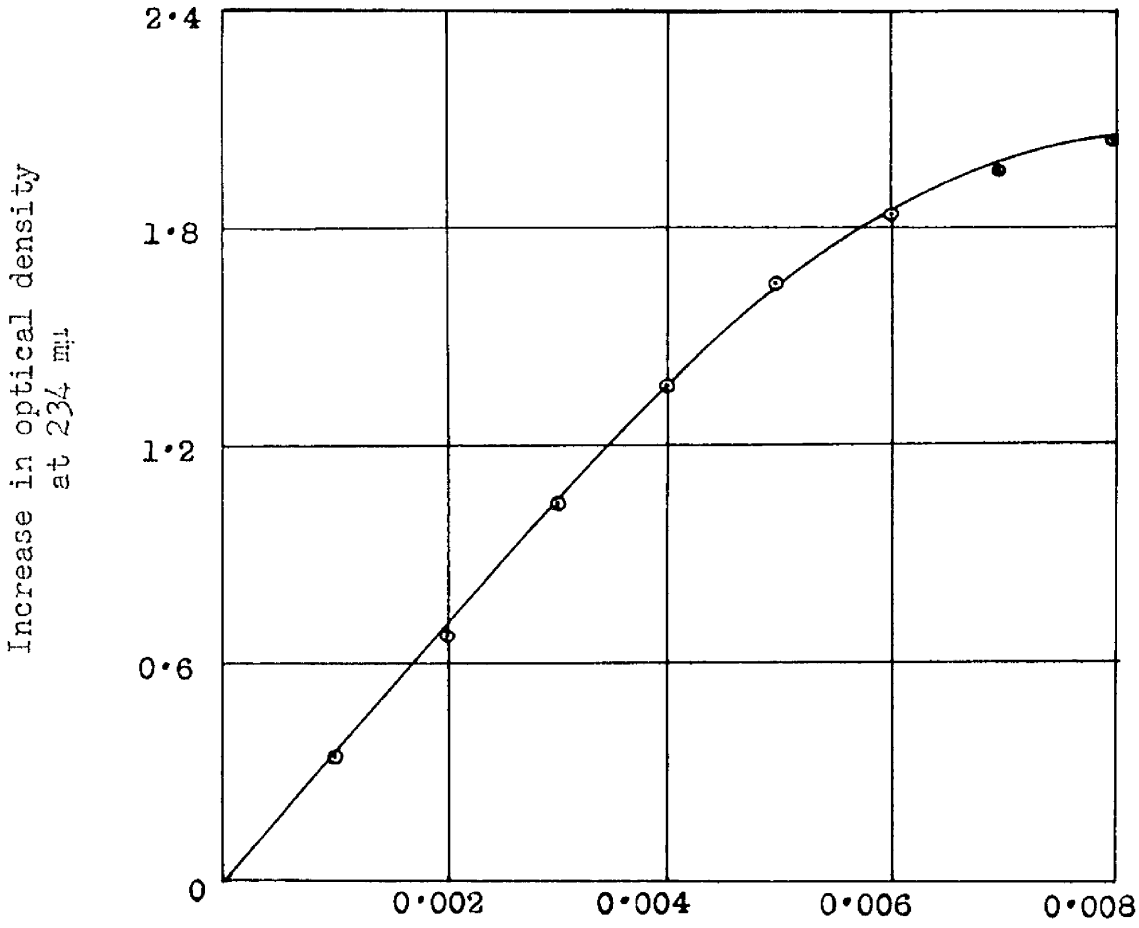


Fig. 9.- Effect of Enzyme Concentration on the Initial Reaction Velocity at pH 9.

Sodium linoleate concentration in reaction system : $1{\cdot}4 \times 10^{-4}$M.

the critical micelle concentration of the soap and suggested that the levelling off of lipoxidase activity above the maximum might be caused by the limited availability of the free soap ions.

It would appear from Figure 7 that a sodium linoleate concentration of $1 \cdot 4 \times 10^{-4}$M, the level chosen for the present reaction system, supplies an adequate level of substrate for the quantities of enzyme normally used.

Effect of Time on Lipoxidase Action.

Under the adopted conditions, lipoxidase activity at pH 9, as shown in Figure 8, was found to be linear with time over a period of 2-3 minutes, except for a very short initial lag in activity.

The occurrence of an induction period in the course of the lipoxidase-catalysed reaction has recently been verified by Haining and Axelrod (70).

Effect of Enzyme Concentration on the Initial Reaction Velocity.

Figure 9 shows the range of linearity of the system at pH 9 with enzyme concentration. The falling-off in activity observed with higher levels of enzyme in the system may have been caused by substrate becoming the limiting factor.

Effect of pH on Lipoxidase Activity.

Considerable controversy regarding the pH optimum of soya lipoxidase has appeared in the literature. For maximum enzyme

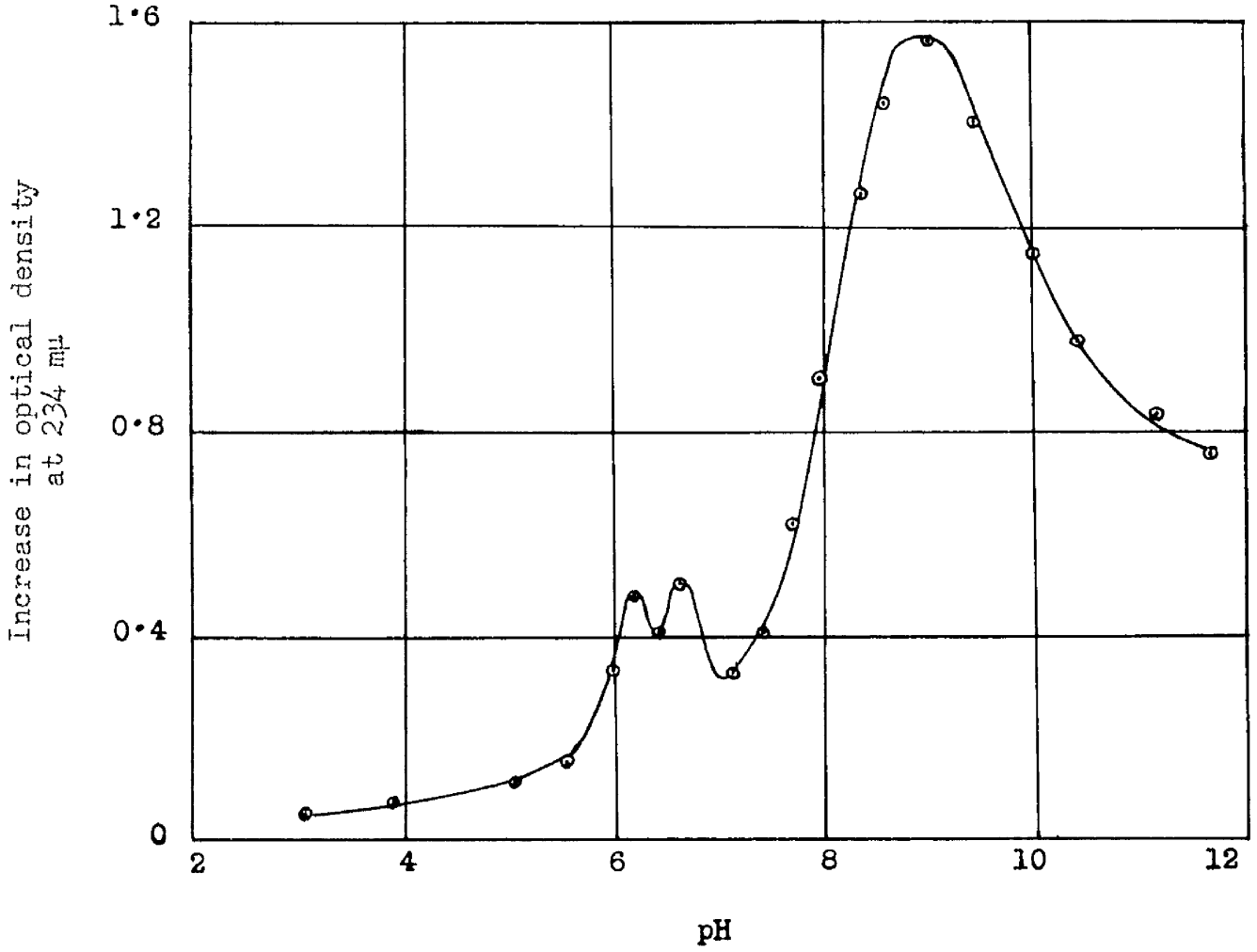


Fig. 10. - Effect of pH on Lipoxidase Activity.

Reaction mixture : 0·005 mg./ml. lipoxidase.
$1{\cdot}4 \times 10^{-4}$ M sodium linoleate.
Reaction time : 60 seconds.

activity pH values of 6·5 and 9 have been reported. (11, 15, 27, 92, 94, 102, 103).

This variation in pH optimum is due to the different systems used in the lipoxidase assay, and to different substrate solubilities under various conditions. Reaction rates vary with substrate availability, which in turn is a function of pH in the heterogeneous systems used. With crystalline lipoxidase and using a pure linoleate substrate, Holman found that the optimum pH was 9 (103).

Under the reaction conditions of the adopted system, a definite optimum for the 94-fold enriched lipoxidase was observed at pH 9, as shown in Figure 10. At the more alkaline pH levels, activity was found to decrease markedly, although not to the same extent as was observed below pH 9.

Two smaller increases in activity were noted at pH 6·2 and pH 6·5. As these two minor peaks appeared with all active solutions examined by the assay system, they may beexplained by differences in substrate dispersal between pH 6 and pH 7.

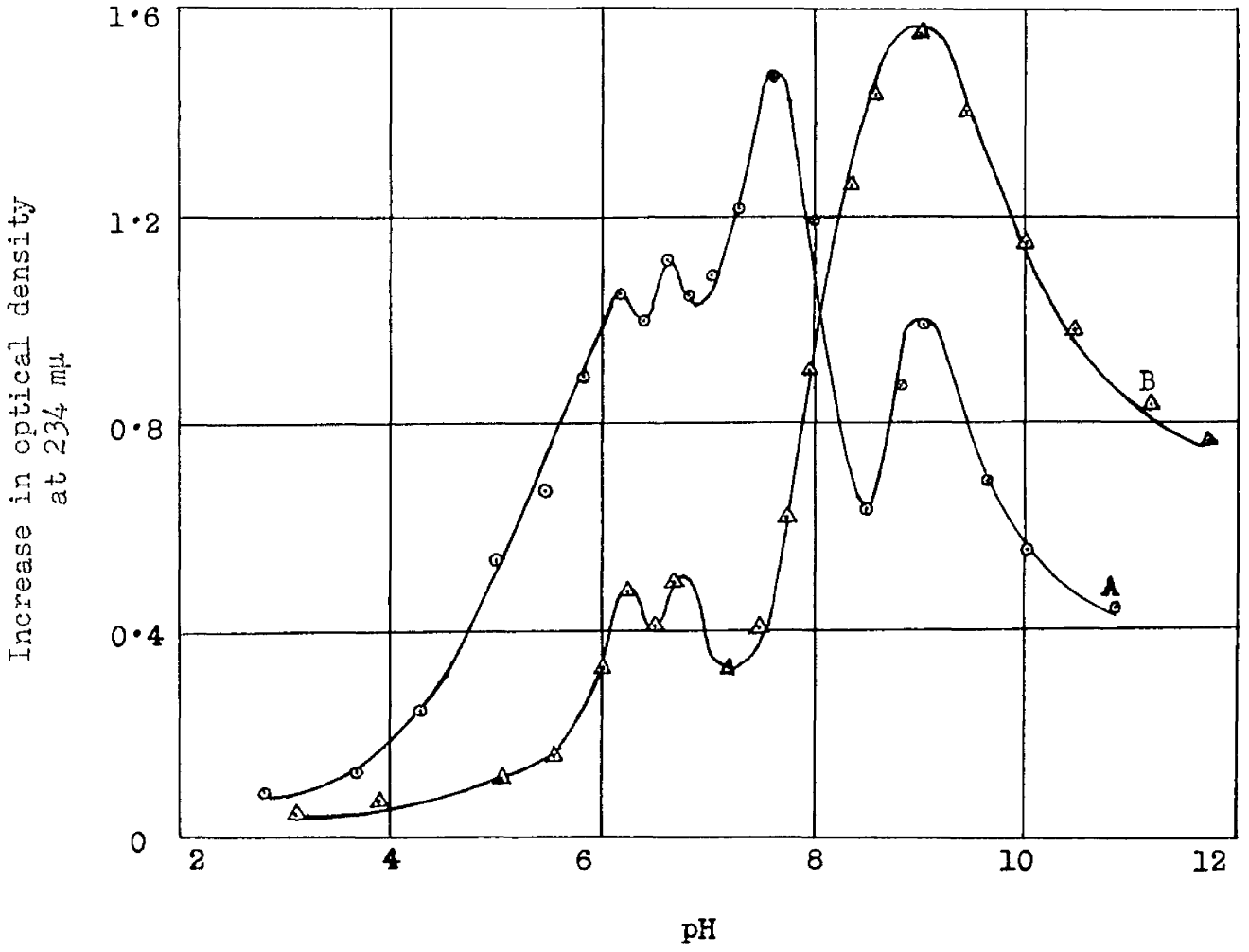


Fig. 11. - The pH-activity curves of Crude and Purified Soya Lipoxidase.

A - 20% aqueous Nigerian soya extract. (0·1ml. used.)
B - purified lipoxidase. (0·005mg./ml.)

Sodium linoleate concentration : $1{\cdot}4 \times 10^{-4}$M.

5. The Lipoxidase Activity of Crude Soya Extracts.

During the course of studies on purified soya lipoxidase, a report by Koch et al. (89) became available, which produced evidence as to the existence in soya-beans of two types of lipoxidase. Examination of the lipoxidase activity of a crude soya extract at several pH levels had indicated the possibility that two different enzymes were present. Subsequent fractionation of crude soya extracts by these workers enabled them to separate two enzyme fractions, which were found to differ, particularly in optimum pH.

In view of these findings, it was of considerable interest to examine the pH-activity curves of crude soya extracts and to compare them with that of the purified lipoxidase-fraction.

Comparison of the pH-activity Curves of Purified Lipoxidase and Crude Soya Extracts.

The lipoxidase activity of a 20% aqueous extract of defatted Nigerian soya, prepared as described in Appendix I, was determined over the pH range 2-12 under the usual reaction conditions. The results are shown in Figure 11.

Apart from the two minor peaks found between pH 6-7, which have been discussed earlier, the general form of the pH-activity curve of the crude extract was found to show two pH maxima, the principal one occurring around pH 7·6 and the other at pH 9.

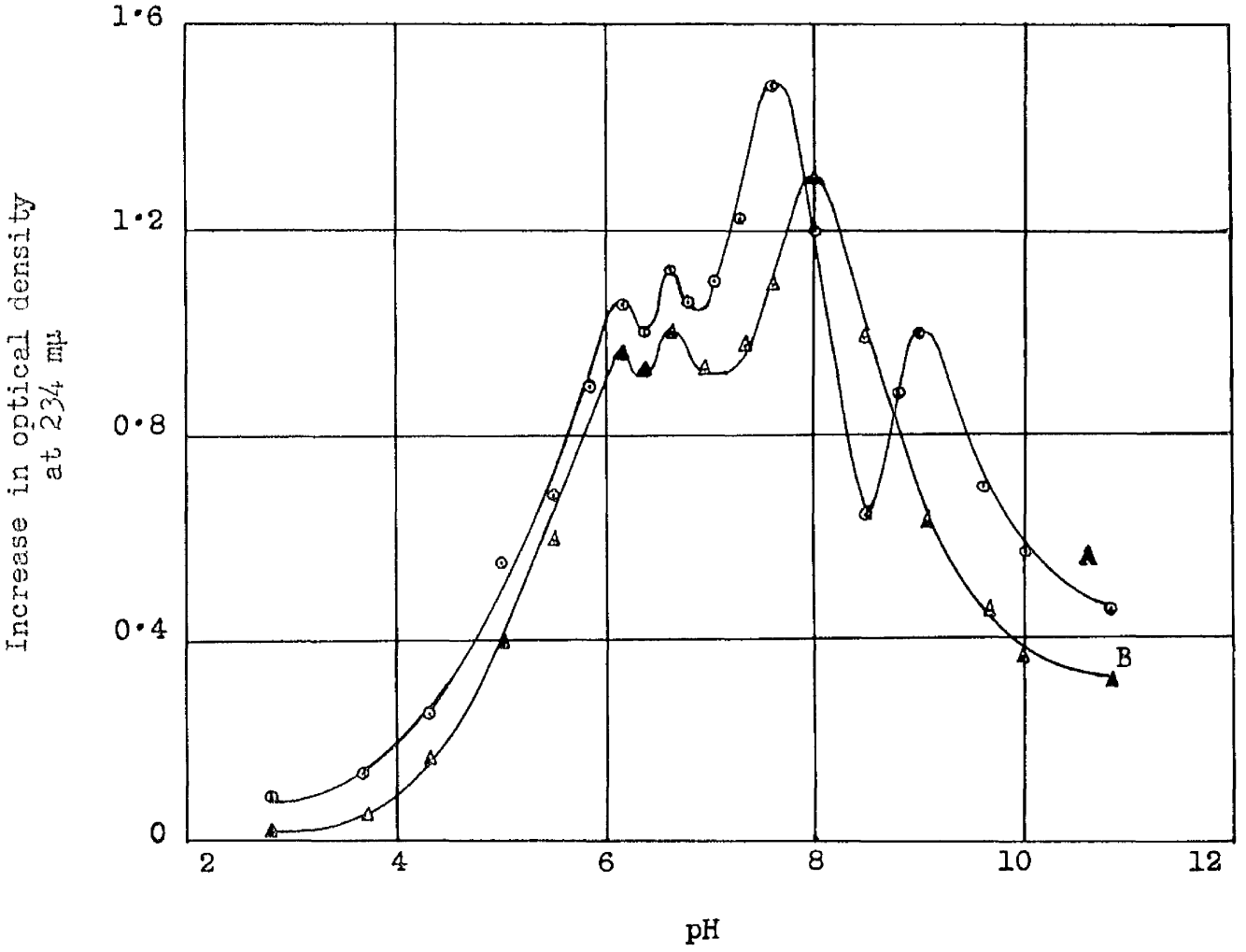


Fig. 12. - The pH-activity curves of Crude Nigerian Soya Extracts.

A - 20% aqueous soya extract.

B - 20% pH 4·5 buffer soya extract.

Reaction mixture : 0·1 ml. soya extract added to system containing $1·4 \times 10^{-4}$ M sodium linoleate.

Reaction time : 60 seconds.

The appearance of two sharply defined pH maxima suggested there to be two linoleate—oxidizing systems present in the crude aqueous extract . Comparison of the pH-activity curves of crude and purified lipoxidase extracts indicated, moreover, that the minor component of the two linoleate-oxidizing systems had been purified during fractionation of the aqueous soya extract .

In view of Holman's observation (83) that a pH 4·5 buffer extract is a much purer preparation of lipoxidase than an aqueous one , it was also considered of interest to examine the pH-activity curve of a pH 4·5 buffer extract .

Accordingly, the pH-curve of a buffer extract of Nigerian soya was determined under identical conditions to those used in the case of the aqueous soya extract .

As shown in Figure 12, the overall activity of the extract made at pH 4·5 was found to be lower than that of the corresponding aqueous extract . In the case of the buffer extract, however, only one peak was observed, at pH 8 .

One possible explanation for the apparent differences in the configurations of the pH-activity curves of the two crude extracts was that there were two lipoxidases present in the Nigerian soya-beans, both acting on sodium linoleate in the described system, one showing maximum activity between pH 7·6 and pH 8, and the other, the less active of the two, having a pH optimum of 9 .

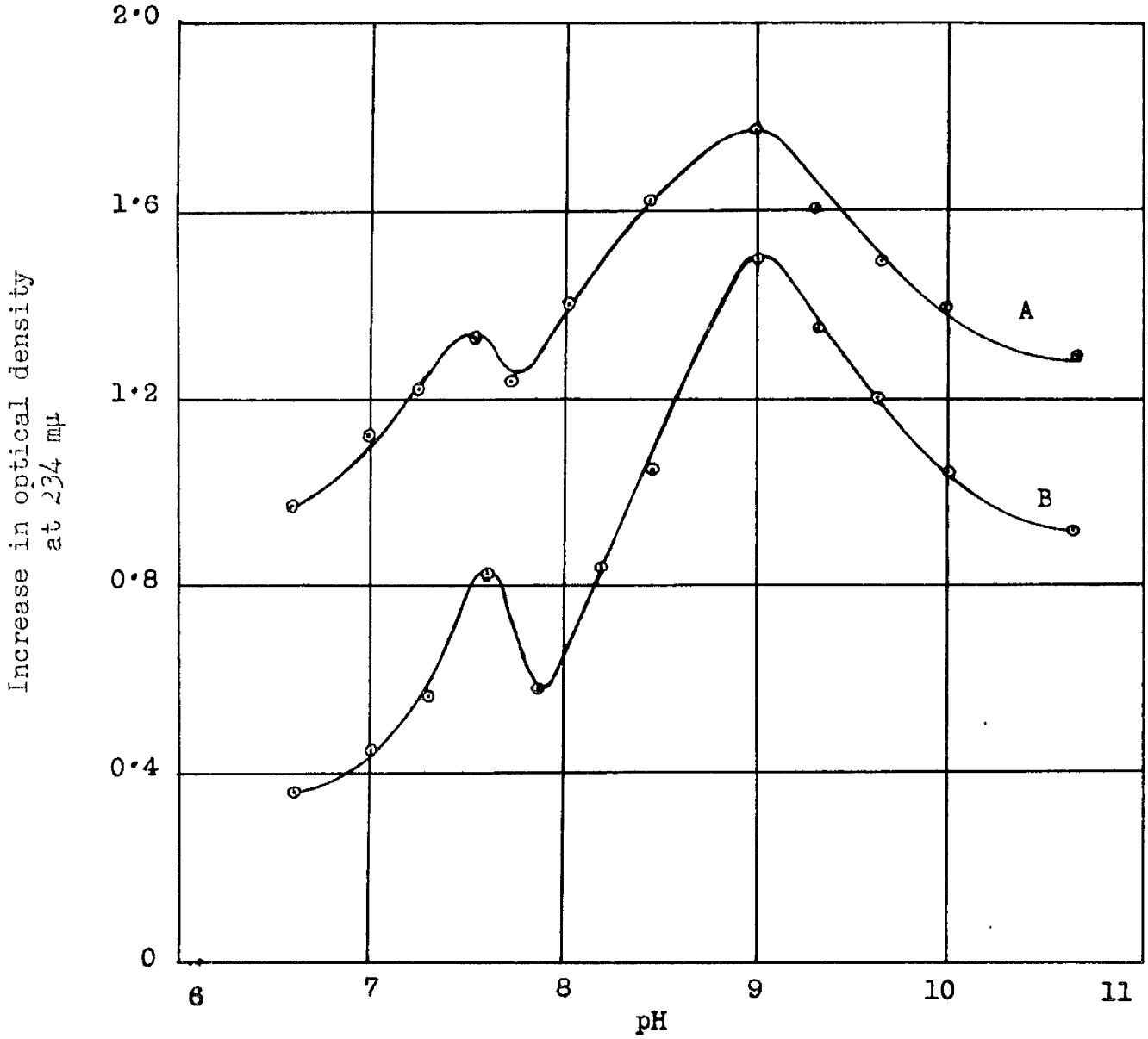


Fig. 13. - The pH-activity curves of Crude Chinese Soya Extracts.

A - 5% aqueous soya extract.

B - 5% pH 4·5 buffer soya extract.

From the results, as shown in Figure 12 , the method of extraction appeared to be selective in determining the relative amounts of the two systems obtained .

The pH-activity relationships of aqueous and buffer extracts of defatted Chinese soya flour were determined under similar conditions and the results are shown in Figure 13 . The activity of the defatted Chinese soya appeared to be considerably higher than that of Nigerian soya , and in order to obtain spectrophotometric readings comparable to those obtained for Nigerian soya extracts , it was found necessary to use 5% extracts of the defatted material .

The pH-activity curves of the Chinese soya extracts both showed two pH maxima , one at pH 9 and the other at pH 7·6 . The major peak in both curves , however , was observed at pH 9 , whereas in the case of the Nigerian soya extracts , the principal maximum had been observed around pH 7·6 .

These results indicated the possibility of the presence of two lipoxidases in soya flour extracts , and suggested that soyas from different sources differ proportionately as to the activity of the two systems .

On the assumption that the two pH maxima of the pH-activity curves of the soya extracts were caused by two enzymes attacking the linoleate substrate at different optimum pH values , attempts to separate the two systems were undertaken .

Fractionation of an Aqueous Soya Extract

by the method of Koch et al.

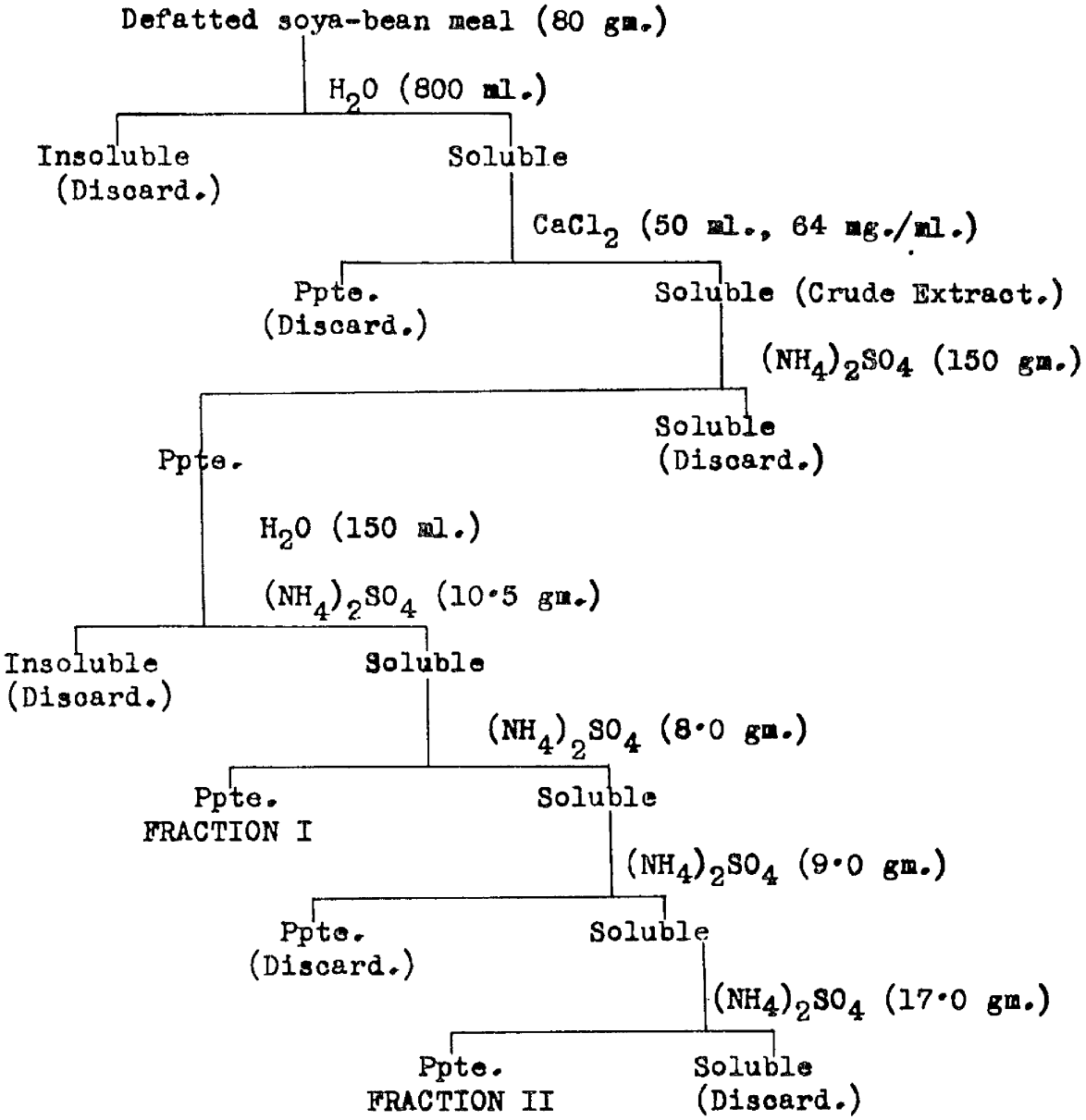


Fig. 14.

Further Studies of the Fractionation of Crude Soya Extracts.

The first attempts to separate two lipoxidases from Nigerian soya-beans followed the method of Koch et al. (89), involving separation from a crude, aqueous soya extract by fractionation with ammonium sulphate. The procedure is shown in Figure 14.

Inactive protein was removed from the crude soya extract by precipitation with calcium chloride. The supernatant was then fractionated with ammonium sulphate and two active fractions were precipitated out, one at a low level and the other at a high level of saturation.

Both fractions obtained by this procedure, however, showed maximum activity at pH 9, as found earlier in the case of the 94-fold enriched lipoxidase preparation.

As separation of a second lipoxidase could not be effected by this means, it was decided to attempt its isolation by fractionation of a pH 4·5 buffer extract of Nigerian soya, in which, according to the configuration of the pH-activity curve as shown in Figure 12, the second linoleate-oxidising system appeared to be the principal factor present.

Due to the diminishing supply of Nigerian soya-beans, it was found impossible to use large quantities of material for this fractionation, and since working with small volumes inevitably results in large losses of activity, it was thought advisable to concentrate the activity of the enzyme at a suitable stage during

Fractionation of a pH 4·5 buffer extract of Nigerian Soya.

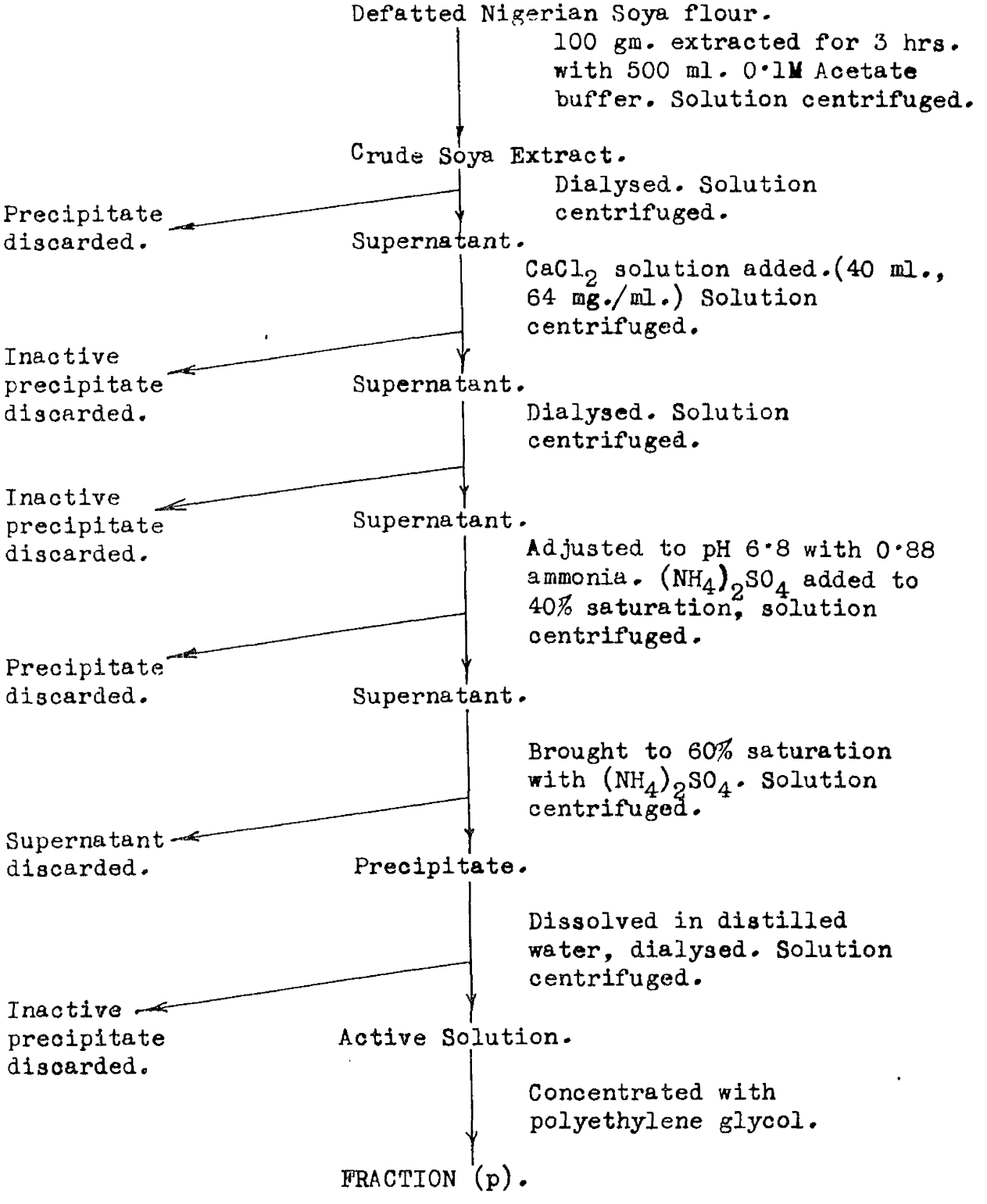


Fig. 15.

the fractionation . For this purpose , the relatively simple yet effective method devised by Kohn (104) was chosen . The method , based on the principle of dialysis against substances of high molecular weight , entailed the removal of water from the enzyme solution by a hydrophilic agent , polyethylene glycol , which has a high absorptive capacity .

The scheme of fractionation employed is shown in Figure 15. Removal of inactive protein from the crude extract was accomplished both through dialysis of the buffer extract and by precipitation with calcium chloride . The active fraction , precipitated from the supernatant between 40-60% saturation with sulphate , was dissolved in 50 ml. distilled water and dialysed until salt-free . The active solution , prior to further concentration , was placed in a 100 ml. measuring cylinder .

The polyethylene glycol (Carbowax 20M, G.T.Gurr and Co.) a waxy , flaky substance of molecular weight 20,000 was broken up and an appropriate quantity of the dry , granulated material was poured into Visking dialysis tubing of 1/4 inch inflated diameter . The proportion of Carbowax used was about 1 part to 5 parts enzyme solution .

The dialysis tubing containing the polyethylene glycol was then bent double and placed in a glass cylinder containing the enzyme solution . The length of the tubing was such as to allow

for the rising column of water inside it. The level to which the tubing was pushed down inside the glass cylinder depended on the final concentration required, the concentrating process ceasing when the fluid containing protein reached the bottom of the tubing.

By this means, concentration of the enzyme solution from 50 ml. to 10 ml. was achieved in 24 hours. The concentrating process was carried out in the cold room, and under these conditions no loss of activity due to denaturation was observed.

A number of such concentrated fractions (p) were combined and refractionated with ammonium sulphate. The active material obtained between 45-50% saturation was dissolved in distilled water, then dialysed until salt-free and finally, concentrated using polyethylene glycol.

The specific activity of the final fraction was found to be 20 times higher than that of the crude buffer extract. From the pH-activity curve of the solution it was observed that the pH-optimum of the 20-fold enriched extract was not, as was expected, pH 8, but pH 9.

By closely following each step in the fractionation procedure, it was discovered, as shown in Table X, that the pH optimum of the supernatant activity collected at the earlier stages of the fractionation was pH 8, whereas that of the supernatant activity collected nearer the end of the process was

Table I

Fractionation of the Lipoxidase Activity of a pH 4.5 buffer extract of Nigerian soya.

Fraction	Lipoxidase Activity (units/ml.) at different pH levels.						
	pH 7.2	pH 7.6	pH 8.0	pH 8.5	pH 9.0	pH 9.5	pH 9.8
Crude Extract	64.0	82.3	116.5	85.5	43.5	39.5	35.5
Dialysate	61.5	79.8	108.5	82.3	42.5	37.8	30.5
Supernatant after addition of $CaCl_2$	58.6	72.5	102.0	79.6	39.7	35.5	26.8
Supernatant at 40% saturation	50.0	70.5	91.8	83.0	64.5	37.9	20.0
Precipitate obtained at 40% saturation	8.8	11.8	15.7	19.5	29.8	21.5	17.5
Supernatant at 60% saturation	16.0	24.0	33.0	41.5	48.0	32.0	24.0
Precipitate obtained at 60% saturation	36.0	47.5	63.0	80.5	99.5	88.2	60.5
Final fraction (20-fold enriched)	80.2	98.7	115.6	132.5	150.4	138.0	119.5

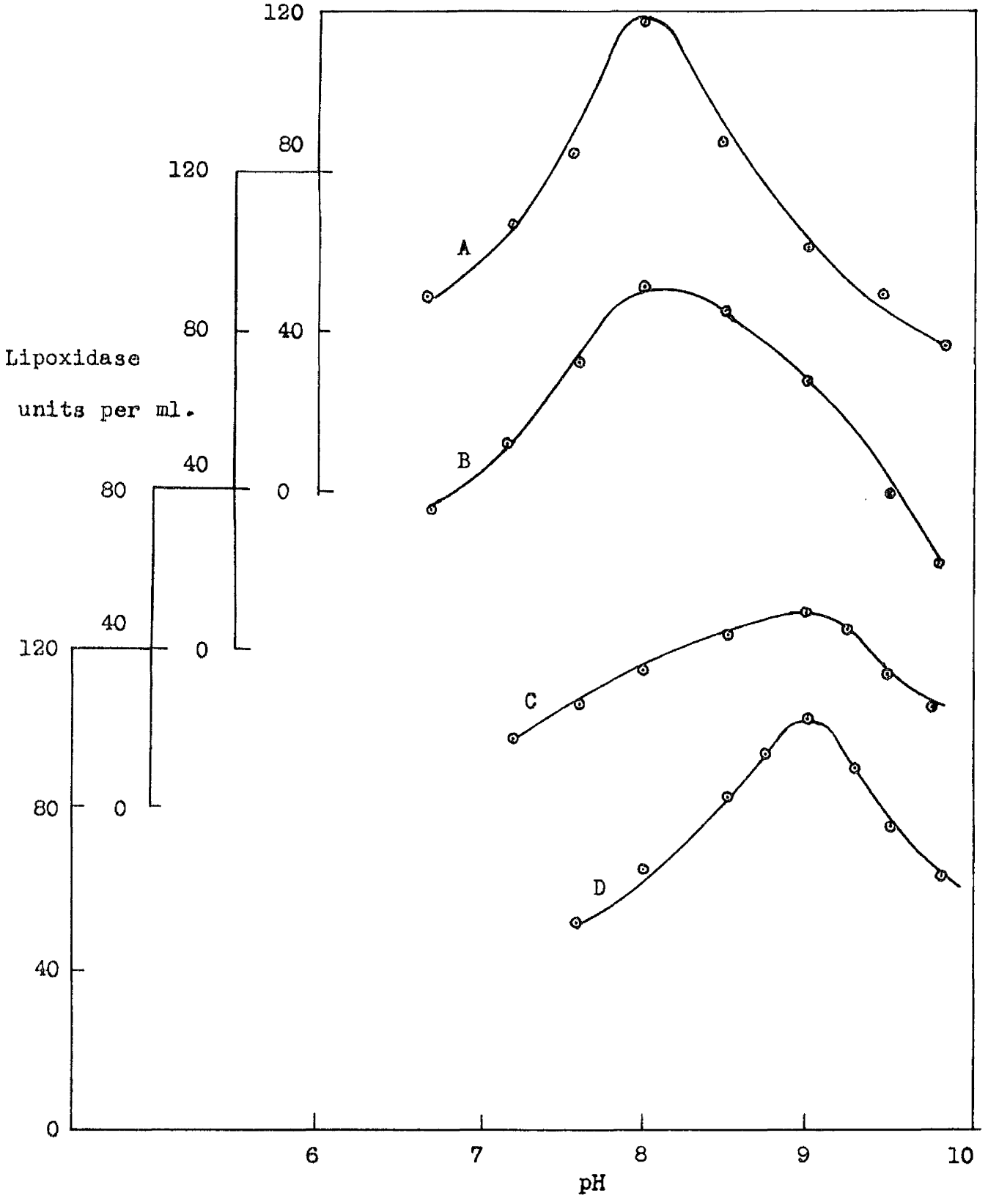


Fig. 16. - Effect of Fractionation on the pH optimum of a pH 4·5 buffer extract of Nigerian Soya.

A - Crude Soya Extract.
B - Supernatant at 40% saturation.
C - Supernatant at 60% saturation.
D - Precipitate at 60% saturation.

pH 9. The precipitated activity was found, in all fractions examined, to have a pH optimum of 9.

This observed change in pH optimum of the lipoxidase activity of a pH 4.5 buffer extract of Nigerian soya is clearly indicated in Figure 16.

Since repeated fractionation of the crude soya extracts had failed to achieve separation of the two pH maxima, it appeared unlikely that the occurrence of two pH maxima in the pH-activity curves of crude soya extracts was caused by two enzymes.

To account for the apparent change in pH optimum of a buffer soya extract during its fractionation, two possible explanations could be put forward,

(a) the alteration in the pH characteristics might have been the result of molecular rearrangement of the enzyme protein caused by a denaturing effect of the salt, or

(b) the effect might have been due to the removal of a component from the crude extract during its purification.

This latter observation gave further interest to the supposition that the peculiar configurations of the pH-activity curves of crude soya extracts might possibly be the result of haematin, present in these extracts, interacting with lipoxidase in the reaction system and modifying the characteristics of the lipoxidase system .

In order to investigate this question, it was decided that a systematic study of the influence of haematin compounds on the behaviour of purified lipoxidase in the adopted system should be undertaken . For the purpose of this investigation , it seemed desirable to employ mixtures of the two purified systems in evenly balanced proportions, such as might be found in crude soya extracts .

EXPERIMENTAL

PART II: THE CHARACTERISTICS OF LIPOXIDASE AND HAEMATIN SYSTEMS

THE CHARACTERISTICS OF LIPOXIDASE AND HAEMATIN SYSTEMS.

1. Linoleate Oxidation Catalyzed by Haematin Compounds.

It seemed desirable before a study of lipoxidase-haematin interaction was begun to establish a pattern for haematin action by means of the assay system used previously to study lipoxidase activity.

The catalytic activity of the haematin compounds has been shown to depend on the presence of preformed peroxide in the system (55, 69). On finding the total peroxide concentration of the system to be linearly related to the concentration of conjugated dienes, Maier and Tappel (106), in order to follow the initiation reactions of haematin catalysis, developed a spectrophotometric technique based on the decrease in diene conjugation of linoleate hydroperoxide when catalytically decomposed by haemoglobin.

Whereas these workers found that haemoglobin destroys hydroperoxide at pH 9, Blain and Styles (87) recently found that cytochrome c can promote formation of conjugated hydroperoxide from freshly prepared sodium linoleate at pH 5·4, although only at linoleate concentrations exceeding $1{\cdot}5 \times 10^{-4}$M.

On this evidence it was of considerable interest to

ascertain whether haematin could, under the conditions of the adopted system, simulate lipoxidase activity by causing the formation of conjugated diene.

Action of Haematin Compounds on Fresh and Peroxidized Linoleate.

The pure haematin compounds used in this study included crystalline haemin (British Drug Houses, Ltd.), crystalline bovine haemoglobin (Sigma Chemical Co., U.S.A.) and crystalline liver catalase (Sigma Chemical Co.) All of these haematin compounds were assayed for their content of ferroporphyrin by conversion to pyridine haemochrome, which was measured by its spectral absorption at 560 mμ. Crystalline haemin was used as the standard in this assay method. All data were thus compared in terms of the active catalyst, ferroporphyrin, and were expressed in equivalent moles of crystalline haemin.

Using the spectrophotometric method of assay described in Appendix I, the action of haemin, catalase and haemoglobin at pH 9 on freshly prepared sodium linoleate at concentrations varying from 10^{-4}M to 10^{-3}M, was examined over periods of one minute and longer.

No reaction whatsoever was observed under these conditions, however, none of the haematin compounds, at the different levels examined, being found to promote formation of conjugated diene, contrary to the observations of Blain and Styles (87).

It was decided, therefore, that a study of haematin action in the presence of preformed peroxide should be made.

In this series of experiments a peroxidised substrate was employed in the reaction system , peroxidised linoleate being prepared by exposing fresh sodium linoleate solution (2 mg.per ml.) to the air for a few days .

The hydroperoxide concentration in the reaction system was determined by measuring spectrophotometrically the optical density of the test solution at 234 mμ against a control solution containing freshly prepared sodium linoleate at an initial level identical to that in the test solution . A molecular extinction coefficient of 25600 was used to convert optical density to moles of conjugated diene (11) .

The action of haematin in the modified system was determined by following , over periods of a minute and longer , changes in the hydroperoxide concentration of the system occurring on interaction of peroxidised linoleate with the haematin catalyst .

Using this technique , it was found that the haematin compounds examined did not effect an increase in the diene conjugation of the system , but rather , caused a significant decrease in diene conjugation of the linoleate hydroperoxide in the system , thus confirming the observations of Maier and Tappel (106) .

Characteristics of the Lipoxidase-free Haematin System .

The characteristics of the lipoxidase-free haematin system were determined by the above method and are shown in Figures 17 to20 .

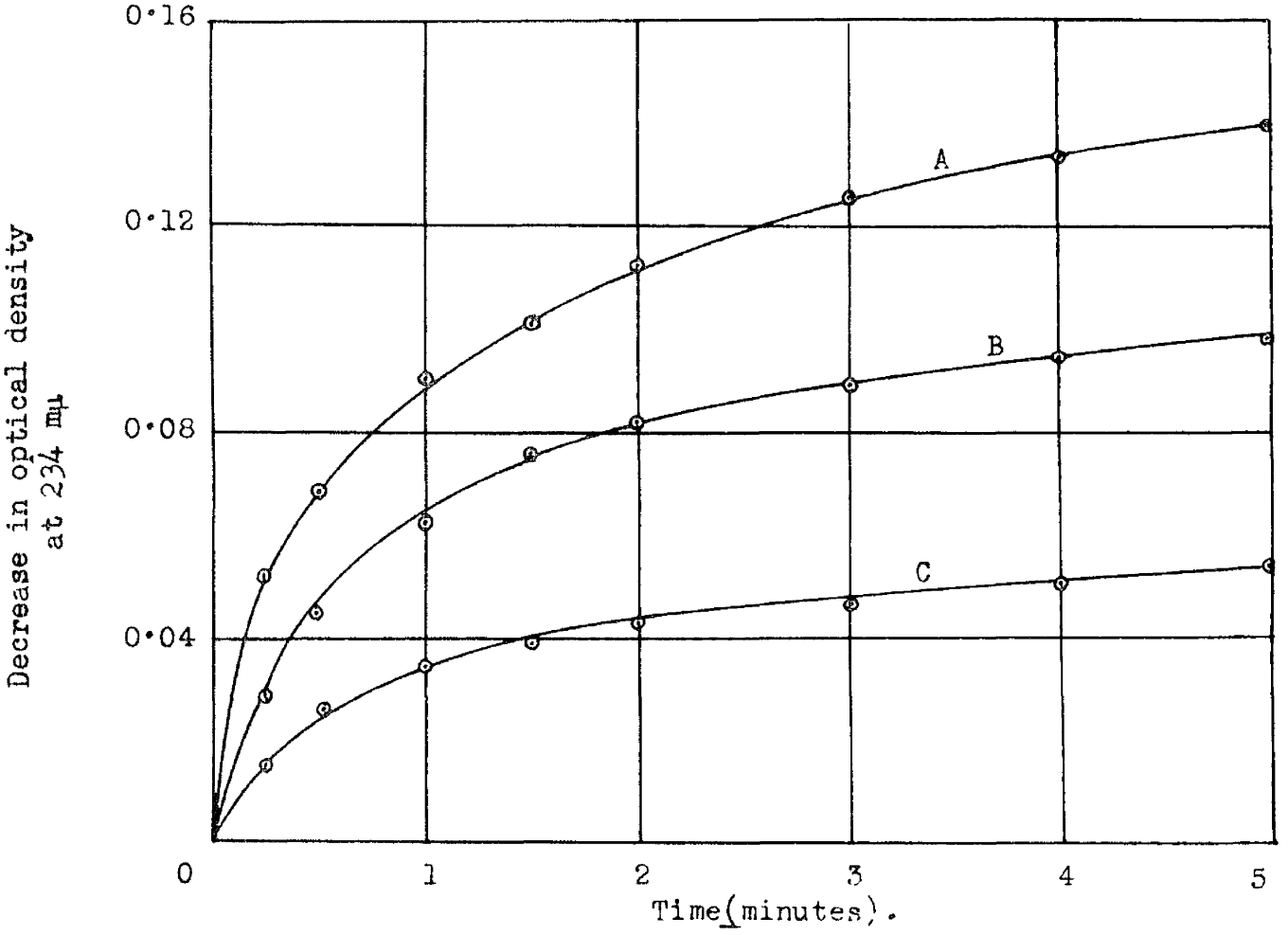


Fig. 17. - Comparative Activities of Haematin Compounds as Catalysts for Linoleate Hydroperoxide Decomposition at pH 9.

A - $0{\cdot}75 \times 10^{-6}$M Haemin.
B - $0{\cdot}75 \times 10^{-6}$M Haemoglobin.
C - $0{\cdot}75 \times 10^{-6}$M Catalase.

Total linoleate concentration : $1{\cdot}4 \times 10^{-4}$M, containing 14×10^{-6}M linoleate hydroperoxide.

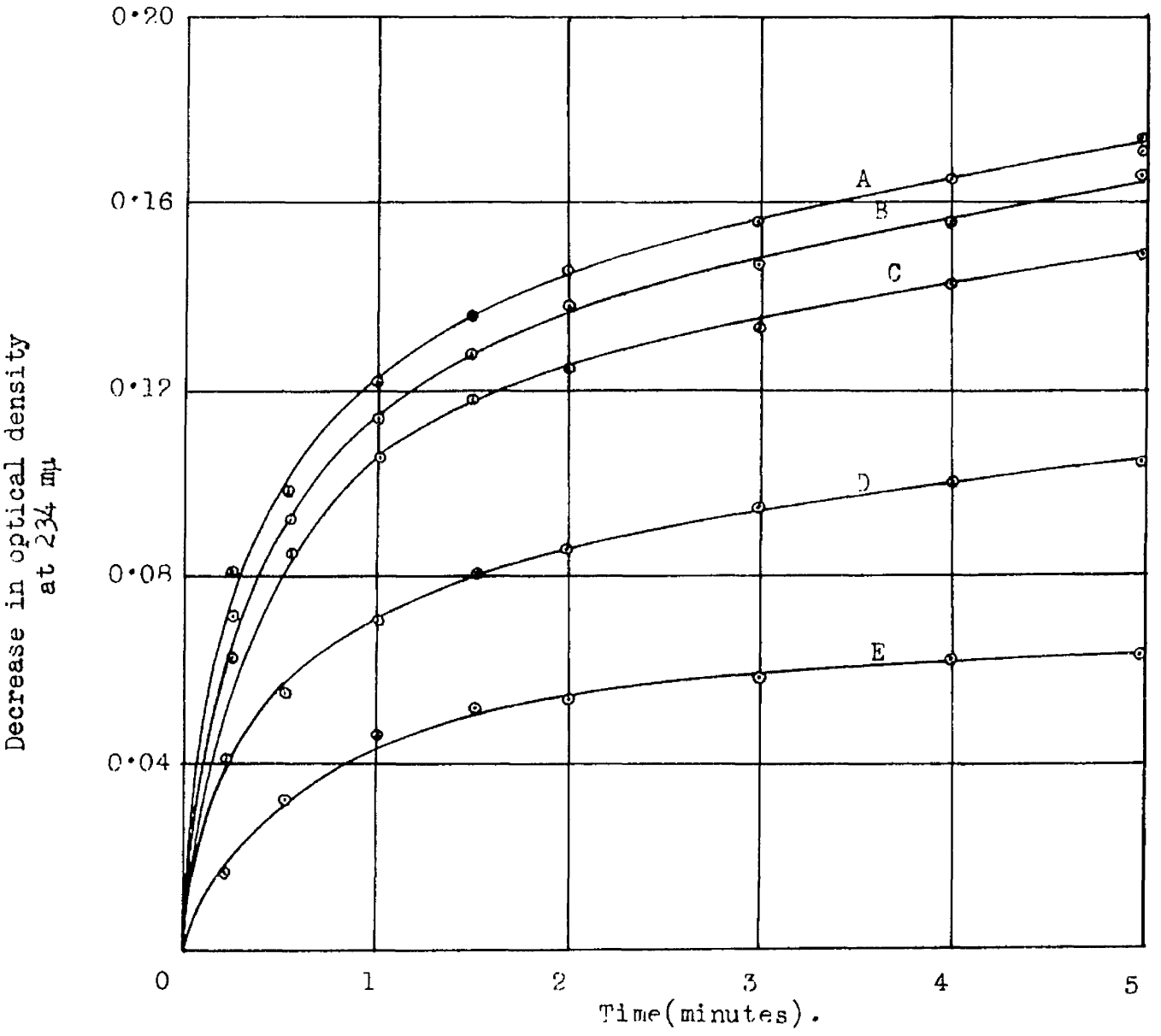


Fig. 18. - Diene Destruction/Time Curves at pH 9 with substrate containing variable quantities of preformed peroxide.

A - 30 x 10^{-6}M linoleate hydroperoxide.
B - 20 x 10^{-6}M
C - 14 x 10^{-6}M
D - 7·0 x 10^{-6}M
E - 3·5 x 10^{-6}M

Total linoleate concentration : 1·4 x 10^{-4}M.

Haemoglobin concentration : 2 x 10^{-6}M.

It was found impossible, owing to the complexity of the process of linoleate autoxidation, to conduct all the experiments with substrate containing equal quantities of preformed peroxide. Under the imposed reaction conditions, however, the experiments were found to be quantitatively reproducible and the results are therefore considered as giving a general pattern of behaviour of the haematin system.

The comparative activities of the various haematin compounds in equivalent concentrations are shown in Figure 17. The course of diene destruction was found to follow the same pattern for each haematin examined, namely, a rapid initial reaction followed by a subsequent decline in reaction rate.

The falling-off in the reaction rate appeared to be due to either peroxide or the haematin catalyst becoming used up.

Relative activities were observed to be in the order haemin > haemoglobin > catalase, in agreement with previous observations made by Tappel (69).

The influence of hydroperoxide concentration on the rate of the haemoglobin-catalysed reaction at pH 9 is shown in Figure 18. The five different sets of results were obtained with substrate solutions which had been exposed to the air for varying lengths of time. The rate of the haemoglobin-catalysed reaction, as clearly indicated, was found to increase with increasing hydroperoxide concentration up to a level of 14×10^{-6}M, above which

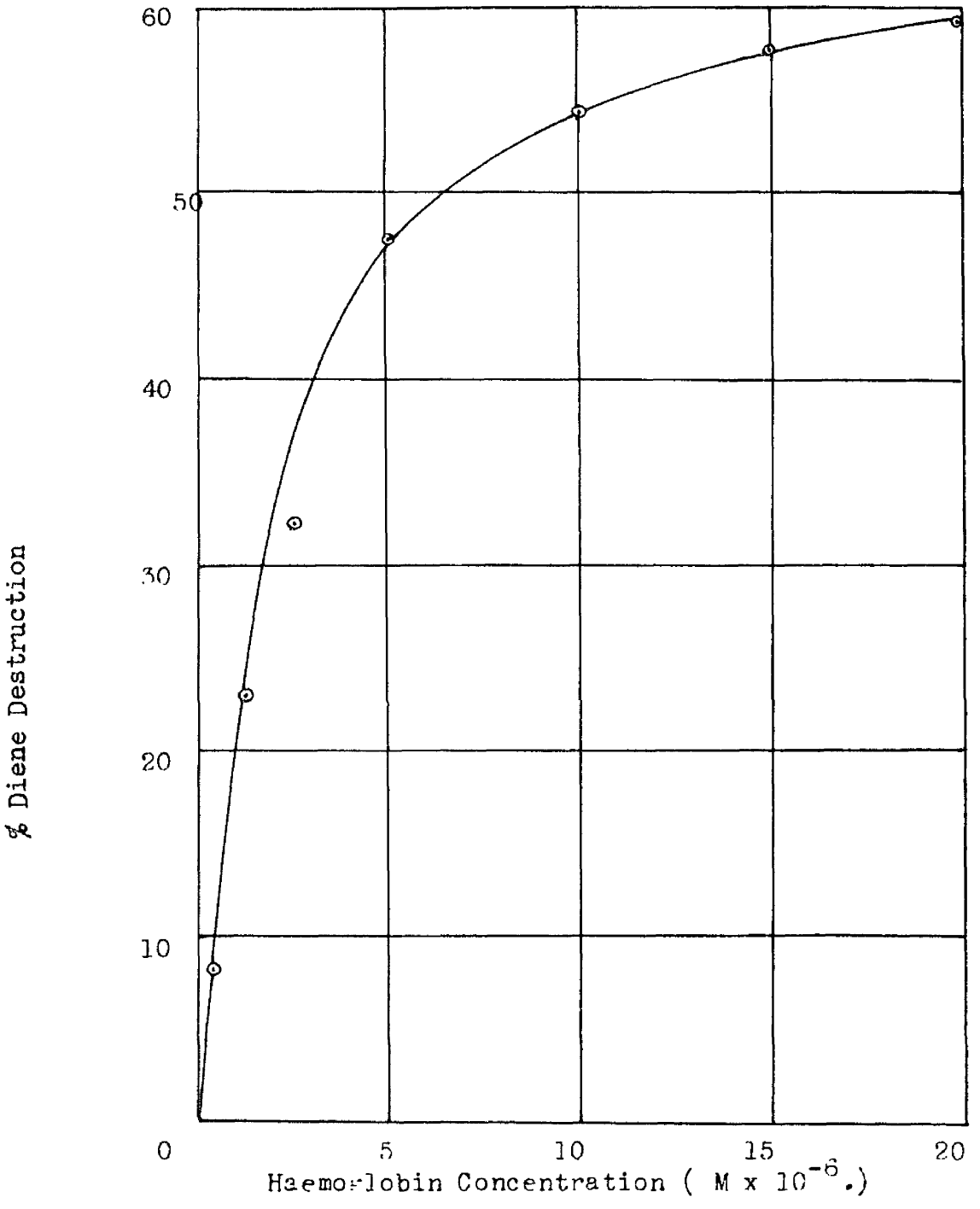


Fig. 19. - Effect of haemoglobin concentration on diene destruction at pH 9.

Total linoleate concentration : $1{\cdot}4 \times 10^{-4}$M, containing 16×10^{-6}M linoleate hydroperoxide.

Reaction time : 60 secs.

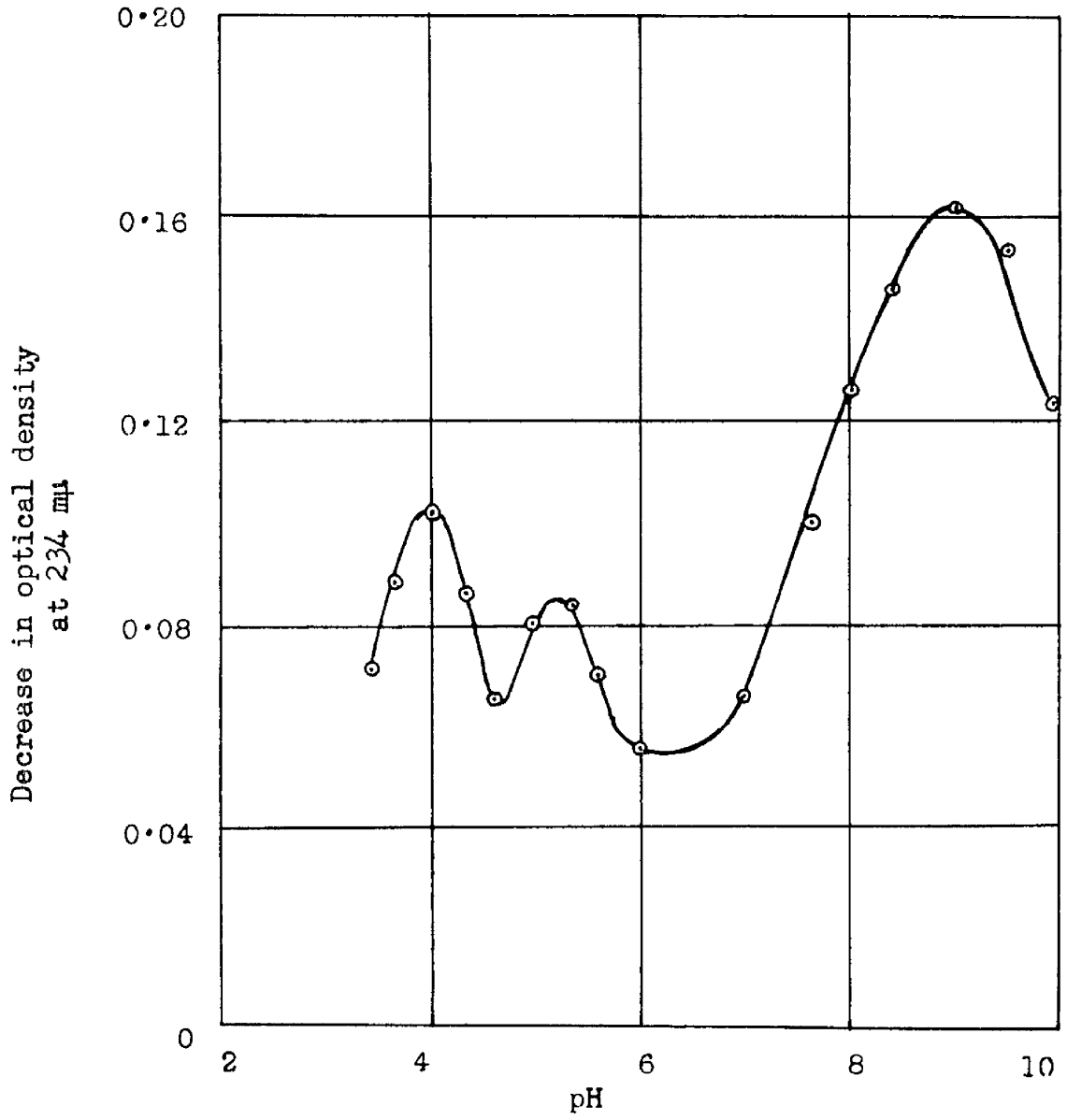


Fig. 20. - Effect of pH on the Haemoglobin-Catalysed Reaction.

Concentration of haemoglobin in the reaction system : 2×10^{-6}M.

Total linoleate concentration : $1{\cdot}4 \times 10^{-4}$M, containing 20×10^{-6}M linoleate hydroperoxide.

Reaction time : 60 secs.

a diminished response to hydroperoxide was observed.

Since the degree of action of the haematin compound was related to the degree of peroxidation of linoleate, it would appear that, in the spectrophotometric assay system, haematin compounds react only on linoleate hydroperoxide and not on linoleate, and that in this respect haematins differ from soya lipoxidase which reacts on linoleate forming hydroperoxide.

Similar to the mechanism described for haematin catalysis, linoleate oxidation catalysis by copper involves a decomposition of the linoleate peroxides (61). The unsaturated-fat oxidase activity of haematin compounds in the system used here would, therefore, appear to resemble the action of a metal catalyst than of an enzyme.

Figure 19 shows the effect of haemoglobin concentration on the initial rate of the reaction at pH 9. The rate of diene-destruction appeared to be directly proportional to haemoglobin concentration over a limited range.

The effect of pH on the haemoglobin-catalysed reaction is shown in Figure 20. The pH optimum for the system was observed to be pH 9. Above this level, however, catalytic activity remained fairly high. A sharp decrease in activity was noted below pH 8, the diene-destroying effect of the haematin being particularly weak around pH 7. Two smaller increases in activity were found at pH 4 and pH 5·4.

It is noteworthy that whereas maximum haematin activity in the adopted system was found at pH 9, previous investigators have described more acid pH optima as being characteristic of haematin activity (55, 107, 108).

2. Studies of Lipoxidase-Haematin Interaction .

Having established a pattern of behaviour for haematin in the adopted system , it was decided to examine the effect of haematin compounds on the lipoxidase-linoleate system .

The only previous observations on the behaviour of fatty systems containing both lipoxidase and haematin were made by Kies (30) and Tappel (62) . Using oxygen uptake as a measure of the course of the reaction , Kies found that crystalline haemin and cytochrome inhibit lipoxidase completely , whereas catalase , peroxidase and haemoglobin do not . No details of relative concentrations or experimental conditions used by Kies in her investigation were published , however .

Contrary to this observation of decreased catalysis , Tappel , who also used an oxygen uptake method of assay , reported that when crystalline haemin and lipoxidase are mixed, the catalysis is much greater than with lipoxidase alone . The figures recorded by Tappel on this topic are also limited .

The experiments in both these investigations , moreover , were apparently carried out with impure preparations of lipoxidase and the results must therefore be considered inconclusive .

T from pH 6 - 11

from pH 6 - 11

A number of mixtures of purified lipoxidase and pure haemoglobin were prepared by adding together aqueous solutions

of the two systems in such a manner that each combination contained the same level of lipoxidase but a different concentration of the haematin catalyst. This procedure was repeated with the other haematin compounds, haemin and catalase.

Using the spectrophotometric assay described in Appendix 1, and substrate substantially free from preformed peroxide, the relative activities of the lipoxidase-haematin mixtures were determined over the pH range 6 to 11 and at a linoleate concentration of $1 \cdot 4 \times 10^{-4}$M. The pH-activity curves of the mixtures were then compared with a curve obtained under similar conditions for a lipoxidase control solution.

From the results of the preceding section, it might be expected that the presence of haematin in the lipoxidase reaction system would result in the destruction of the conjugated linoleate hydroperoxides formed by lipoxidase and that this "inhibitory" effect would be particularly noticeable around pH 9.

That this is apparently the case may be seen from Figures 21 to 31 for, under the experimental conditions, haemoglobin, catalase and haemin, all, in increasing concentration, were found to diminish lipoxidase activity, particularly between pH 8 to 10. This effect was observed to cause a marked depression of the pH optimum of purified lipoxidase.

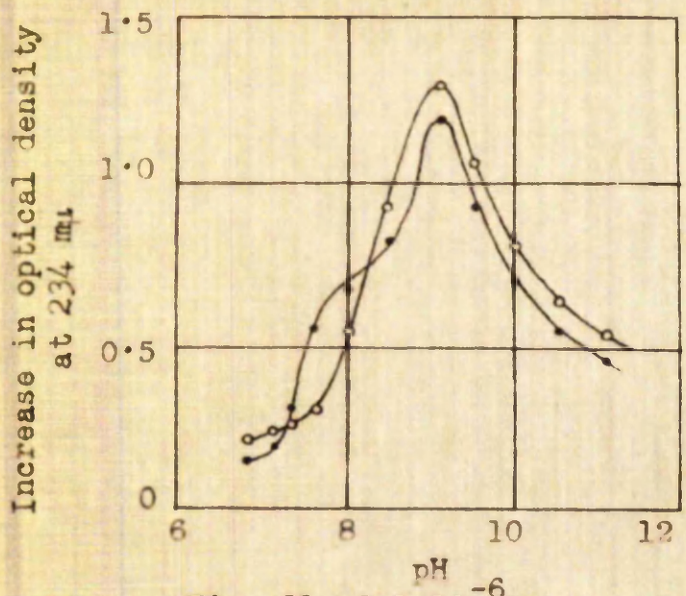


Fig. 21: $0{\cdot}1 \times 10^{-6}$ M haemoglobin.

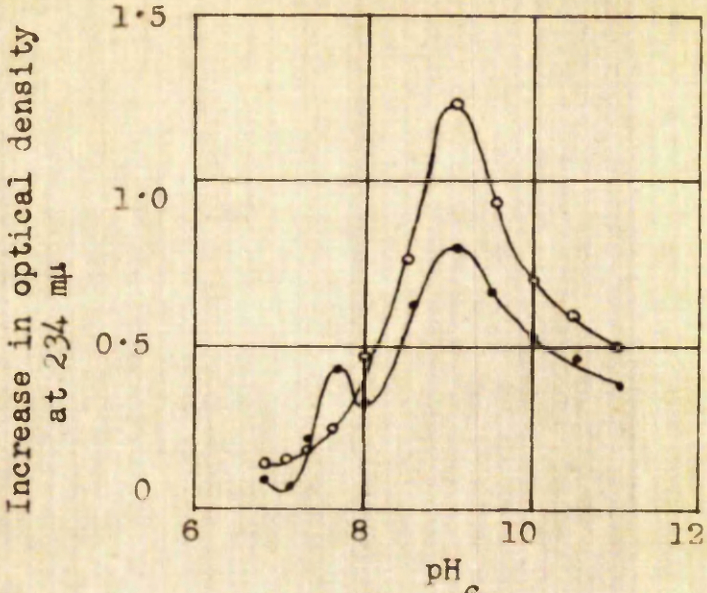


Fig. 22: 2×10^{-6} M haemoglobin.

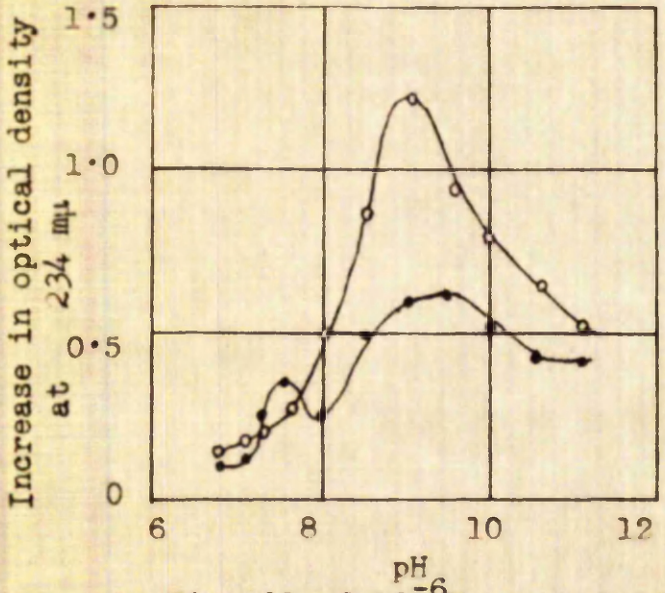


Fig. 23: 8×10^{-6} M haemoglobin.

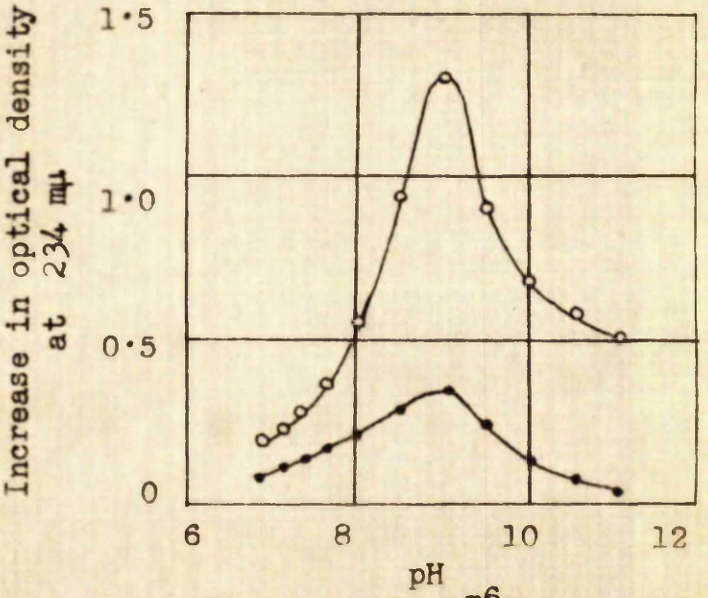


Fig. 24: 17×10^{-6} M haemoglobin.

Figs. 21-24. - Effect of Haemoglobin on the Lipoxidase-Linoleate System between pH 6 and pH 11.

—o—lipoxidase control (0·004 mg./ml.)
—•—lipoxidase+haemoglobin.

Sodium linoleate concentration: 1.4×10^{-4} M.
Reaction time: 60 secs.

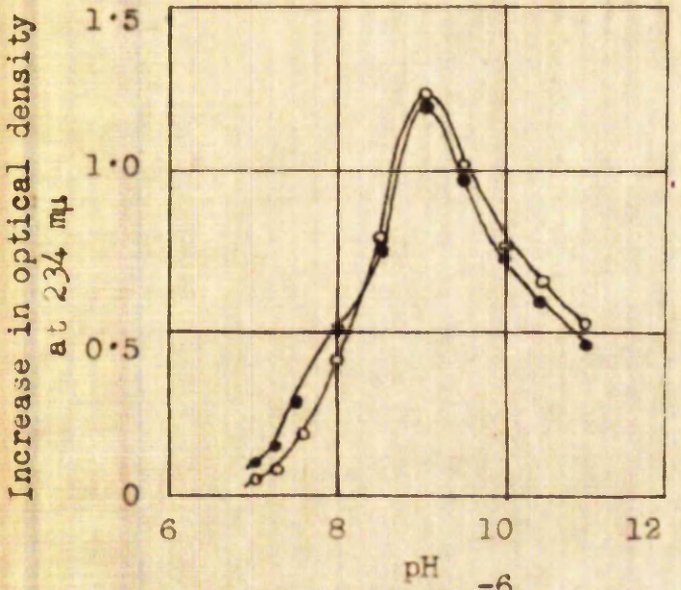


Fig. 25: $0{\cdot}025 \times 10^{-6}$M catalase.

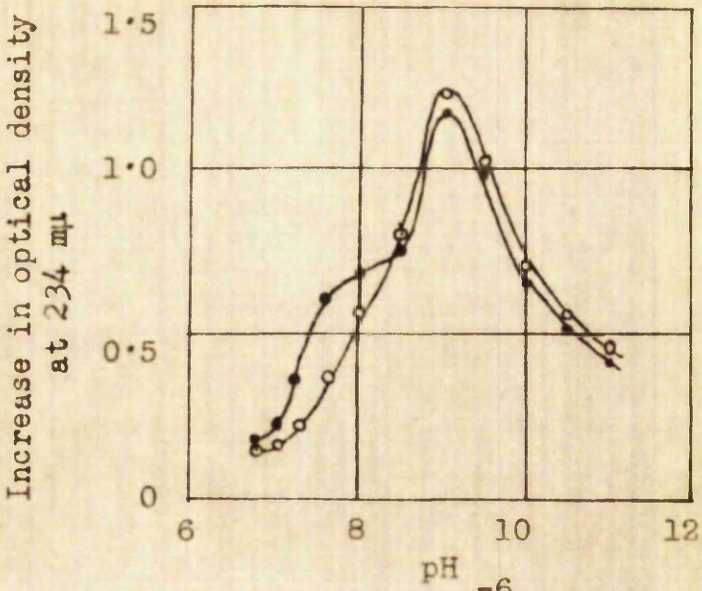


Fig. 26: $0{\cdot}25 \times 10^{-6}$M catalase.

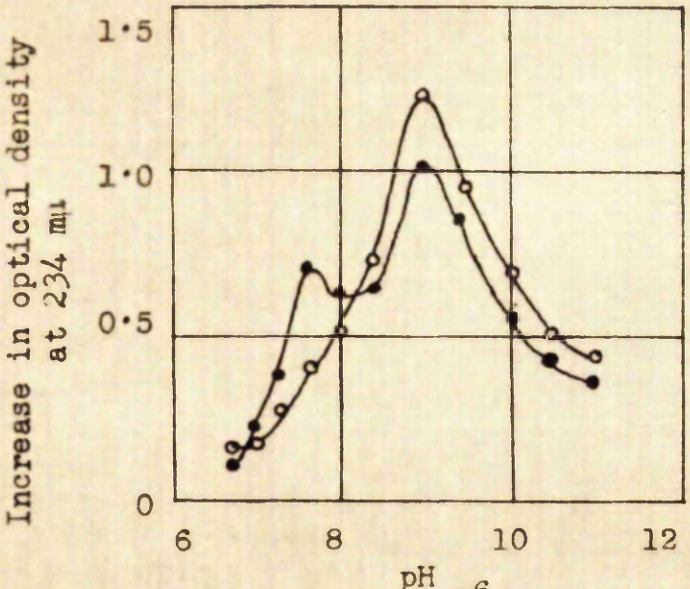


Fig. 27: $0{\cdot}75 \times 10^{-6}$M catalase

Figs. 25-27. - Effect of Catalase on the Lipoxidase-Linoleate System between pH 6 and pH 11.

—o— lipoxidase control (0·004 mg./ml.)
—•— lipoxidase + catalase.

Sodium linoleate concentration : $1{\cdot}4 \times 10^{-4}$M.
Reaction time : 60 secs.

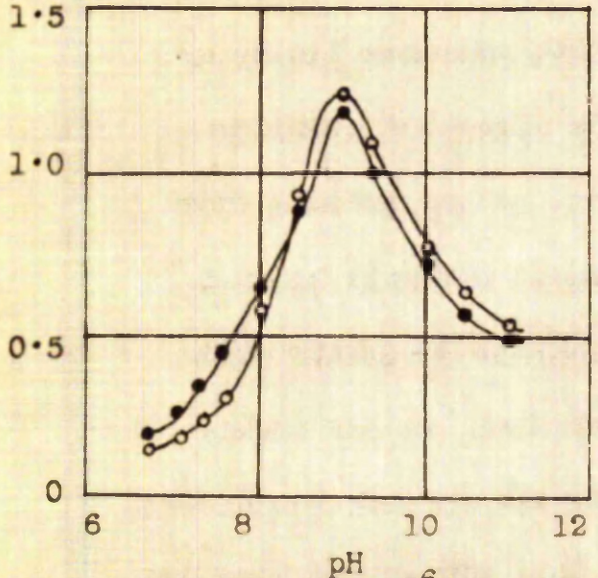


Fig. 28: $0{\cdot}025 \times 10^{-6}$ M haemin.

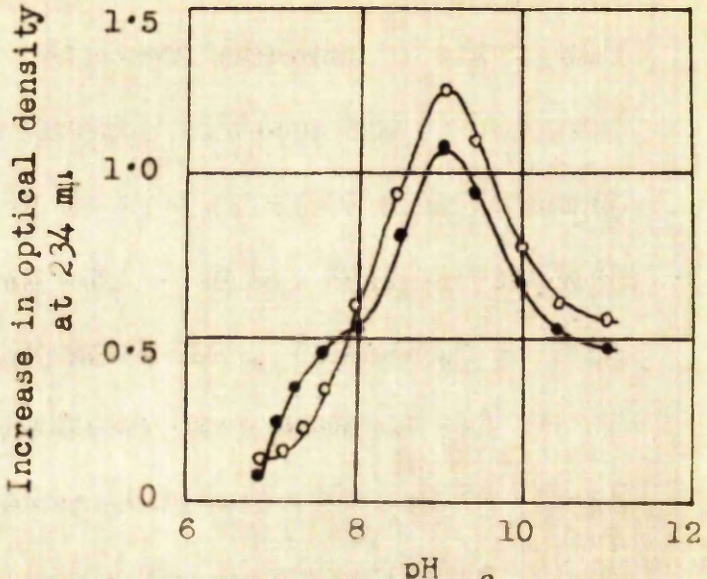


Fig. 29: $0{\cdot}1 \times 10^{-6}$ M haemin.

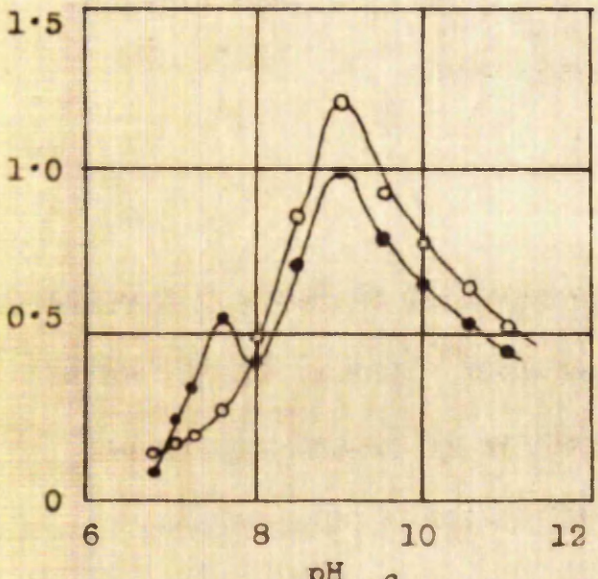


Fig. 30: $0{\cdot}5 \times 10^{-6}$ M haemin.

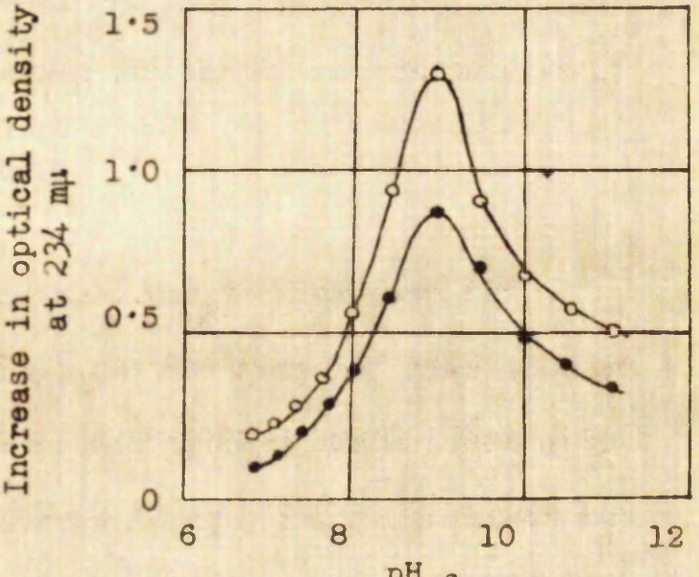


Fig. 31: 2×10^{-6} M haemin.

Figs. 28-31. - Effect of Haemin on the Lipoxidase-Linoleate System between pH 6 and pH 11.

—o—lipoxidase control (0·004 mg./ml.)
—•—lipoxidase + haemin.

Sodium linoleate concentration : $1{\cdot}4 \times 10^{-4}$ M.
Reaction time : 60 secs.

Between pH 7 to 8 , on the other hand, an anomalous increase in lipoxidase activity was observed, but only at the low levels of haematin examined . With narrower levels of haematin in the reaction system, this increased activity appeared as a definite peak at pH 7·6, as may be seen from Figures 22,23,27 and 30 . The increased activity between pH 7 to 8, however , was found to diminish gradually with increasing haematin concentration, so that, at the higher levels of haematin examined, considerable overall inhibition of lipoxidase activity was noted . This effect was observed to be most marked in the presence of 17 x 10^{-6}M haemoglobin, haematin action, as shown in Figure 24 , causing almost complete flattening of the lipoxidase pH-activity curve .

The behaviour of the lipoxidase-haematin mixtures indicates clearly that the expected interactions take place . On the other hand, using diene conjugation as a measure of reaction progress and considering the general concept of haematin compounds as destroyers of diene, the overall pattern is unexpected .

It is worth noting at this point that the irregular increase in diene, in the presence of small amounts of haematin, should occur at a region of pH where haematin had previously been shown to have a very low diene-destroying activity .

The experimental results show that the different effects of

haematin on the lipoxidase system can, in response to pH, produce a pH-activity curve having two pH maxima, as may be seen from Figures 22, 25, 27 and 30. That the addition of haematin to the lipoxidase reaction system should result in a pH curve with maxima at pH 7·6 and pH 9 was of considerable interest since, as mentioned earlier in this thesis, crude soya extracts, under almost identical conditions, had been observed to catalyse linoleate oxidation with two similar pH maxima. It should be borne in mind, however, that the relative heights of the two pH maxima were found to vary with crude extracts made from soya from different sources, so that, in certain cases, the activity at pH 7.6 was higher than at pH 9.

If, it was considered, the relative heights of the two pH maxima of a lipoxidase-haematin mixture could be so altered, support might be given to the explanation of soya extract double-maximal pH-activity curves postulating lipoxidase-haematin interaction. It was, therefore, decided that an examination should be made of the effects on lipoxidase-haematin interaction at the two pH maxima of such variables as reaction time, linoleate concentration and enzyme concentration. Such a study might provide information as to the nature of the interaction at the lower pH maxima, observed to result in increased formation of conjugated diene.

Although haemoglobin was chosen for this series of

experiments on lipoxidase-haematin interaction , it should be pointed out that the action of a selected haematin protein may be regarded as typical of the others , since haematin catalysis has been shown to be dependent on the haematin moiety modified to some extent by the protein to which it is combined (68,69) .

At the outset of these experiments on lipoxidase-haemoglobin interaction , it became clear that the haematin catalyst was being decolourized during the course of the lipoxidase reaction . A closer examination of this effect seemed justified since it was considered that destruction of the haemoglobin in the lipoxidase reaction system might significantly influence the interaction of the two systems .

Destruction of the Haemoglobin in the Lipoxidase-Linoleate System.

The coupled oxidation of haemoglobin and haemin has been shown to occur in haematin-catalysed (54,68) and in lipoxidase-catalysed oxidation of linoleate (30) . These co-oxidations are apparently caused by the interaction of linoleate peroxide radicals and the labile substances (69) .

In order to measure the destruction of haemoglobin in the lipoxidase-linoleate system , a suitable method of assay was required . After several attempts to achieve a workable

method, a modification of the procedure used by Tappel (68) was chosen. The adopted procedure involved measurement of haemoglobin breakdown by two methods,

(a) by following, spectrophotometrically, the disappearance of haemoglobin as acid methaemoglobin, and

(b) by the simultaneous determination of the inorganic iron released as a result of haemoglobin destruction.

The procedure, which is described in detail in Appendix II, although essentially clumsy, gave results which were found to be reproducible. It was found necessary to use a fairly high concentration of haemoglobin in the reaction system in order to ensure that the low levels of iron released during the initial stages of the reaction could be measured with sufficient accuracy.

Using the procedure detailed in Appendix II, the destruction of haemoglobin in the lipoxidase reaction system was determined at pH 9 and at pH 7·6. The initial level of haemoglobin in the system was 10×10^{-6}M and the relative concentrations of lipoxidase and sodium linoleate were 0.004 mg/ml. and $1{\cdot}4 \times 10^{-4}$M, respectively. The results, which are shown in

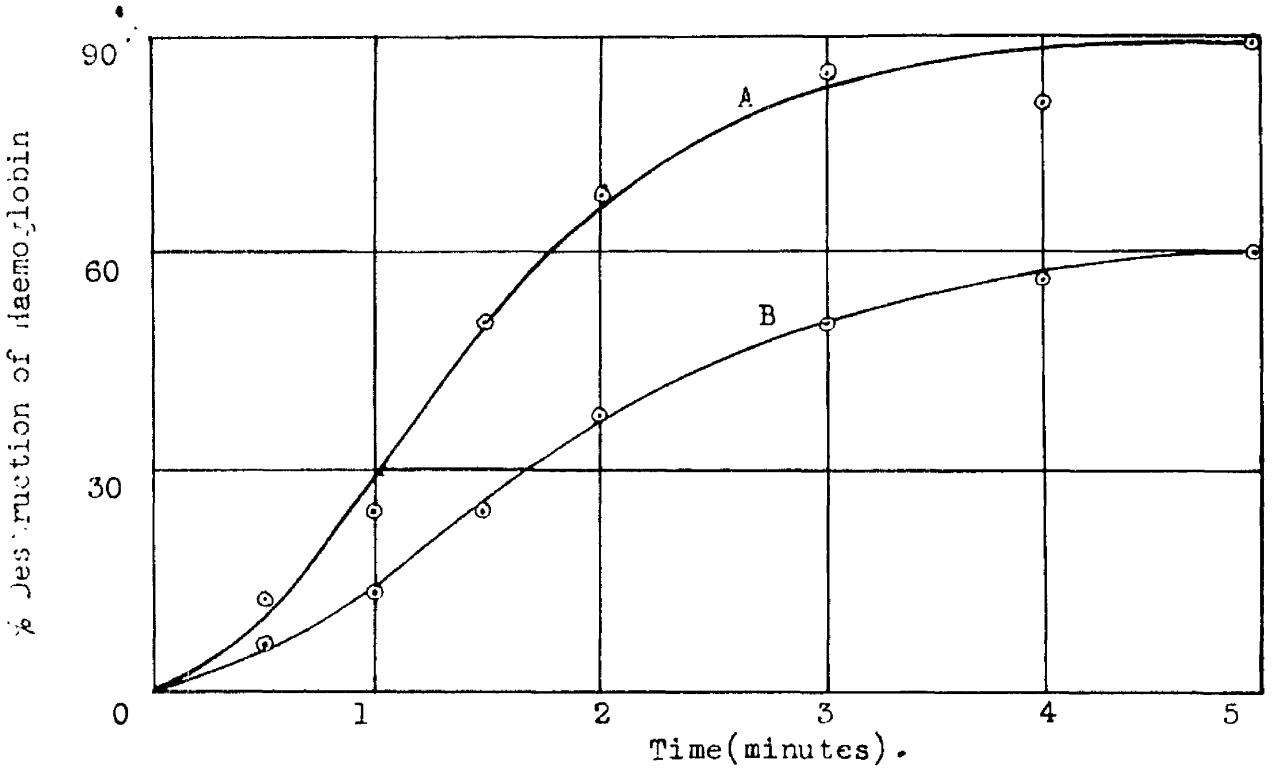


Fig. 32. - The coupled oxidation of haemoglobin in the lipoxidase-linoleate system at pH 9.

A - Disappearance of acid methaemoglobin at 498 mμ.

B - Release of inorganic iron.

Initial level of haemoglobin : 10×10^{-6}M.

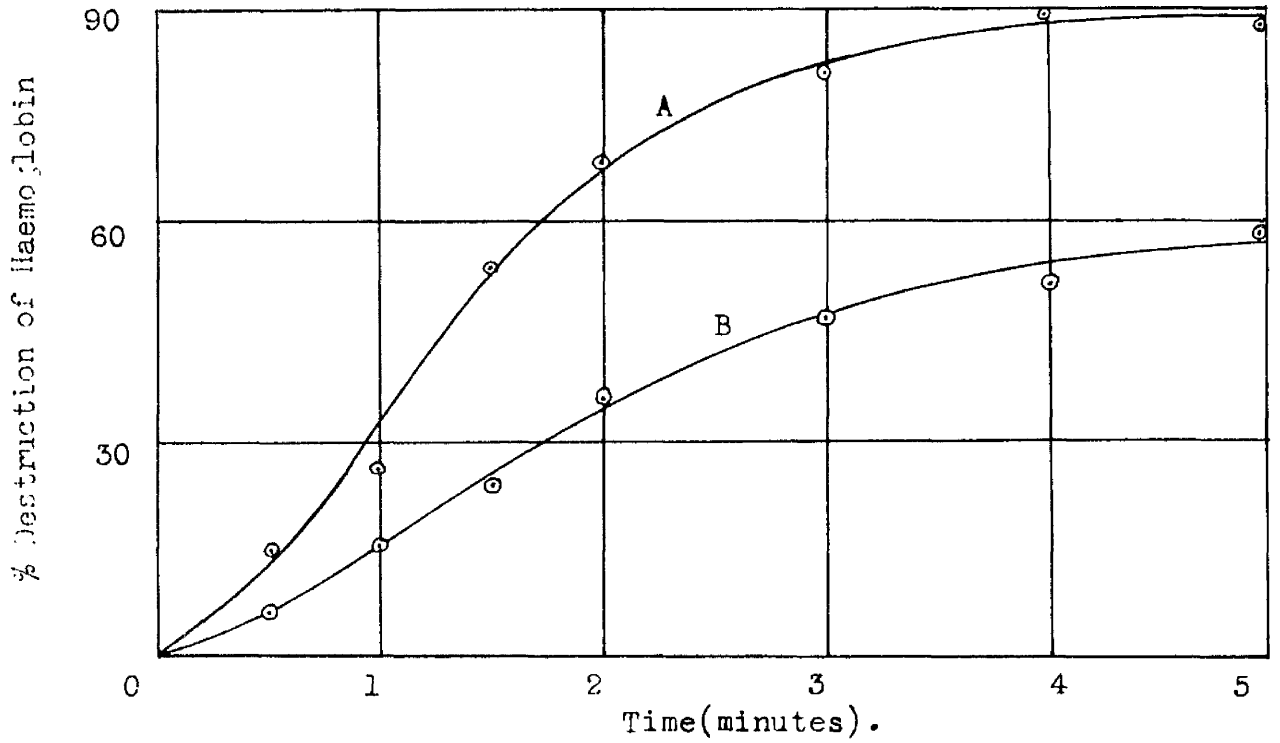


Fig. 33. - The coupled oxidation of haemoglobin in the lipoxidase-linoleate system at pH 7·6.

A - Disappearance of acid methaemoglobin at 498 mμ.

B - Release of inorganic iron.

Initial level of haemoglobin : 10×10^{-6}M.

Figures 32 and 33, are typical of those obtained from repeated experiments.

The pattern of destruction of haemoglobin at both pH levels was found to be almost identical. Determination of haemoglobin as acid methaemoglobin indicated a rapid loss of the haemoglobin molecule. The simultaneous determination of the iron ions released showed that the decrease in haemoglobin could only be partly accounted for by the iron set free.

Having established that haemoglobin is decomposed in the lipoxidase reaction system, studies of lipoxidase-haemoglobin interaction under a variety of conditions were continued.

Effect of Time on Lipoxidase-Haemoglobin Interaction at pH 9 and pH 7.6.

Using the lipoxidase assay procedure, the activities of lipoxidase-haemoglobin mixtures were determined over periods of one minute and longer at pH 9 and at pH 7·6. This procedure was repeated, under identical conditions, for a lipoxidase control solution. The level of sodium linoleate employed in these experiments was $1{\cdot}4 \times 10^{-4}$M.

Figure 34 shows the effect of 2×10^{-6}M haemoglobin on the rate of the lipoxidase reaction at pH 9. The expected diminishing effect of haemoglobin on diene conjugation was noticeable only during the initial phases, for at the later stages of the reaction, the haematin catalyst appeared to have

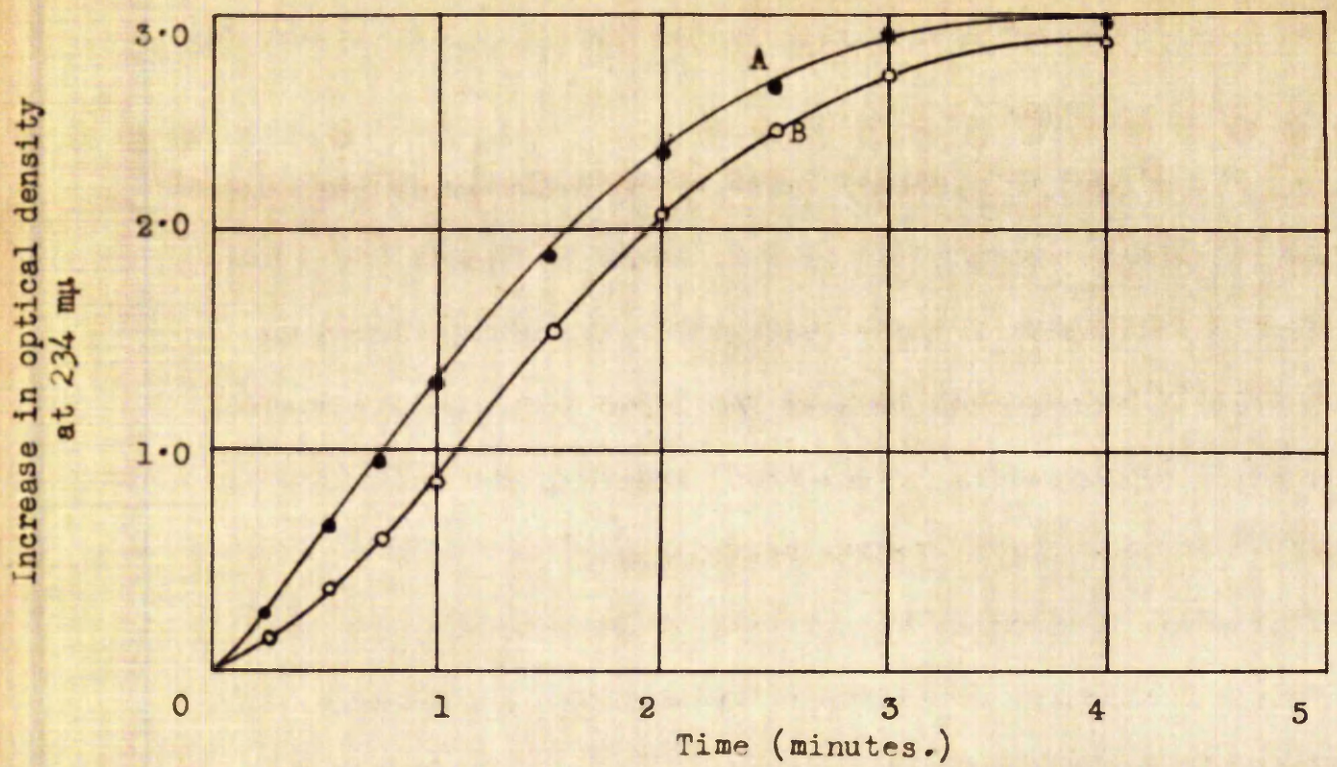


Fig. 34. - Effect of haemoglobin on the rate of the lipoxidase reaction at pH 9.

A - Lipoxidase control (0·004 mg./ml.)

B - Lipoxidase + 2 x 10^{-6}M haemoglobin.

Sodium linoleate level : 1·4 x 10^{-4}M.

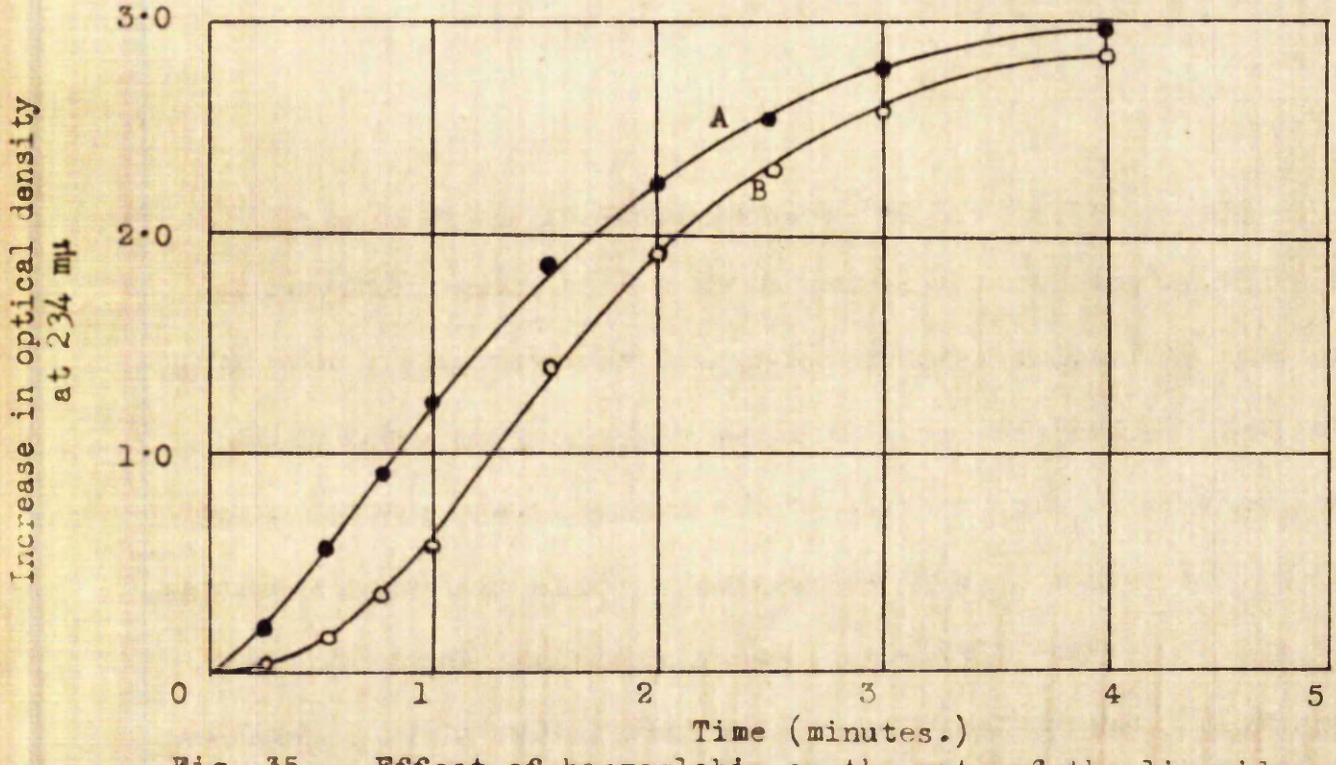


Fig. 35. - Effect of haemoglobin on the rate of the lipoxidase reaction at pH 9.

A - Lipoxidase control (0·004 mg./ml.)

B - Lipoxidase + 10 x 10^{-6}M.

Sodium linoleate level : 1·4 x 10^{-4}M.

little or no effect. As reference to Figure 32 will indicate, the destruction of haemoglobin in the system would account for this loss in haematin action.

The effect of a higher level of haemoglobin on the rate of the lipoxidase reaction at pH 9 is shown in Figure 35. The overall inhibition of diene conjugation was more evident at 10 x 10^{-6}M haemoglobin than at the lower level in the system, as would be expected. Once more, however, the "inhibitory" effect of haemoglobin became increasingly less as the reaction progressed, presumably due to loss of haemoglobin from the system. Although breakdown of haemoglobin was clearly taking place, it would appear that at the later stages of the reaction there was still sufficient haemoglobin present to cause a noticeable decrease in diene conjugation.

The effect of 2 x 10^{-6}M haemoglobin on the rate of the lipoxidase-catalysed reaction at pH 7·6 is shown in Figure 36. At this pH level haemoglobin appeared to cause only a very slight, initial "inhibition" of lipoxidase action, after which diene conjugation in the presence of the haematin catalyst increased to a rate beyond that of the control. This increased formation of diene was most distinct at reaction periods exceeding three minutes. Taking into account the information already obtained

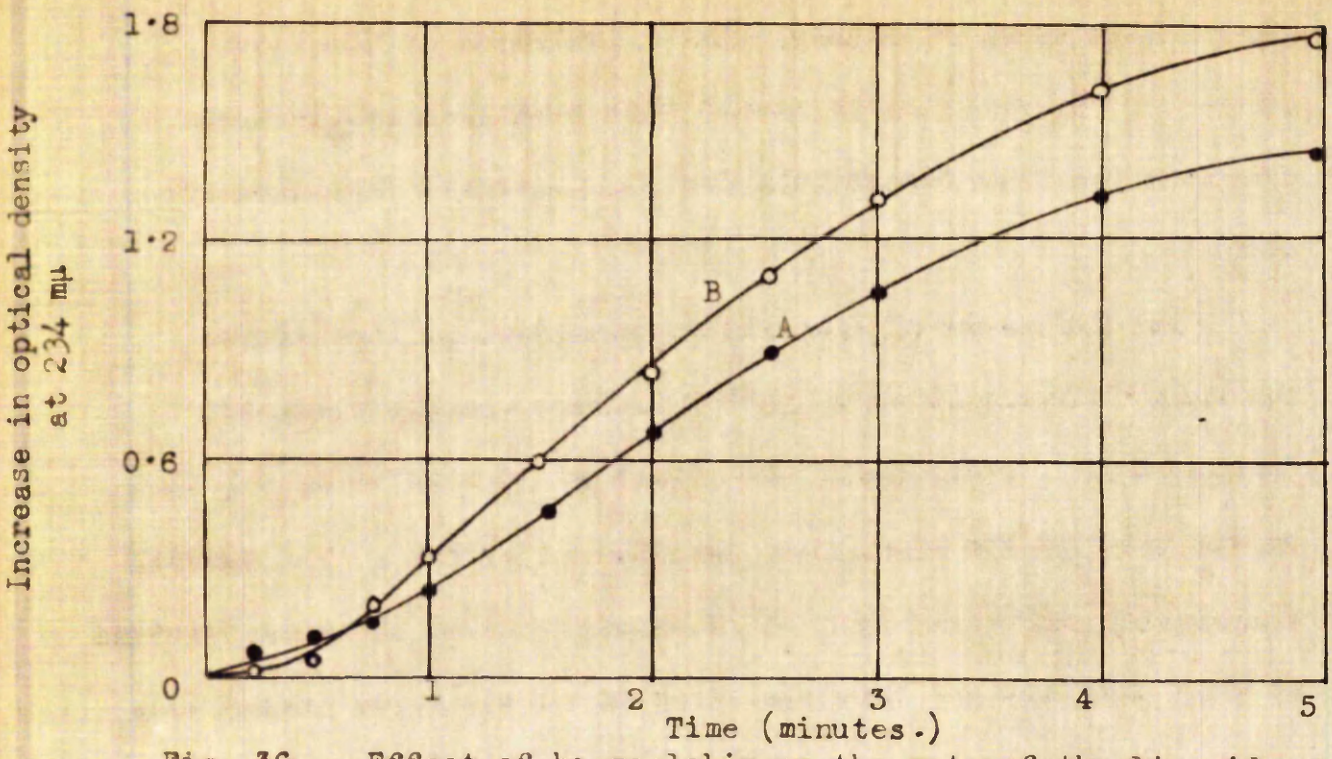


Fig. 36. - Effect of haemoglobin on the rate of the lipoxidase reaction at pH 7·6.

A - Lipoxidase control (0·004 mg./ml.)

B - Lipoxidase + 2 x 10^{-6} M haemoglobin.

Sodium linoleate level : 1·4 x 10^{-4} M.

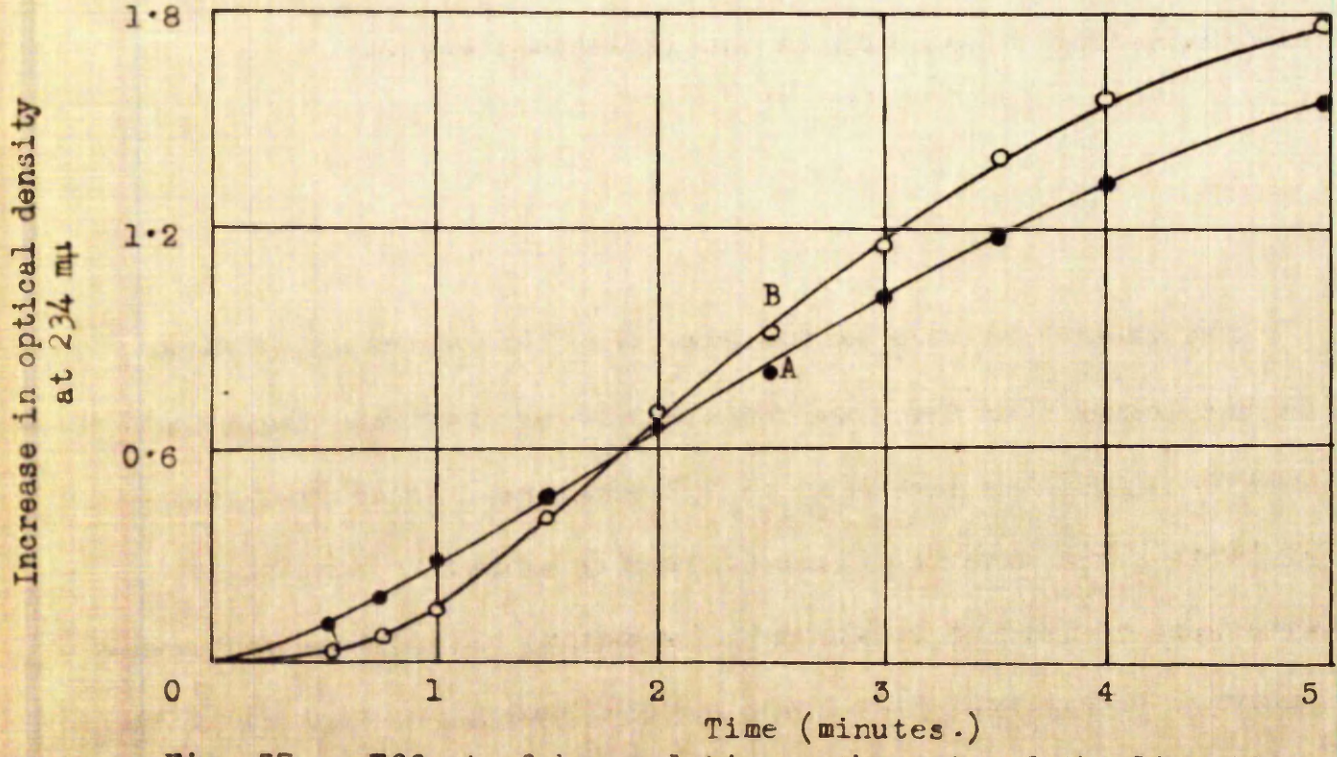


Fig. 37. - Effect of haemoglobin on the rate of the lipoxidase reaction at pH 7·6.

A - Lipoxidase control (0·004 mg./ml.)

B - Lipoxidase + 10x10^{-6} M haemoglobin.

Sodium linoleate level : 1·4 x 10^{-4} M.

that a high level of haemoglobin is destroyed at the later stages of the reaction it seemed that haemoglobin-breakdown products might be responsible for the increased formation of diene .

The influence of haemoglobin-breakdown on lipoxidase-haemoglobin interaction at pH 7·6 is more clearly indicated in Figure 37 , which shows the effect of 10 x 10^{-6}M haemoglobin on the rate of the lipoxidase reaction at pH 7·6 . The initial diminishing effect by 10 x 10^{-6}M haemoglobin on diene conjugation is much more obvious than was observed at the lower haemoglobin level. Again , however , this effect rapidly lessened as , apparently , the haemoglobin was being destroyed , and after two minutes a marked increase in diene was noted , which , as reference to Figure 33 will show , was concurrent with a high degree of destruction of haemoglobin in the lipoxidase system .

The general pattern of the results of the above experiments is consistent with the observations made earlier that haematin retards lipoxidase action at pH 9 but augments it at the lower pH level , and that the diene-destroying action of haematin increases at both pH levels with increasing haematin concentration . Although it appeared from these results that lipoxidase at pH 7·6 is a more effective catalyst for diene conjugation in the presence

of haemoglobin, the activities of the lipoxidase-haemoglobin mixtures at this pH level did not exceed those observed at pH 9 over the reaction periods employed.

The reaction rate curves shown in Figures 36 and 37, taken together with the data on the destruction of haemoglobin in the system, provide evidence to suggest that the increase in diene observed on addition of haemoglobin to the lipoxidase system at pH 7·6, is caused by some intermediary product of haemoglobin-breakdown catalysing the formation of conjugated diene.

On the other hand, this concept does not, at first, seem entirely adequate in that the increased formation of diene was not observed, under identical conditions, at pH 9, and at which pH level destruction of the haematin catalyst had been shown to occur to almost the same extent as at pH 7·6.

However, taking into consideration the previously obtained information that the pH optimum for the diene-destroying action of haematin in the adopted system is pH 9, the above hypothesis still appears tenable, since it is conceivable that the failure to observe the stimulated increase in diene at pH 9 was the result of unaffected haemoglobin in the system simultaneously counteracting the action of the breakdown product.

In keeping with this line of argument, the apparent ineffectiveness of the intact haemoglobin in the system at pH 7·6 may be explained by the fact that, as indicated earlier in this thesis, the diene-destroying action of the haematin

catalyst at this pH level is very weak , which effect would not , therefore , be expected to be great enough to hinder formation of diene promoted by the haemoglobin-breakdown product . Moreover , since lipoxidase at pH 7.6 is far removed from its pH optimum , it seems not unlikely that the stimulation effect of the haemoglobin-breakdown product would , therefore , become more manifest at this pH level .

Assuming that low levels of the haematin catalysts in the system are effectively destroyed within reaction intervals of one minute , the above considerations would account adequately for the observations already made in this section on lipoxidase-haematin interaction at several pH levels and at the same concentration of linoleate , but over the shorter reaction periods .

Effect of Linoleate Concentration on Lipoxidase-Haemoglobin Interaction .

The previous experiments had been carried out at a linoleate concentration of $1{\cdot}4 \times 10^{-4}$M . The effect of haemoglobin on the courses of the lipoxidase reactions at pH 9 and at pH 7·6 was examined at two other levels of linoleate , viz.,$0{\cdot}35 \times 10^{-4}$M and $2{\cdot}8 \times 10^{-4}$M. The relative concentrations of lipoxidase and haemoglobin used were , respectively ,0·004 mg.per ml. and 2×10^{-6}M.

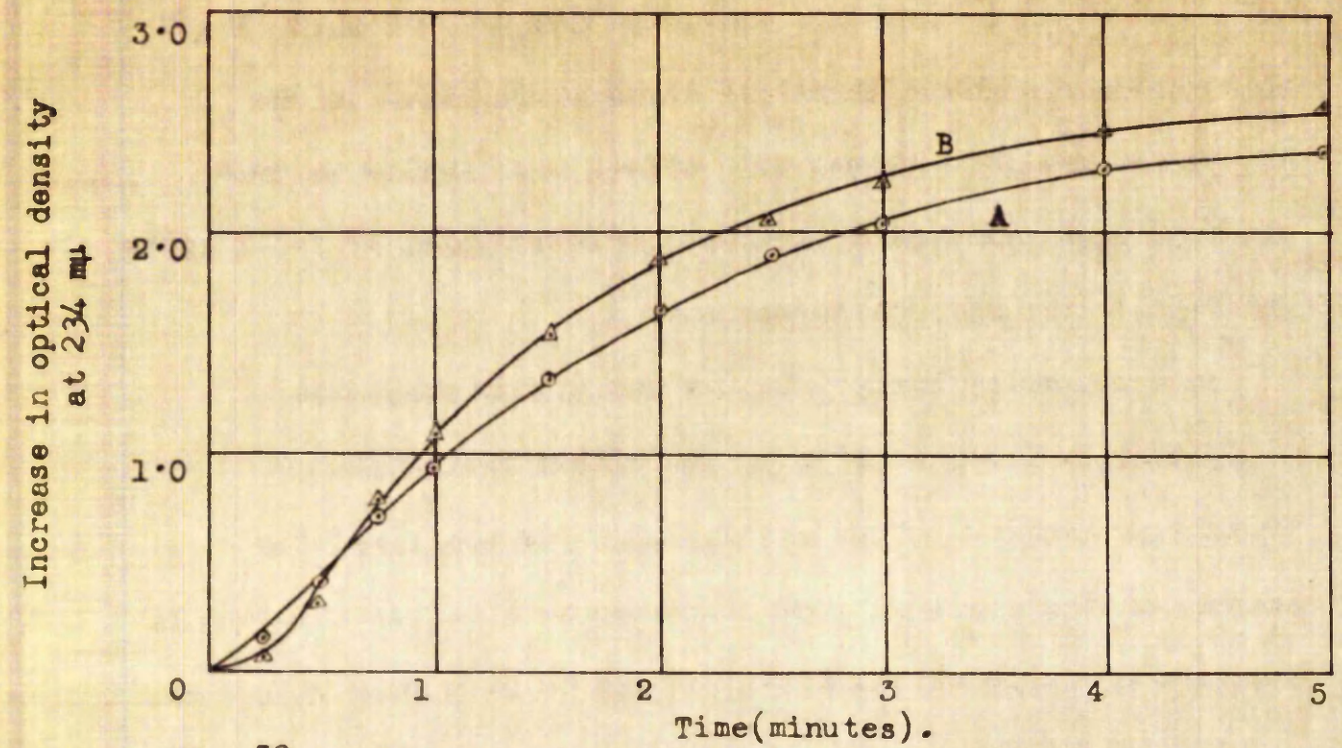


Fig. 38 : - Effect of haemoglobin on the rate of the lipoxidase reaction at pH 9, at a linoleate level of $2 \cdot 8 \times 10^{-4}$M.
A - Lipoxidase control (0·004 mg./ml.)
B - Lipoxidase + 2×10^{-6}M haemoglobin.

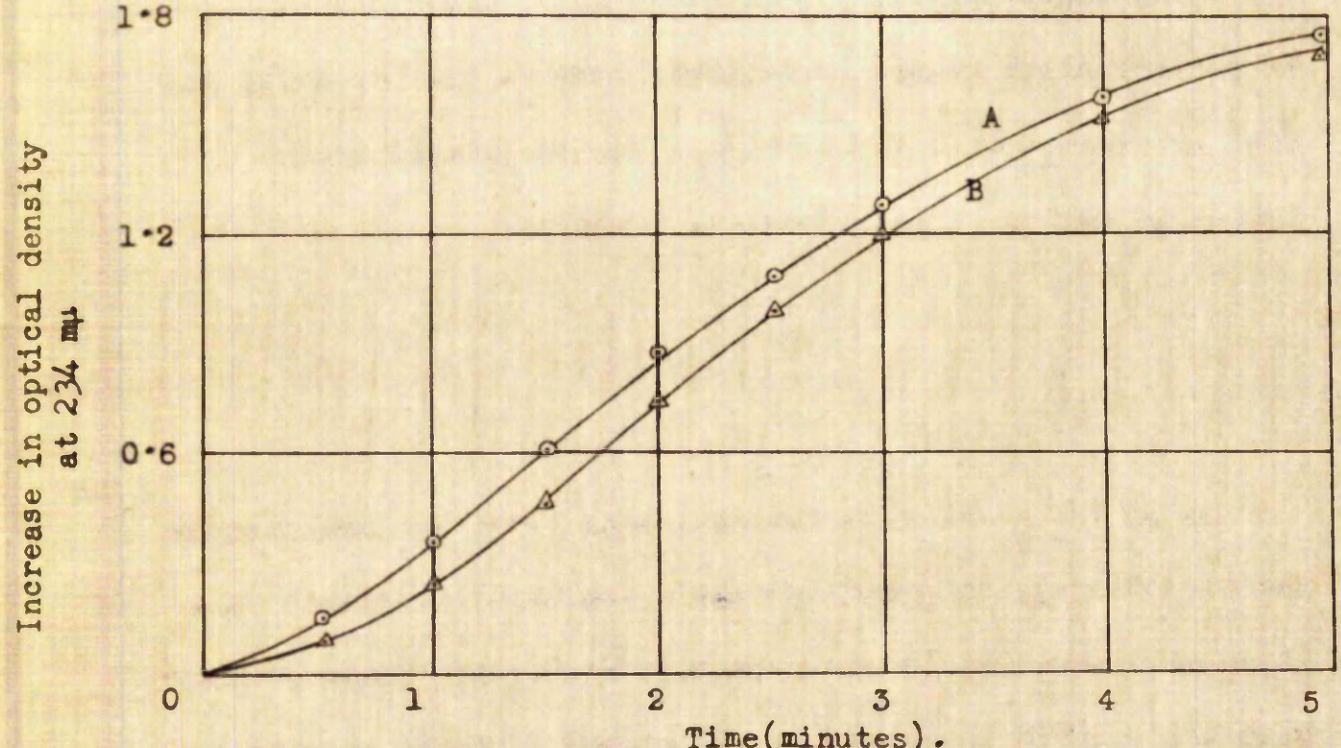


Fig. 39 : - Effect of haemoglobin on the rate of the lipoxidase reaction at pH 7·6, at a linoleate level of $0 \cdot 35 \times 10^{-4}$M.
A - Lipoxidase control (0·004 mg./ml.)
B - Lipoxidase + 2×10^{-6}M haemoglobin.

At pH 9 , at a linoleate concentration of $0 \cdot 35 \times 10^{-4}$M , haemoglobin was found to retard diene conjugation in the lipoxidase system . Since this effect was similar to that observed previously at a linoleate concentration of $1 \cdot 4 \times 10^{-4}$M, the results are not reproduced here .

At the same pH level , but at the higher substrate concentration ($2 \cdot 8 \times 10^{-4}$M), the slight inhibition of lipoxidase action , which was observed for the first few seconds of the reaction , was followed by a marked increase in the rate of diene conjugation , as may be seen from Figure 38.

This observation seemed to reflect the more complete destruction of the haemoglobin in the system at the higher substrate concentration , that is to say , there did not appear to be sufficient intact haemoglobin left in the system at the high substrate level to counteract the postulated diene-producing action of its breakdown products.

At pH 7·6 , and with the same high level of linoleate in the reaction system , haemoglobin appeared to accelerate the lipoxidase reaction in a manner similar to that found earlier with $1 \cdot 4 \times 10^{-4}$M linoleate in the system . These results are also , therefore , not reproduced here.

When the linoleate concentration in the system at pH 7·6

was reduced to $0 \cdot 35 \times 10^{-4}$M, haemoglobin, as shown in Figure 39, was found to exert a slight but noticeable inhibition of diene conjugation.

These observations made at pH 7·6 would appear to support the suggestion already implied by the data obtained at pH 9, that haemoglobin is more effectively destroyed in the lipoxidase system at high linoleate concentrations.

While the results of the above experiments indicated the behaviour of haemoglobin in the lipoxidase-linoleate system to be dependent on the level of linoleate in the system, the response to linoleate concentration appeared to be influenced somewhat by the pH of the reaction system.

To obtain further information concerning the influence of linoleate concentration on the interaction of the two systems, a closer examination of the substrate-activity relationships of lipoxidase-haematin mixtures was undertaken.

Under the usual conditions, substrate-activity curves were plotted for a lipoxidase-haemoglobin mixture at pH 9 and at pH 7·6. This procedure was repeated with a lipoxidase control solution.

The results, which are shown in Figures 40 and 41, indicate that linoleate concentration influences the interaction of

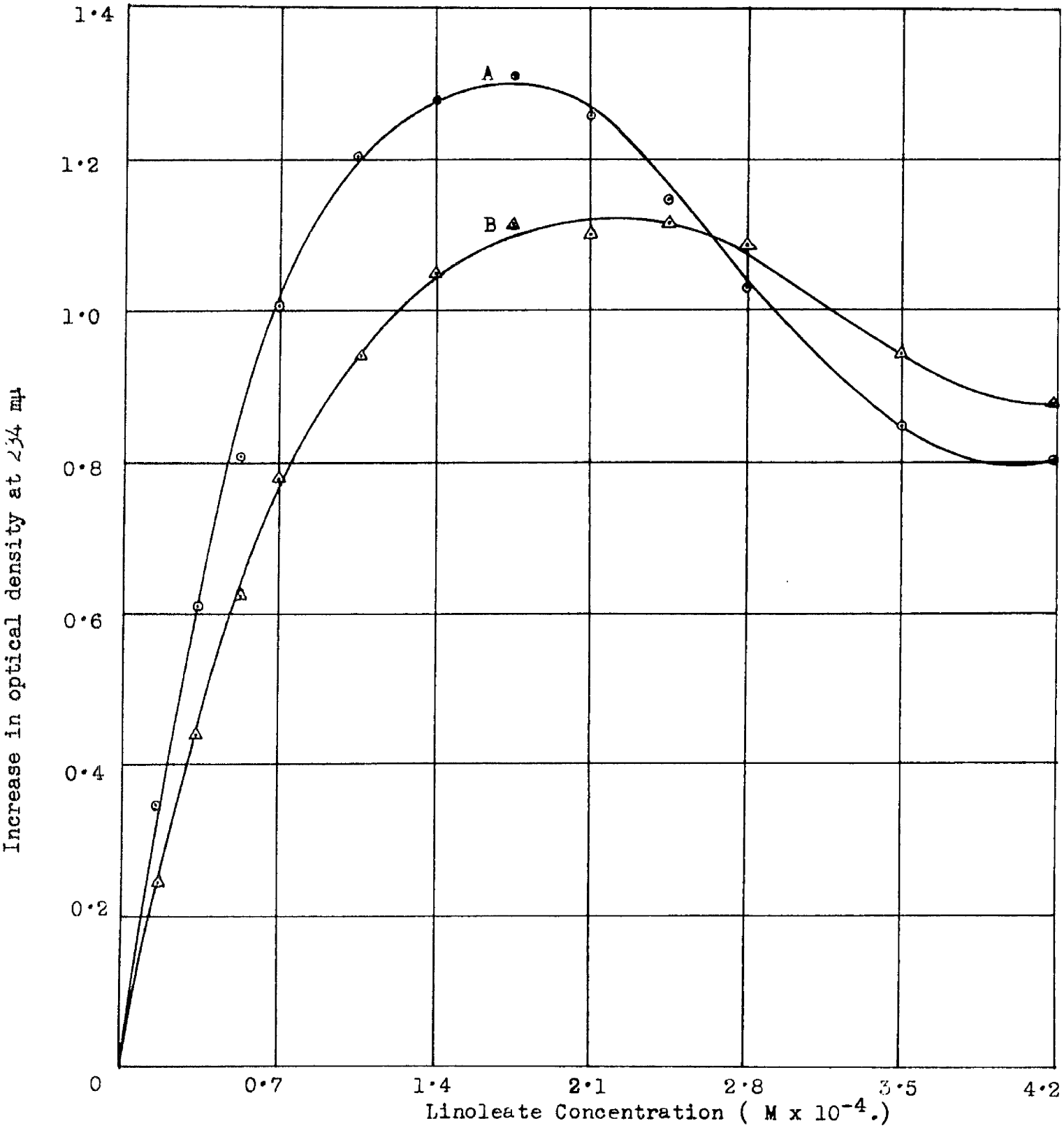


Fig. 40. - Effect of haemoglobin on lipoxidase activity at pH 9 at various levels of linoleate.

A - Lipoxidase control (0·004 mg./ml.).
B - Lipoxidase + 2×10^{-6}M haemoglobin.

Reaction time : 60 seconds.

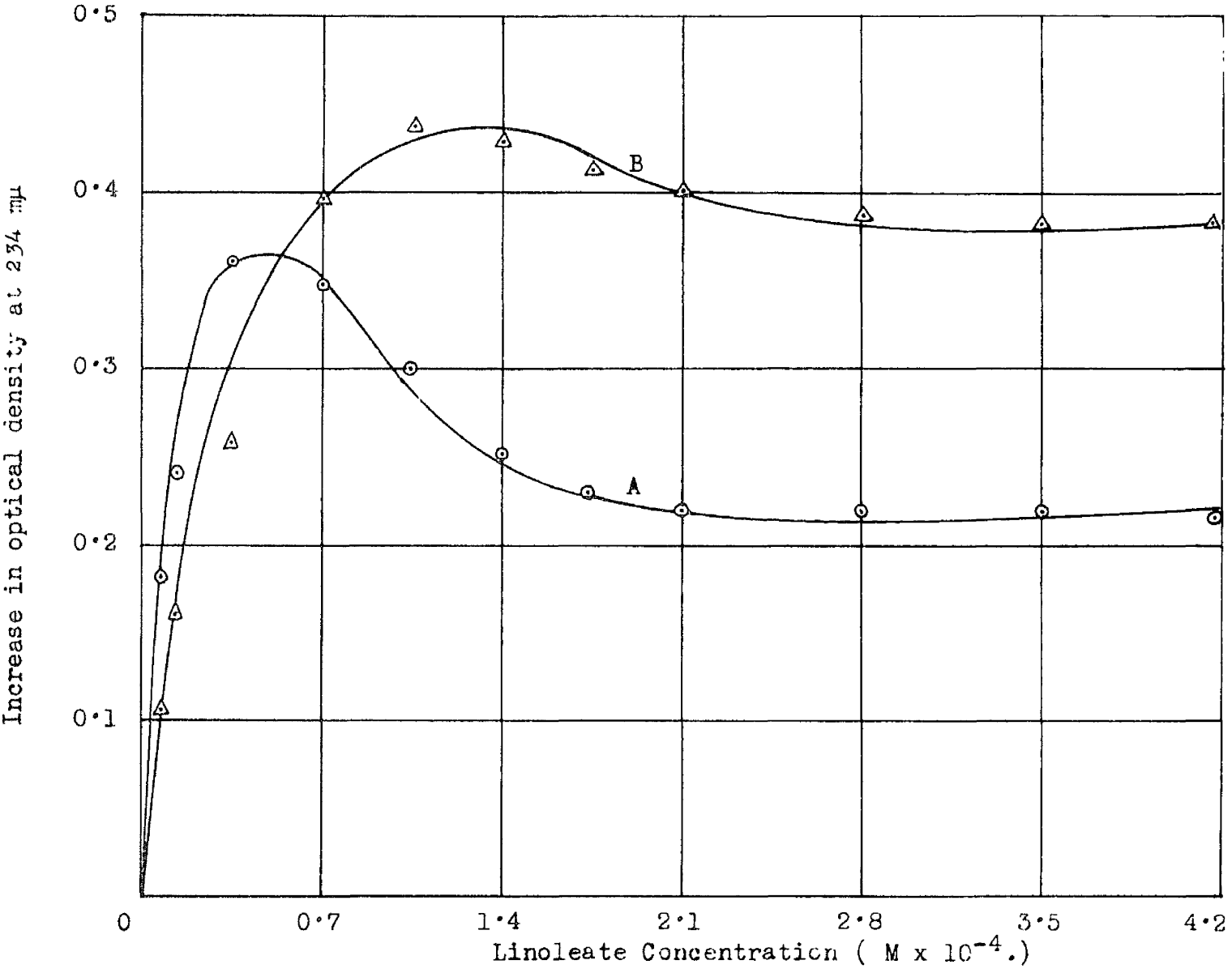


Fig. 41. - Effect of haemoglobin on lipoxidase activity at pH 7·6 at various levels of linoleate.

A - Lipoxidase control (0·004 mg./ml.).
B - Lipoxidase + 2×10^{-6}M haemoglobin.

Reaction time : 60 seconds.

lipoxidase and haemoglobin , confirming the previously obtained evidence leading to this conclusion . The general pattern of the results at both pH levels was found to be essentially similar ,diene apparently being diminished in the presence of haemoglobin at the lower levels of linoleate , but becoming enhanced at relatively higher linoleate concentrations . The increased formation of conjugated diene observed at higher substrate levels was more evident at pH 7·6 than at pH 9.

The curves shown in Figures 40 and 41 are in keeping with the view that a more complete breakdown of haemoglobin occurs, rapidly , at high linoleate concentrations .

The influence on haemoglobin behaviour of the linoleate level in the lipoxidase system , and the apparent dependence of the lipoxidase-linoleate intercourse on pH , would account for the different effects observed previously at the two pH levels with the same linoleate concentration in the reaction systems.

The apparent differences in the substrate-activity relation--ships at the two pH levels may be explained by variations in substrate availability in the different reaction systems due, possibly , to dissimilar substrate solubilities at the two pH levels.

The data described here on the effect of linoleate on haematin action in the lipoxidase system are analogous to the findings of Blain and Styles (87) who , in a study of haematin catalysis of linoleate oxidation , observed that cytochrome c , at pH 5·4, destroys the small amount of diene present at low levels of freshly prepared sodium linoleate , but conjugates diene at high linoleate levels .

Since destruction of the haematin catalyst has been shown to occur in haematin-catalysed oxidation of linoleate (54,68), and in view of the fact that the possible decomposition of cytochrome c in their reaction system was not considered by Blain and Styles , it would seem not unlikely that the increased formation of conjugated diene observed by these workers at the high linoleate concentrations was due to breakdown products of the haematin compound rather than to cytochrome c itself , promoting the formation of conjugated linoleate hydroperoxide .

Effect of Lipoxidase Concentration on the Interaction of the two Systems.

The effect of lipoxidase concentration on lipoxidase-haemoglobin interaction at the two pH levels was determined by measuring the relative activities of solutions of varying concentrations of lipoxidase and comparing the results with those obtained , under similar conditions , for solutions containing the same range of enzyme concentrations but with haemoglobin added in constant proportion.

Sodium linoleate was used in these experiments at a level of $1{\cdot}4 \times 10^{-4}$M .

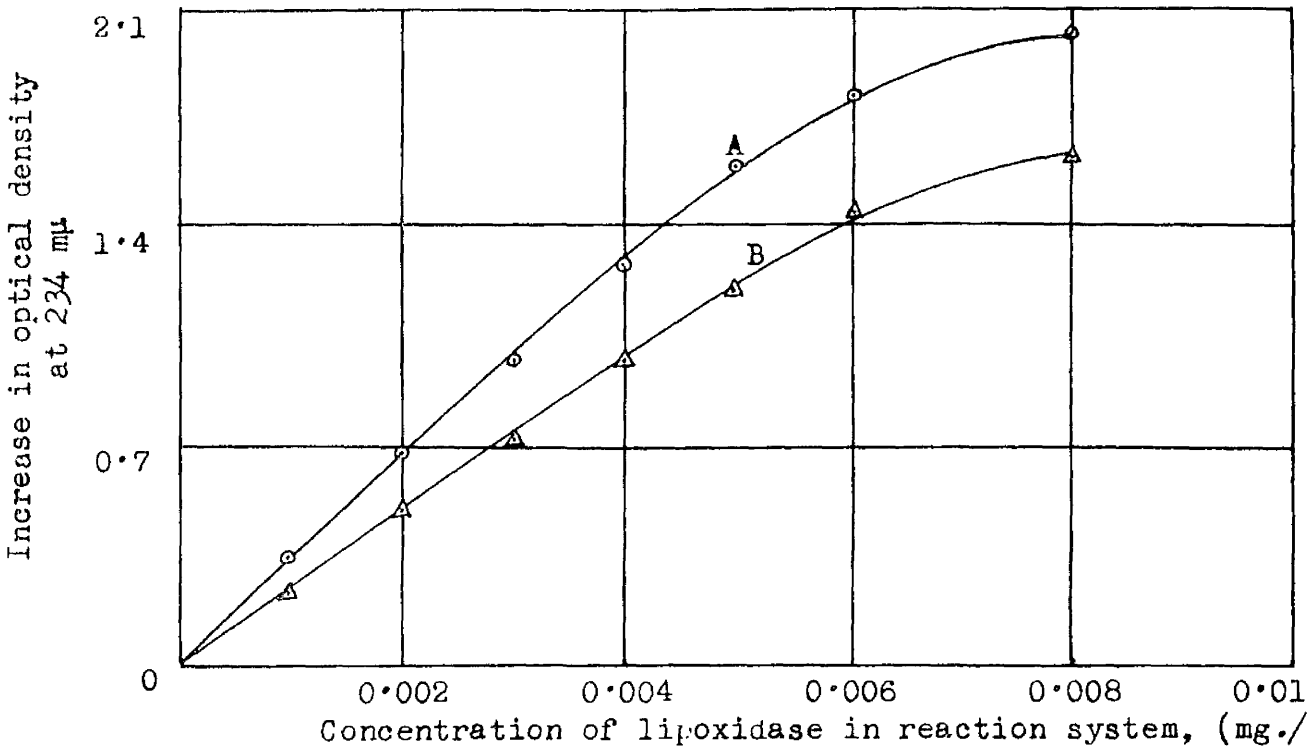


Fig. 42. - Effect of lipoxidase concentration on lipoxidase--haemoglobin interaction at pH 9.

A - Lipoxidase control.

B - Lipoxidase+haemoglobin in constant proportion.
Sodium linoleate level : $1 \cdot 4 \times 10^{-4}$M.
Reaction time : 60 seconds.

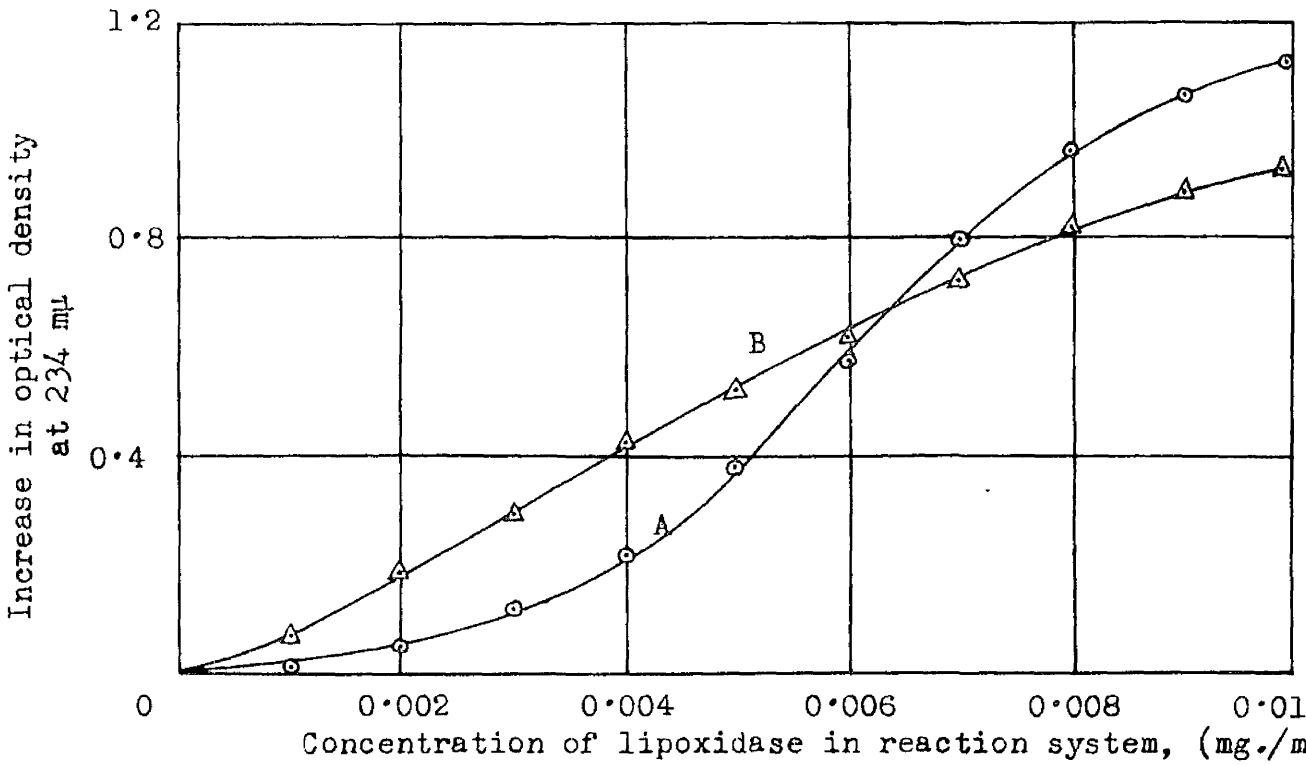


Fig. 43. - Effect of lipoxidase concentration on lipoxidase--haemoglobin interaction at pH 7·6.

A - Lipoxidase control.

B - Lipoxidase+haemoglobin in constant proportion.
Sodium linoleate level : $1 \cdot 4 \times 10^{-4}$M.
Reaction time : 60 seconds.

From the considerations already discussed on lipoxidase-haematin interaction, it might be expected, since haemoglobin was being added to the system along with lipoxidase, that the haematin-effected destruction of conjugated diene would increase with increasing lipoxidase concentration . That this was apparently the case at pH 9 may be seen from Figure 42.

At pH 7·6,on the other hand, the expected inhibition of diene conjugation was observed only at the highest enzyme concentrations employed, as shown in Figure 43, for with low levels of both catalytic factors in the system, a marked increase in the diene was noted. However, since,as reasoned earlier, the diene-destroying action of the very low levels of haemoglobin present in the reaction system at one minute would be hardly noticeable at pH 7·6, the increase in lipoxidase activity observed at high dilution at pH 7·6 may be explained, in agreement with the supposition already made, as due to catalysis of the formation of conjugated diene by haemoglobin-breakdown products.

The addition of further amounts of haemoglobin to the system, in the presence of lipoxidase, would account adequately for the gradual lessening of this effect and for the resulting decrease in diene observed at the very high enzyme concentrations examined .

The lack of proportionality observed between concentration of lipoxidase and diene conjugation at pH 7·6 is to be expected

since the enzyme was acting in a system at a pH level well below its optimum. It is interesting to note from Figure 43 that a more nearly linear relationship, such as was found at pH 9, was restored in the presence of the haematin compound.

Attempts to Vary the Relative Heights of the Two pH Maxima.

As described earlier in this section, the interaction of lipoxidase and haematin in the lipoxidase assay system was found to result in a pH-activity curve with two pH maxima, the smaller of the two peaks occurring at pH 7·6. One of the intentions of this study was to determine suitable reaction conditions under which the relative heights of the two pH maxima might be changed.

Evidence from the two preceding parts of this section suggested that changes in the ratio of activity at the two pH maxima might, through the differential effects on either maximum by haematin, be produced by suitable adjustment of both linoleate concentration and the level of lipoxidase in the reaction system.

Accordingly, the substrate-activity relationships at the two pH levels for lipoxidase-haemoglobin mixtures containing different amounts of lipoxidase were determined and compared. Repeated observations showed the most significant change in the ratio of activity at the two pH levels to occur, in the presence of 10^{-6}M haemoglobin, at a sodium linoleate concentration of $0{\cdot}7 \times 10^{-4}$M and with a lipoxidase level in the system of 0.002 mg. per ml.

From the substrate-activity curves for this level of lipoxidase, as shown in Figures 44 and 45, the activity of the

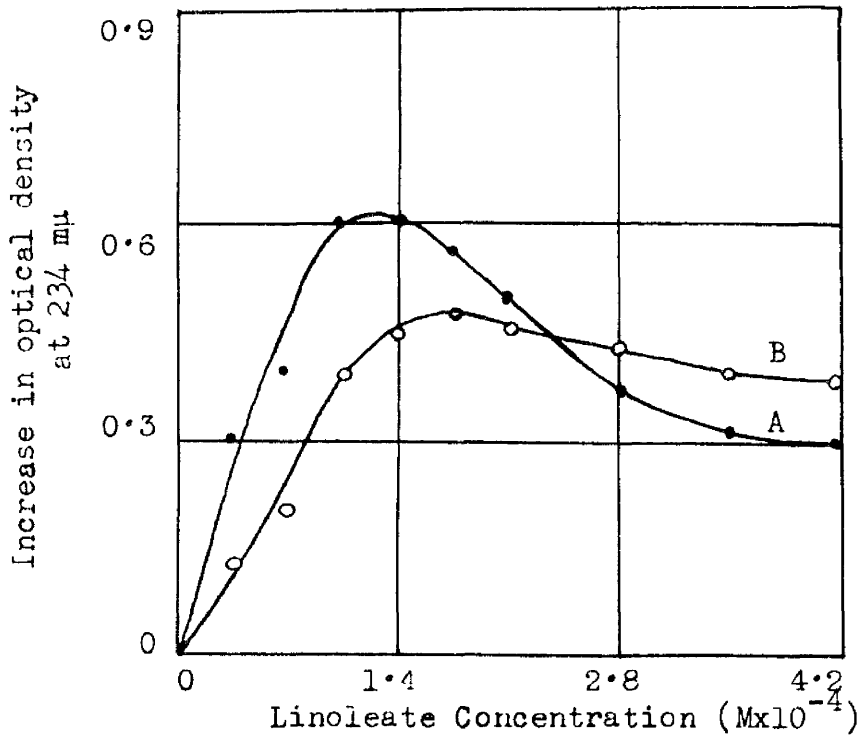


Fig. 44. - Substrate-Activity relationship of a lipoxidase-haemoglobin mixture at pH 9.
A - lipoxidase control (0·002 mg./ml.)
B - lipoxidase + 10^{-6}M haemoglobin
Reaction time : 60 secs.

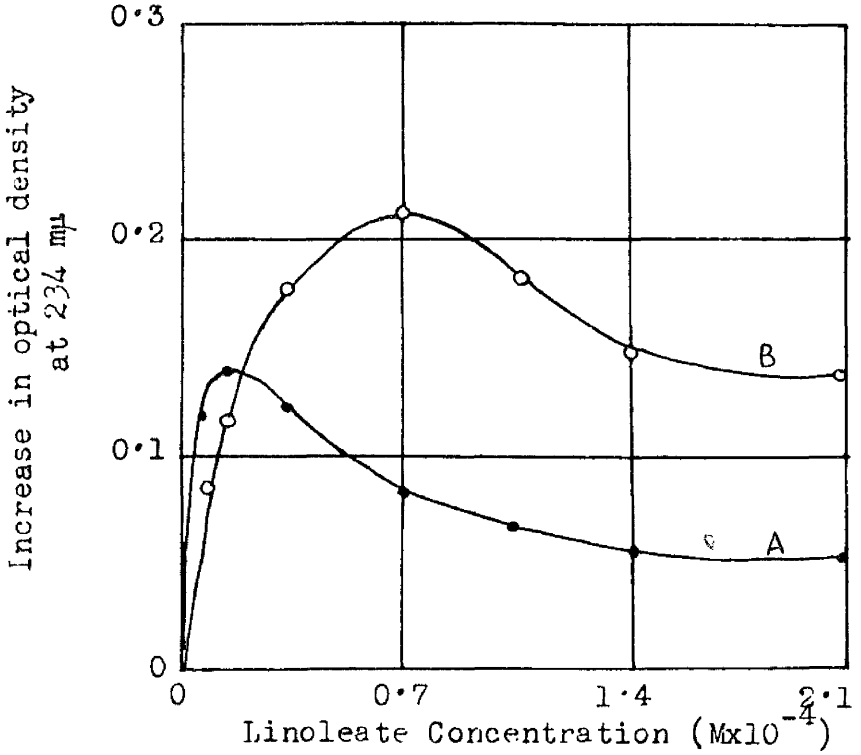


Fig. 45. - Substrate-Activity relationship of a lipoxidase-haemoglobin mixture at pH 7·6.
A- lipoxidase control (0·002 mg./ml.)
B - lipoxidase + 10^{-6}M haemoglobin.
Reaction time : 60 secs.

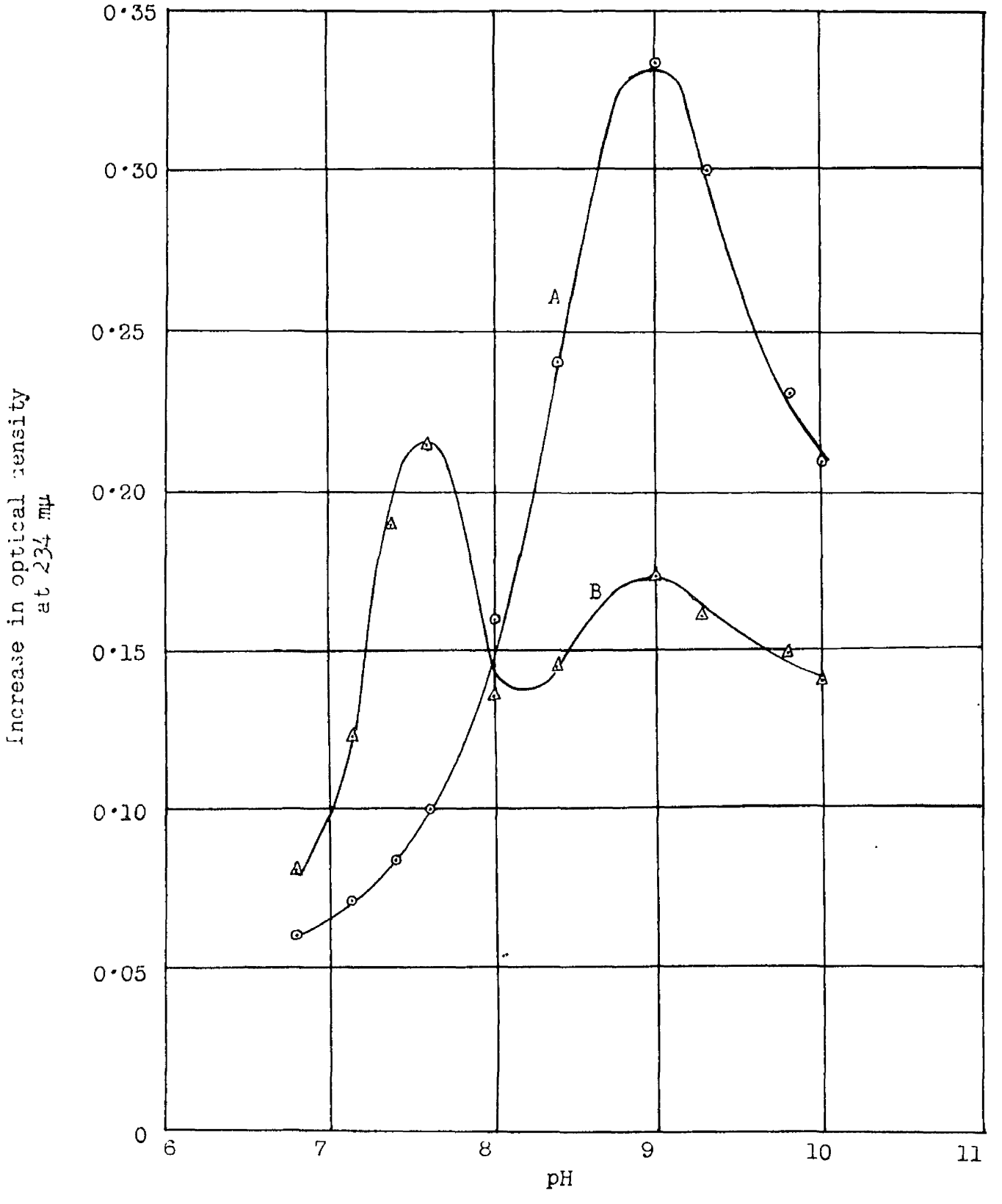


Fig. 46. - pH-activity curves of lipoxidase and of a lipoxidase-haemoglobin mixture.

A - Lipoxidase control, (0·002 mg./ml.).

B - Lipoxidase+10^{-6}M haemoglobin.

Sodium linoleate level : $0{\cdot}7 \times 10^{-4}$M.

lipoxidase-haemoglobin mixture at a linoleate concentration of $0{\cdot}7 \times 10^{-4}$M appeared to be higher at pH 7·6 than at pH 9.

In view of the implications of these results, the pH-activity curve of the lipoxidase-haemoglobin mixture was determined, using the method described in Appendix 1, at a substrate concentration of $0{\cdot}7 \times 10^{-4}$M. The relative concentrations of lipoxidase and haemoglobin used in this experiment were identical to those described above.

Under these conditions, as may be seen from Figure 46, the change in lipoxidase activity produced by the action of the haematin compound in response to pH, resulted in a pH-activity curve with two pH maxima, the larger of the two appearing at pH 7·6.

Although, under the adopted conditions, the overall activity of the lipoxidase-haematin combination was unavoidably low, the configuration of the pH-curve was found to be reproducible.

3. Lipoxidase-Haematin Interaction in Linoleate Oxidation catalysed by Crude Soya Extracts .

The work described in the preceding section was concerned with the interaction of purified lipoxidase and pure haematin in the lipoxidase assay system . In this context the possible involvement of haematin in linoleate oxidation catalysed by crude plant extracts could not be overlooked .

Little or no previous study has been made of the possible mutual interaction of lipoxidase and haematin with regard to catalysis by crude plant extracts . Holman (49), however , from studies on soya-bean germination , considered there to be a relationship between lipoxidase and catalase in germinating soya-beans. The results of these studies led Holman to suspect that the presence of catalase complicates the behaviour of lipoxidase .

Recent work by Blain and Styles (87, 108) suggests that in crude extracts of plant material haematins do play a role in catalysis of fat oxidation . These workers noted that in the linoleate-coupled oxidation of carotene catalysed by crude soya extracts , two factors are apparently involved , one having a true lipoxidase action in bleaching carotene by concurrent oxidation of linoleate and the other behaving like haematins in using preformed hydroperoxide to bleach carotene . Blain and Styles found this haematin-like factor , which they term lipoperoxidase, to predominate in a pH 4·5 buffer extract of defatted soya .

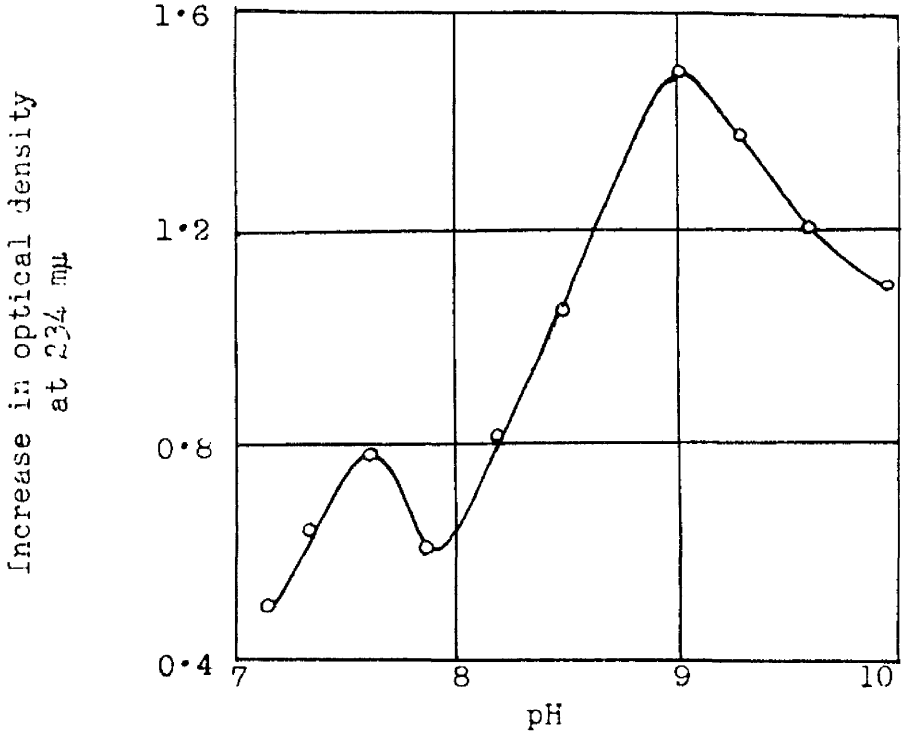


Fig. 47. - pH-Activity curve of a pH 4·5 5% buffer extract of Chinese soya.

linoleate concentration: $1 \cdot 4 \times 10^{-4}$ M.

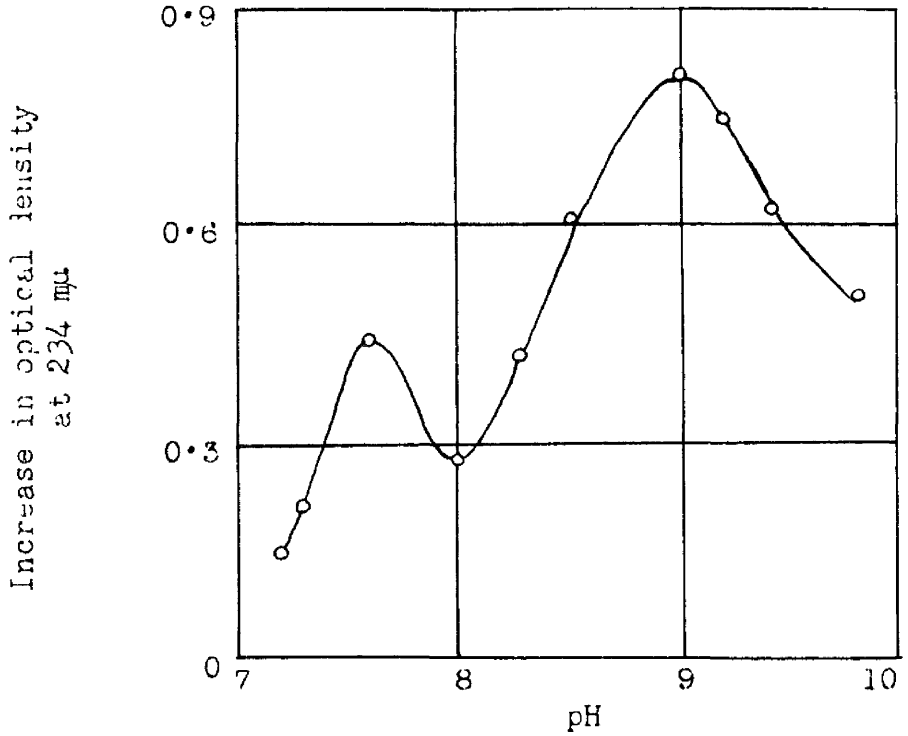


Fig. 46. - pH-Activity curve of a lipoxidase-haematin mixture.

Reaction mixture:- lipoxidase: 0·004 mg./ml.
haemoglobin: 2×10^{-6} M.
linoleate: $1 \cdot 4 \times 10^{-4}$ M.

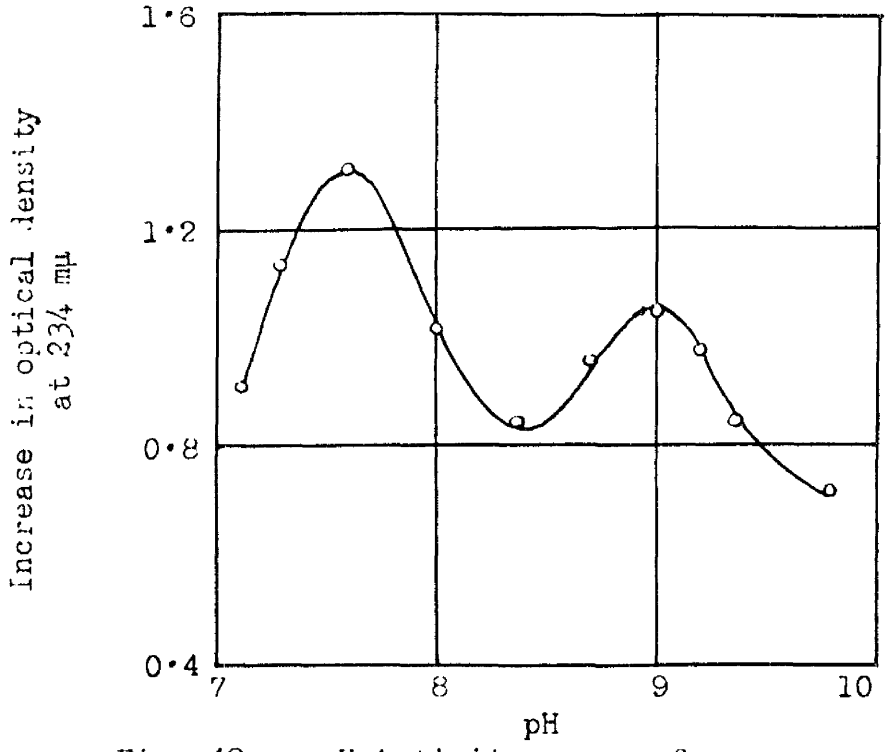


Fig. 49. - pH-Activity curve of an aqueous 20% extract of Nigerian soya.

linoleate concentration: $1 \cdot 4 \times 10^{-4}$M.

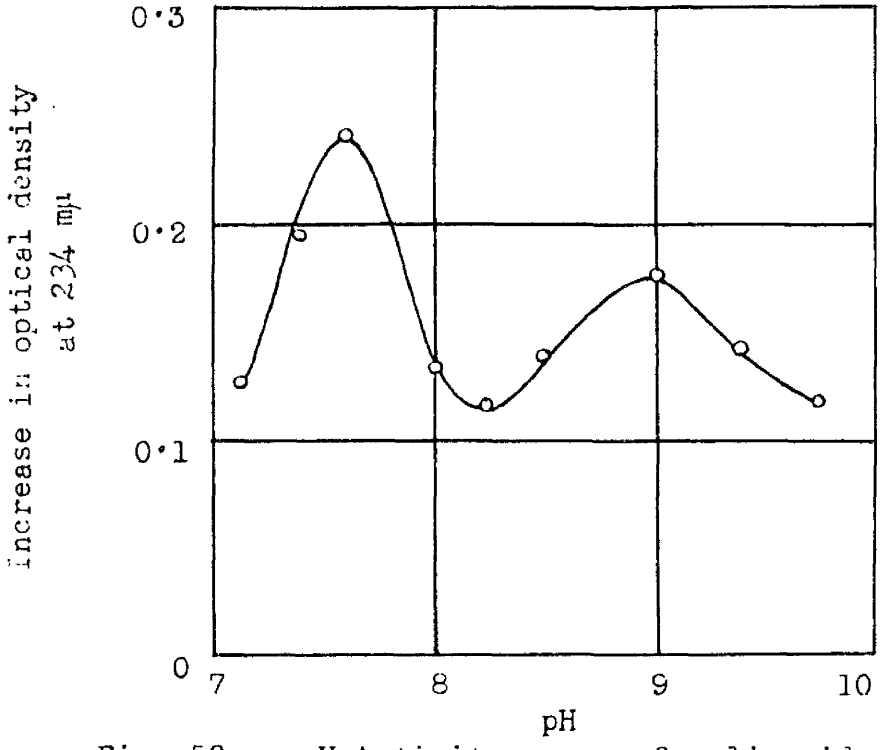


Fig. 50. - pH-Activity curve of a lipoxidase-haematin mixture.

Reaction mixture:- lipoxidase: 0·002 mg./ml.
haemoglobin: 10^{-6}M.
linoleate: $0 \cdot 7 \times 10^{-4}$M.

In considering the meagre evidence available, it occurred to the writer that the question of the possible interplay between lipoxidase and haematin in crude extract catalysis might be more adequately resolved by the use of model enzyme systems.

The final experimental section of this thesis is therefore intended to contribute towards an answer to this problem by a comparative study of the behaviour of crude soya extracts with that of model enzyme systems under identical conditions.

Comparison of the pH-activity Curves of Crude Extracts and Model Systems.

[illegible] course of these investigations that the activity of lipoxidase-haematin mixtures, under certain conditions, responded to pH in a fashion similar to that shown by crude soya extracts. The comparable configurations of the pH-activity curves of soya extracts and lipoxidase-haematin mixtures are shown in Figures 47 to 50.

The striking similarities of the pH-activity curves of crude and model systems suggested that a lipoxidase-haematin

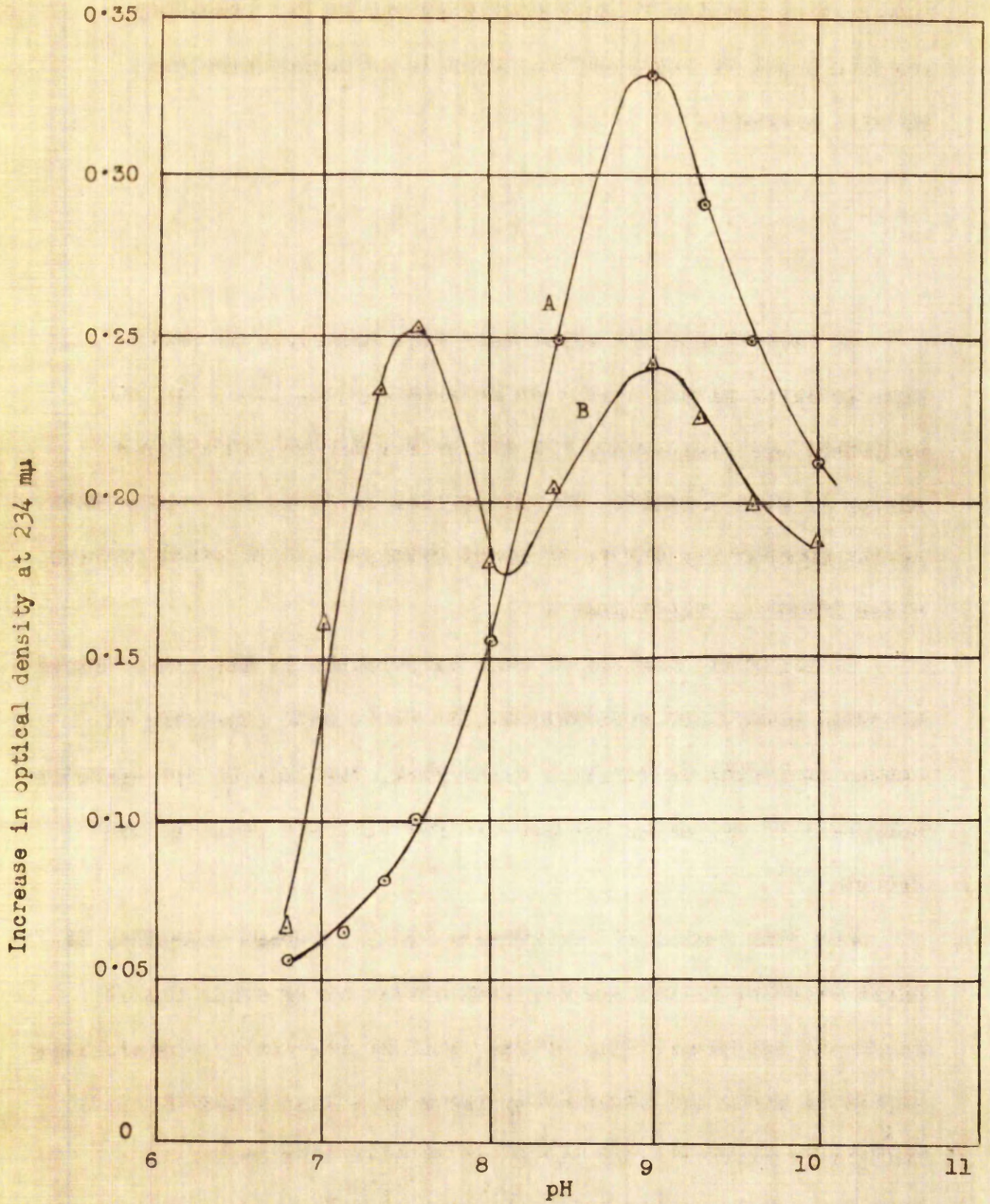


Fig. 51. - pH-activity curves of lipoxidase and of a lipoxidase-catalase mixture.

A - Lipoxidase control. (0·002 mg./ml.)

B - Lipoxidase + $0{\cdot}5 \times 10^{-6}$M catalase.

Sodium linoleate level : $0{\cdot}7 \times 10^{-4}$M.

Reaction time : 60 secs.

interaction similar to that characterised in the preceding section might be involved in linoleate oxidation catalysed by soya extracts.

To investigate the hypothesis that catalysis by crude soya extracts involves such an interaction and that haematin compounds are responsible for the double-maximal pH-activity curves of soya extracts, it was decided to carry out experiments on the pH-activity curves of crude extracts and of model systems under identical conditions .

In order to simulate as near as possible in the model enzyme systems, conditions occurring in the crude soya extracts, it seemed desirable to employ a known plant haematin as the haematin component of the model system, and for this end catalase was chosen.

For this series of experiments it also seemed essential to adopt reaction conditions for each system under which the pH maxima of the pH-activity curves would be critically discernible. Figure 51 shows the pH-activity curve of a lipoxidase-catalase mixture in which the two peaks are clearly distinguished. Here the substrate level is half that used in the case of a crude soya extract to give a comparable pH-curve configuration as shown in Figure 49.

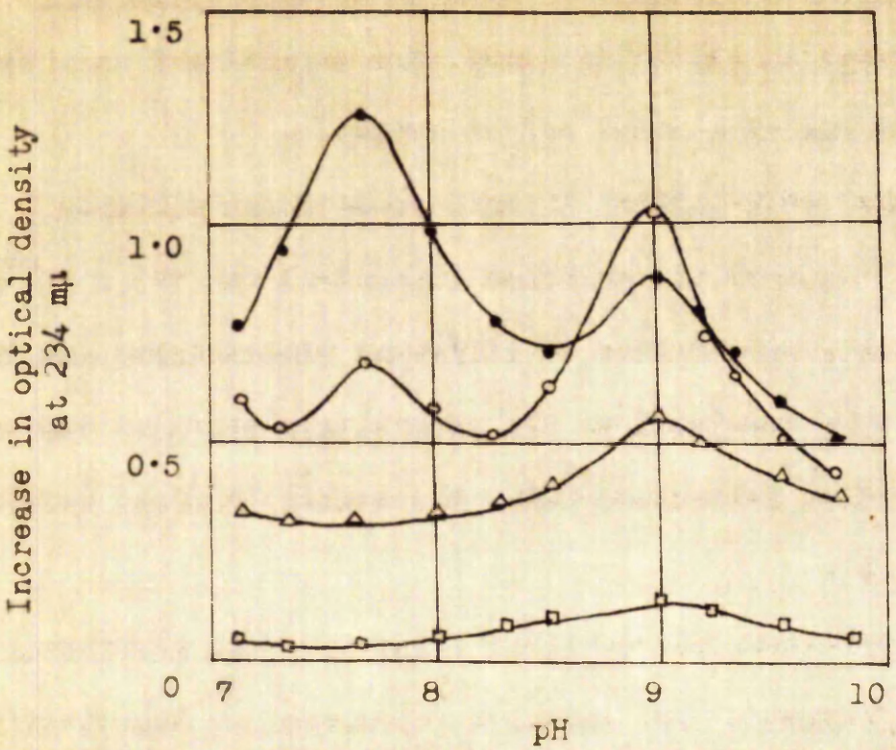


Fig. 52. - Effect of Heat on the Activity of a 20% aqueous Nigerian Soya extract.

Linoleate concentration : $1 \cdot 4 \times 10^{-4}$ M.

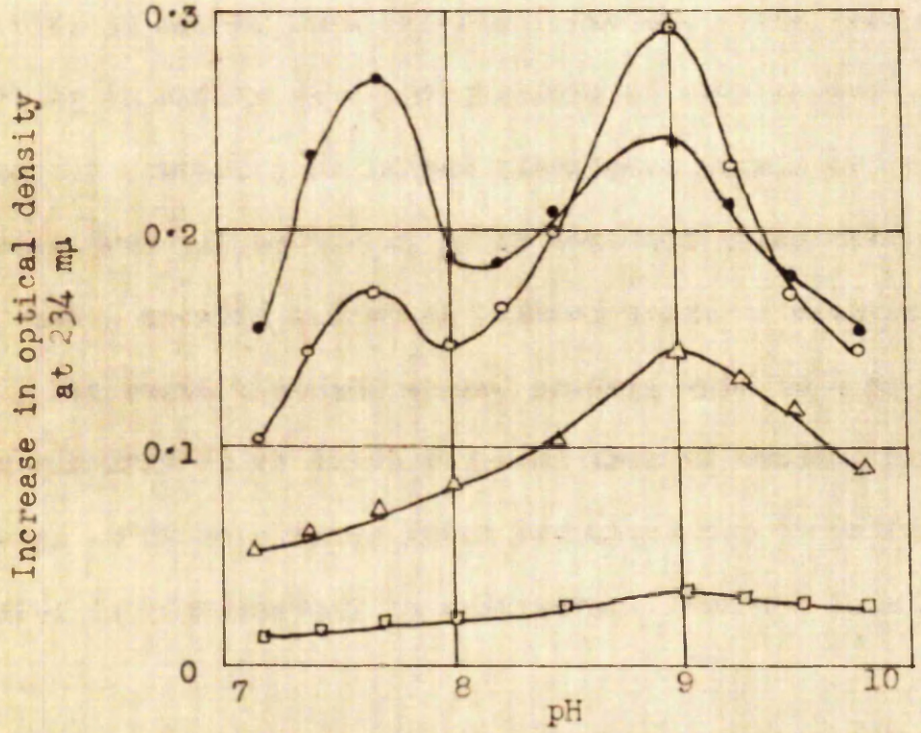


Fig. 53. - Effect of Heat on the Activity of a Lipoxidase--Catalase mixture.

Reaction mixture : lipoxidase: 0·002 mg./ml.
catalase : $0 \cdot 5 \times 10^{-6}$ M.
linoleate: $0 \cdot 7 \times 10^{-4}$ M.

—●— Enzyme control
—○— After 2 minutes at 55° C.
—△— After 2 minutes at 60° C.
—□— After 2 minutes at 70°C.

Heat Inactivation Studies of Crude and Model Systems .

Some heat inactivation tests were carried out on a crude soya extract and on a model enzyme system.

A number of identical lipoxidase-catalase mixtures , containing 1 mg. per ml. purified lipoxidase and $2{\cdot}5 \times 10^{-4}$M pure catalase , were heated to different temperatures and the effects of this treatment on the pH-activity curve of the model system were then determined using the method of assay detailed in Appendix 1 .

This procedure was repeated under the same conditions with 20% aqueous Nigerian soya extracts , prepared as described in Appendix 1 , and the results of the experiments are shown in Figures 52 and 53 .

When both crude and model systems were heated to 55°C for 2 minutes , the maximum in activity for each system at pH 7·6 was found to be almost completely inhibited , whereas the peak at pH 9 in both cases appeared to be significantly activated by this treatment . After heating to 60°C., however , the maximum at pH 9 of both systems became markedly depressed , while the lower pH maximum in each case was found to be entirely removed . Further heating of the crude and model systems to 70°C. appeared to cause almost complete destruction of the activity of both systems.

These heat inactivation experiments were carried out a number of times and in each case the results were found to be

similar to those shown.

The remarkably similar effects of heating on the pH-activity curves of crude and model systems indicated that the hypothesis under consideration might be correct; that is to say, it appeared likely that haematin, present in the crude extract, was interfering with lipoxidase action in the system both by retarding diene conjugation at the more alkaline pH maximum and by causing, indirectly, the increased formation of diene at the lower pH maximum of the soya extract.

The apparent instability of catalase towards heat, as evidenced by the activating and inhibiting effects of the lower temperature on the two pH maxima of the model system, is of interest since it is well known that cytochrome c, another haematin compound ubiquitous in plant and animal tissues, is remarkably heat-stable.

Salt Effects on the pH Maxima of both Systems.

During studies of the fractionation of crude soya extracts, it had been noted that the presence of fairly high salt concentrations in the enzyme extracts caused a marked alteration in the forms of the pH-activity curves. It was, therefore, of considerable interest to examine and compare the effects of salt on the pH-characteristics of both crude and model systems.

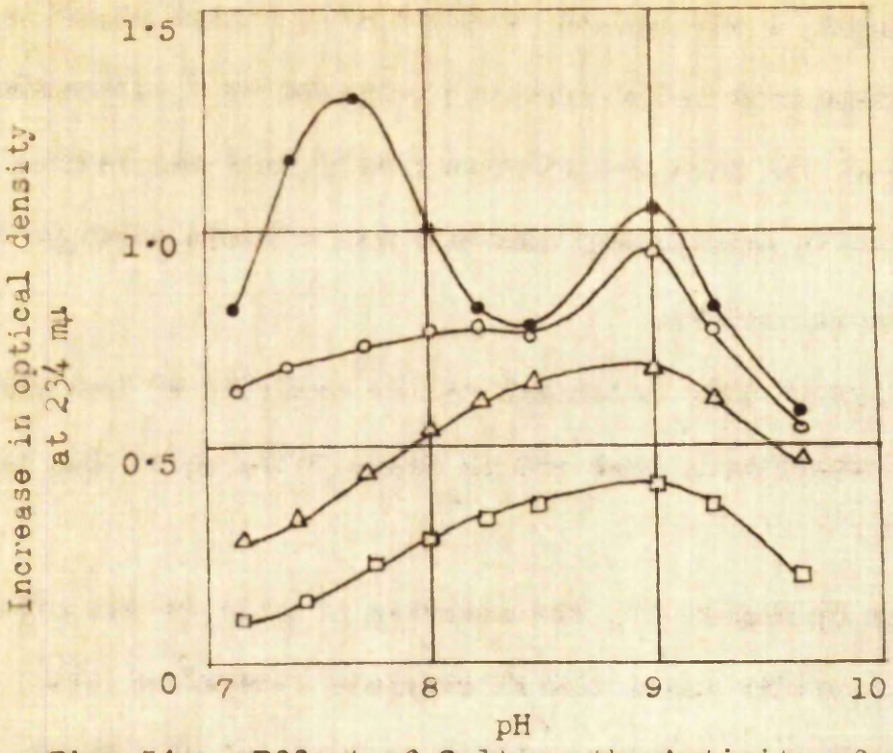


Fig. 54. - Effect of Salt on the Activity of a 20% aqueous Nigerian Soya extract.

linoleate concentration : $1{\cdot}4 \times 10^{-4}$M.

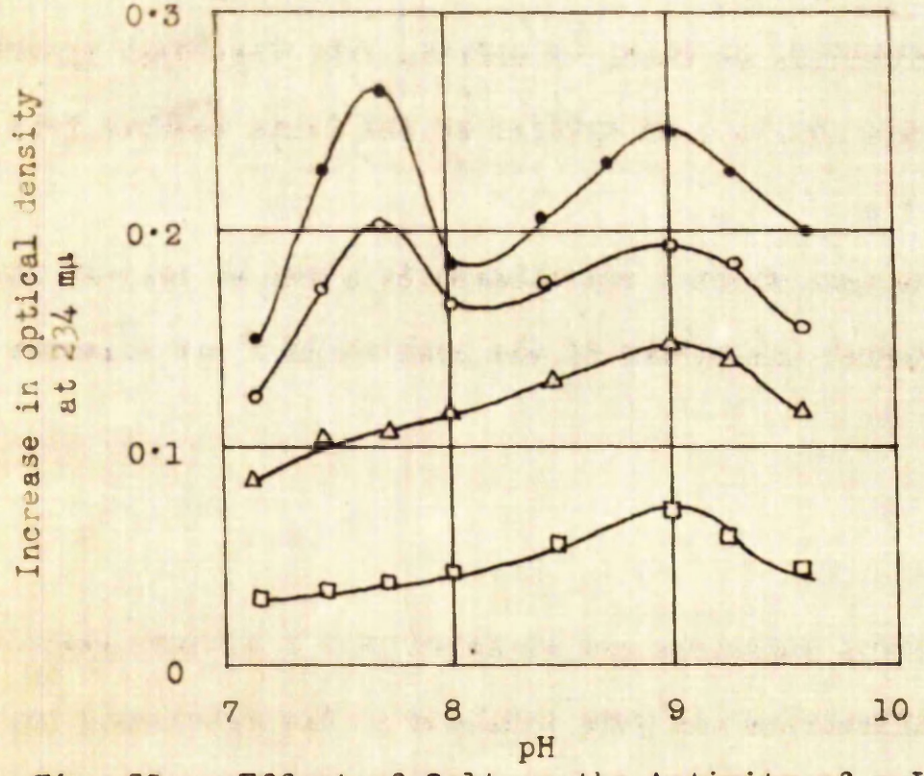


Fig. 55. - Effect of Salt on the Activity of a Lipoxidase--Catalase mixture.

Reaction mixture : lipoxidase : 0·002 mg./ml.
catalase : $0{\cdot}5 \times 10^{-6}$M.
linoleate : $0{\cdot}7 \times 10^{-4}$M.

—•— Enzyme control. —o— Enzyme solution at 40% satn.
—▲— 40% satd soln after 20 hrs. at 0-4°C. —□— Soln after 48 hrs. at 0-4°C.

Accordingly, a 20% aqueous extract of defatted Nigerian soya was treated with solid ammonium sulphate to a saturation level of 40% . The salt was stirred slowly into the active solution to avoid unnecessary denaturation effects through local high salt concentrations.

The effect of this treatment on the activity of the soya extract was determined, over the pH range 7-11, under the usual conditions.

As shown in Figure 54, the addition of salt to the soya extract was found to cause almost complete removal of the maximum at pH 7·6, suggesting either partial separation or inactivation of the component responsible for this high activity. The maximum at pH 9, on the other hand, was observed to be only slightly affected, so that, in effect, this treatment appeared to change the absolute pH optimum of the crude extract from pH 7·6 to pH 9 .

As the soya extract was allowed to stand in contact with the salt, a gradual diminution of the peak at pH 9 was observed .

The above procedure was repeated with a mixture containing purified lipoxidase and pure catalase . The relatively low activity of the lipoxidase-catalase mixture necessitated careful handling of the solution, and when precautions were taken to ensure

that undue losses of activity did not occur on treatment with salt , the results , shown in Figure 55, were found to be reproducible .

Addition of ammonium sulphate to the mixture did not appear to result in the complete removal of the maximum at pH 7·6, as was observed with soya extracts , for the two pH maxima were still fairly well delineated . However , prolonged contact of the lipoxidase-catalase mixture with salt was found to cause transformation of the pH-activity curve in a manner closely resembling that described in the case of the soya extract. The appearance of the curves shown in Figures 54 and 55 suggests that this effect may have been due to removal of the catalase or other haematin from solution by the salt .

The apparently analogous behaviour towards salt of the model and crude systems strongly indicated that a lipoxidase-haematin interaction occurs in linoleate oxidation catalysis by soya extracts , thus reinforcing the evidence leading to this conclusion already derived from the heat inactivation experiments.

Effects of Cyanide on the Lipoxidase Activity of Model and of Cr<u>Effects of Cyanide on the Lipoxidase Activity of Model and of Crude Systems</u>.

Mapson and Moustafa (31), in 1954, reported two factors to be involved in the linoleate-coupled oxidation of glutathione catalysed by crude extracts of pea lipoxidase, one of which, being cyanide-sensitive, appeared to behave like a haematin compound (71).

In considering these findings, it seemed that further evidence of the involvement of haematin, and of the nature of its role in crude extract catalysis, might be obtained by comparing the effects of cyanide on crude and model systems.

The effect of cyanide was determined, firstly, on the activity of purified lipoxidase over the pH range 7 to 11 and over reaction periods of one minute and longer. The lipoxidase concentration and the substrate level used were similar to those adopted for the study of the model systems. Potassium cyanide, added to the reaction system at a level of 10^{-3}M, was found to have no effect whatsoever on lipoxidase activity under these conditions, in agreement with Holman's findings (11).

Having established that the purified lipoxidase system is not affected by cyanide, it seemed likely that any consequent change in lipoxidase behaviour observed in the presence of

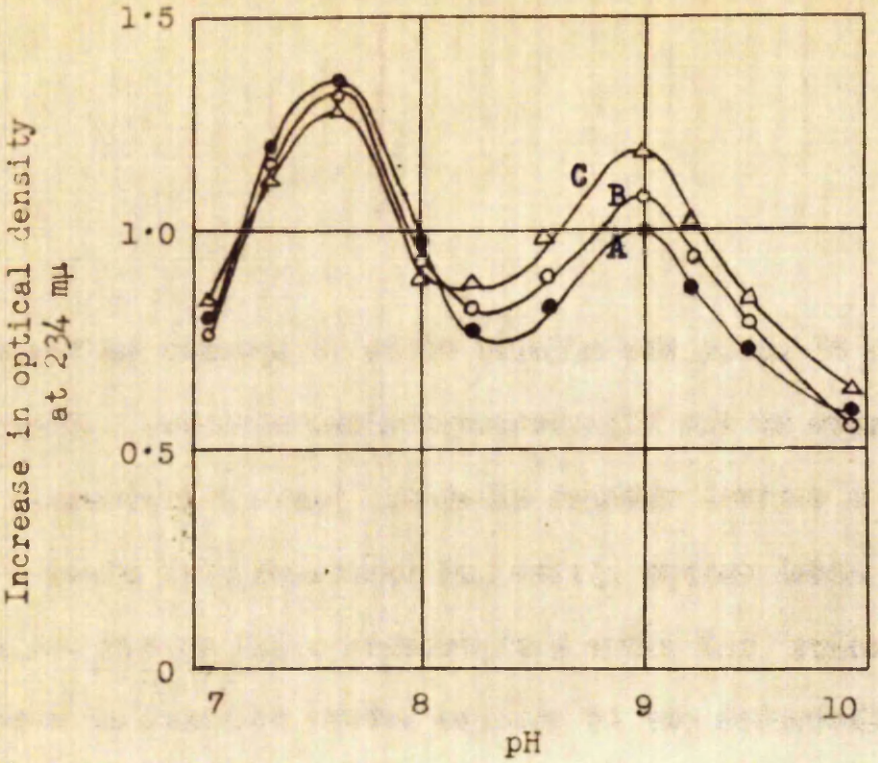


Fig. 56. - Effect of Cyanide on the Activity of a 20% aqueous Nigerian Soya Extract.
linoleate concentration : $1 \cdot 4 \times 10^{-4}$M.
level of cyanide in system : 10^{-3}M. (KCN.)

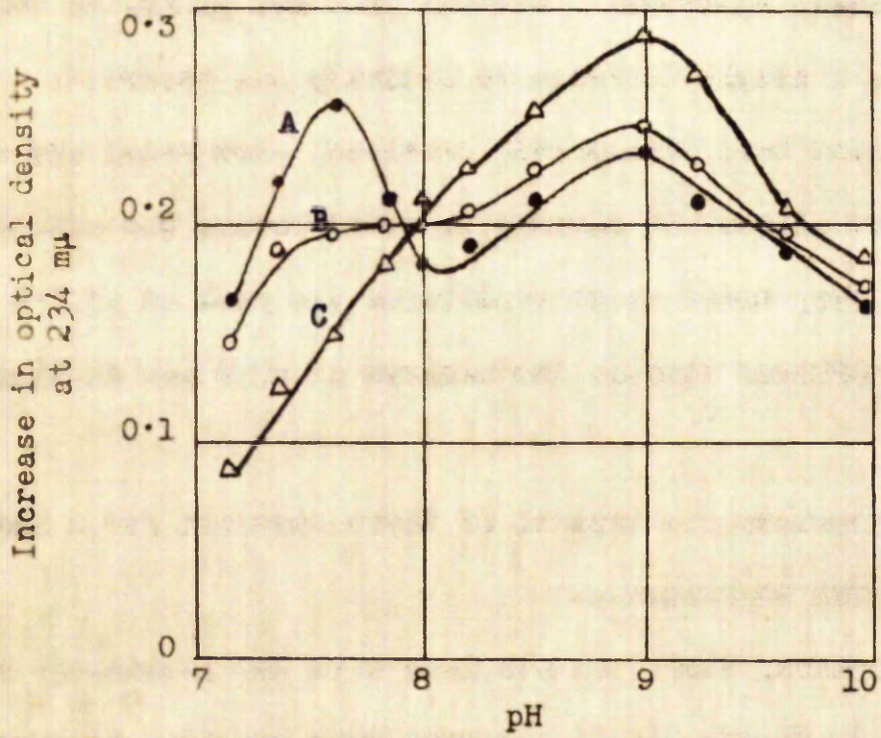


Fig. 57. - Effect of Cyanide on the Activity of a Lipoxidase--Catalase mixture.
Reaction mixture : lipoxidase : 0·002 mg./ml.
catalase : $0 \cdot 5 \times 10^{-6}$M.
linoleate : $1 \cdot 4 \times 10^{-4}$M.

—●— Enzyme control. —○— Enzyme, substrate, cyanide mixed simultaneously.
—△— Enzyme, cyanide equilibrated 10 minutes before substrate added.

catalase would reflect the action of cyanide on the haematin component.

Figure 57 shows the effects of 10^{-3}M cyanide on the pH-activity curve of the lipoxidase-catalase mixture. Curve A represents a control without cyanide; curve B represents substrate, model enzyme system and cyanide-buffer mixed simultaneously; and curve C represents model system and cyanide-buffer equilibrated for 10 minutes before addition of substrate.

When model system, cyanide and substrate were mixed simultaneously, a marked inhibition of the maximum at pH 7·6 was immediately apparent. Between pH 8 and pH 10, on the other hand, a slight increase in activity was observed. These effects became more pronounced, moreover, when model system and cyanide were allowed 10 minutes to react before the substrate was added, for, under these conditions the peak at pH 7·6 was entirely inhibited whereas the maximum at pH 9 was distinctly activated.

These results are typical of those observed for a large number of such experiments.

On comparing these observations with the previously obtained data shown in Figure 51, it appeared that cyanide, as expected, had effectively eliminated haematin-interference in the

lipoxidase reaction system.

It was therefore possible, on the basis of these findings, to speculate that cyanide "activation" of soya extract activity would reflect the presence in the crude extracts of a haematin-like inhibitor of diene conjugation. The results suggested, moreover, that if cyanide "inhibition" of crude extract activity could be manifested, support would be given to the postulate that haematins, in the adopted system, contribute to the lipoxidase activity of crude soya extracts.

In view of these considerations, it seemed worth while to determine whether the differential effects of cyanide on the pH maxima of a crude soya extract could be demonstrated.

Accordingly, the effect of 10^{-3}M cyanide on the pH-activity curve of a 20% aqueous Nigerian soya extract was examined under conditions similar to those described for the model enzyme system.

In the case of the crude extract, however, a dissimilar response to cyanide was found. Figure 56 shows that cyanide, added simultaneously to the reaction system, had no effect on the maximum at pH 7·6, but caused a marked increase in activity between pH 8 to pH 10 as was noted earlier in the case of the lipoxidase-catalase mixture. This "activation" effect became more apparent when soya extract and cyanide had been equilibrated

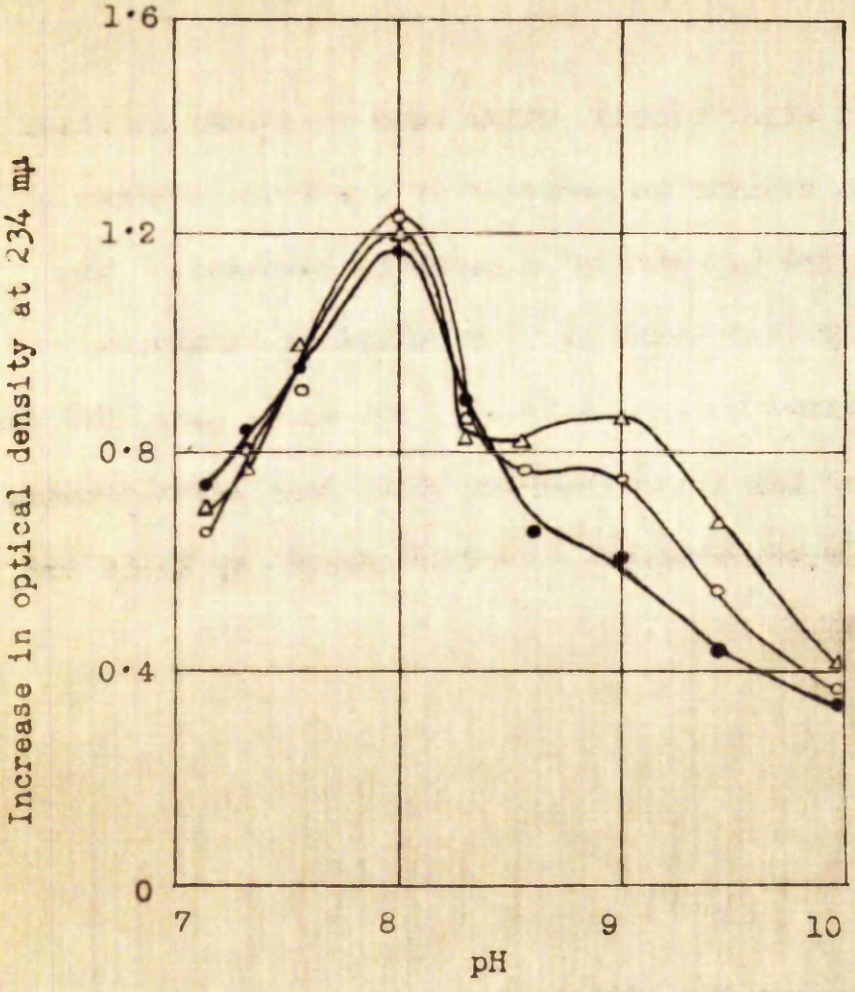


Fig. 58. - Effect of Cyanide on the Activity of a 20% pH 4·5 buffer extract of Nigerian Soya.

linoleate concentration : $1{\cdot}4 \times 10^{-4}$M.
level of cyanide in system : 10^{-3}M (KCN.)

—●— Enzyme control.
—o— Enzyme, substrate, cyanide mixed simultaneously.
—△— Enzyme, cyanide equilibrated for 10 minutes before substrate added.

for 10 minutes before addition of substrate to the system. Under these experimental conditions, cyanide-inhibition of the activity below pH 8 was hardly noticeable.

From these observations, which were repeated, it seems likely that the maximum in activity of the crude extract at pH 7·6 was not the function of a haematin compound. The results do imply that haematin is involved in linoleate oxidation catalysed by soya extracts, but as an agent for the decomposition of linoleate hydroperoxide, thus corroborating the evidence already obtained on other grounds by Blain and Styles (87, 109).

Figure 58 shows the effect of cyanide on the pH-activity curve of a 20% pH 4·5 buffer extract of defatted Nigerian soya. It will be observed that the effect of cyanide on the maximum at pH 8 was very slight and that changes in the activity of the buffer extract in this region were scarcely discernible, as had been found in the case of the corresponding aqueous soya extract. At pH levels above 8 there was an unmistakable increase in

lipoxidase activity, which, it will be noted, was found to result in the appearance of a maximum in activity at pH 9.

This seems to indicate that the apparent absence of a maximum in lipoxidase activity at pH 9 from the pH-curve of a freshly prepared pH 4·5 buffer extract, may be due to the predominance in such as extract of the haematin-like diene destroyer. This observation also tends to support the findings of Blain and Styles (87, 109) that haematin present in crude soya is more soluble in pH 4·5 buffer than in distilled water.

The data presented in this section favour the hypothesis that a mutual interaction exists between lipoxidase and haematin in linoleate oxidation catalysed by crude soya extracts, with haematin effecting the breakdown of the conjugated linoleate hydroperoxides formed by lipoxidase.

DISCUSSION

DISCUSSION.

Owing to the nature of the evidence presented, discussion of detail has already been dealt with in the text of the experimental sections.

The experiments described on lipoxidase-haematin interaction show that the presence of haematin affects the characteristics of the lipoxidase oxidation. While the evidence on haematin-catalysed destruction of conjugated diene hydroperoxide in the lipoxidase system is clear-cut, the data on the haematin-induced increase in diene is not so easily interpreted.

The relationship between lipoxidase and haematin in the adopted system was complicated both by the considerable number of variables in the reaction system and also by the destruction of the haematin compound. Due to the complex nature of the system, a number of rational interpretations may be put forward to explain the anomalous increase in diene observed in the presence of haematin. Nevertheless, the simplest explanation, and the one which fits all the facts concerned, is that the increased formation of conjugated diene was catalysed by a product of haematin decomposition.

The concept of a haematin cleavage product capable of conjugating diene, which has not previously been considered, would account adequately for the observations made recently by Blain and Styles (87) on haematin catalysis of diene formation at

relatively high levels of linoleate. This view is also reconcilable with the recent findings of Maier and Tappel (106), who reported that haemoglobin at pH 7 and pH 9 caused rapid oxygen uptake by linoleate at linoleate concentrations above 10^{-2}M, since this increased rate of oxygen absorption could have been caused by the product of haemoglobin decomposition promoting the formation of conjugated linoleate hydroperoxide in the system at the high linoleate levels. The co-oxidation of the haematin compound, moreover, might also be expected to contribute to this increased oxygen uptake.

In the course of breakdown of the haematin molecule, inorganic iron ions are released. Inorganic iron has been shown to have catalytic activity with regard to the oxidation of unsaturated fats (52, 54) and it might therefore be supposed that the observed increase in diene was due to catalysis of diene formation by iron ions. However, inorganic iron salts, ferrous ammonium sulphate and ferric chloride, when added in concentrations at all commensurate with the amount of iron existing in the system on the breakdown of the haematin compound, were found to have no effect whatsoever on diene conjugation in the adopted system.

It seems likely, therefore, that the factor responsible for the phenomenon of increased production of conjugated diene in the lipoxidase system is a cleavage fragment of the haematin compound. Since this effect was detected not only in the presence of the conjugated haem proteins haemoglobin and catalase, but also in the

presence of crystalline haemin, which is not combined with protein, it would appear that the haematin-breakdown product concerned is probably a fraction of comparatively low molecular weight. The isolation of the breakdown product would provide the essential confirmation of this theory.

The data with respect to similarity of behaviour of crude soya extracts and lipoxidase-haematin mixtures give strong indication that haematin is involved in unsaturated-fat oxidation catalysed by soya extracts. Moreover, subsequent determinations of the catalase and peroxidase levels present in Nigerian and Chinese soya have shown that the relative peak heights of the pH-activity curves of the soya extracts can be related to their haematin contents, for it was found that Nigerian soya, the major pH maximum of which occurred around pH 7.6, contained almost five times the levels of catalase and peroxidase detected in Chinese soya, the pH-activity curves of which exhibited very small peaks at pH 7.6.

The present study with model systems had as its major objective the production of a pattern of behaviour comparable to that of crude soya extracts and it should be pointed out that the levels of haematin required to produce the desired effects were, in all probability, higher than the levels normally found in plant tissues. Certain limitations must also be recognised in the use of model enzyme systems. One cannot be assured of

duplicating the conditions occurring in plant tissue extracts. For example, crude soya extracts, when in high concentrations such as those employed in the present study, contain large amounts of extraneous protein and since, in general, proteins in solution exhibit surface active properties, the possibility exists that the increased lipoxidase activity observed between pH 7 and 8 in the case of soya extracts was the result of lowered surface tension and the consequent increase in substrate availability in the heterogeneous reaction systems existing at these pH levels. This effect does not seem unlikely since extracts of Nigerian soya, which showed high activity in the adopted system around pH 7.6, contained a great deal more soluble material than did those of Chinese soya which was a much richer source of lipoxidase. The results of the experiments on cyanide effects on crude and model systems tend to support the line of argument that the increased formation of conjugated diene observed around pH 7.6 in the case of crude soya extracts was not altogether caused by a haematin compound, or, rather, by a haematin derivative.

The present study, however, does provide strong evidence to indicate that the primary function of haematin in linoleate oxidation catalysed by crude soya extracts is the destruction of the linoleate hydroperoxides formed by the action of lipoxidase.

It seems clear, therefore, that a mutual interaction exists

between lipoxidase and haematin in unsaturated-fat oxidation catalysed by soya extracts. On the basis of the observations described in this thesis, the possibility of casual interference from haematins in work with crude plant extracts cannot be disregarded.

CONCLUSIONS

CONCLUSIONS

1. A satisfactory method for the separation of haematin-free lipoxidase from soya-beans has been developed.
2. The characteristics of the haematin-free lipoxidase system have been defined using the diene conjugation method of assay.
3. The unsaturated-fat oxidase behaviour of haematin compounds has been established. In its diene-destroying action haematin appears to behave more like a metal catalyst than an enzyme.
4. The interaction of purified lipoxidase and pure haematin in the adopted reaction system has been examined and the variables involved have been studied. From the data obtained it is concluded that the presence of haematin complicates the behaviour of lipoxidase.
5. In the course of this interaction haematin is destroyed, and evidence has been obtained to suggest that a cleavage product is capable of conjugating diene.
6. The characteristics of crude soya extracts and lipoxidase-haematin mixtures have been examined and compared. On the basis of the work carried out, the possible interference from haematin present in unsaturated-fat oxidations catalysed by crude plant extracts cannot be overlooked.

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APPENDIX I

LIPOXIDASE ASSAY

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Reagents and Apparatus.

The following reagents and apparatus are required:

Reaction Buffer, pH 9:- 10 ml. N ammonia and 30 ml. ammonium chloride, diluted to 2000 ml. with distilled water.

Alternative reaction buffers, pH 2.0 to pH 12.0:- Where observations over a fairly wide range are required, Walpole's (109) pH 2 to 5.5 acetate buffer system and Britton and Welford's (110) pH 6 to 12 phosphate buffer system have been found convenient. Five volumes of Walpole's mixture of 0.4M sodium acetate and 0.4M hydrochloric acid are diluted to one hundred volumes. The 0.4M potassium dihydrogen phosphate/0.2M sodium hydroxide buffer mixture of Britton and Welford is also diluted one in twenty.

The pH of the reaction buffers are determined in the presence of the appropriate quantity of substrate.

Enzyme extraction buffer, pH 4.5:- 114 ml. of 0.1N acetic acid and 86 ml. of 0.1N sodium acetate.

Aqueous sodium hydroxide/potassium cyanide:- A 20% solution of sodium hydroxide in distilled water, containing 5% potassium cyanide, is used to stop the enzyme reaction.

Alcoholic sodium hydroxide:- 4 gm. caustic soda pellets are dissolved in 5 ml. of warm distilled water and diluted to 100 ml. with ethanol. The solution is standardised in the usual way.

Sodium linoleate substrate:- Methyl linoleate, obtained from the Hormel Institute, Minnesota, is stored in evacuated ampoules at -20°C., each ampoule containing 0.21 gm. of methyl linoleate, which is equivalent to 0.20 gm. of linoleic acid. A slight excess of the alcoholic N sodium hydroxide solution is added to an opened ampoule and mixed. The solution is allowed to saponify overnight, and diluted to 100 ml. with distilled water. One ml. of this solution contains 2 mg. of linoleic acid. The substrate thus prepared is freshly made up for each experiment.

Spectrophotometric measurements:- The Hilger Uvispek spectrophotometer is used with wavelength 234 mμ, slit width 5l/ and 1 cm. cells.

Reaction flasks:- The reaction is carried out in 200 ml. conical flasks. These are thoroughly cleaned by soaking overnight in a strong caustic soda solution. Then they are rinsed thoroughly with tap water and then distilled water before drying for use. Between assays they may be rinsed thoroughly with distilled water and dried. Soaps and detergents should never be used for cleaning glassware, since the presence of traces of such surface-active agents may interfere with the assay.

Method.

The preparation of crude soya extracts:- The coarsely ground soya meal is defatted for six hours in a Soxhlet apparatus with

diethyl ether (b.p.: 34°-36°C.). The defatted material is ground to pass a 60-mesh sieve, and 5 gm. suspended in 25 ml. of either distilled water or pH 4.5 extraction buffer. The mixture is shaken for one hour, then centrifuged for ten minutes at 2000 r.p.m. and decanted through a No. 1 Whatman filter paper.

Assay:- The reaction is carried out in the prepared conical flasks, the additions being made in the following order.

Addition	Reaction Flask	Blank Flask
1.	Reaction Buffer, 50 ml.	Reaction Buffer, 50 ml.
2.	Substrate solution, 1 ml.	Aq. sodium hydroxide/ potassium cyanide, 1 ml.
3.	Enzyme solution, x ml.	Enzyme solution, x ml.
4.	Aq. sodium hydroxide/ potassium cyanide, 1 ml.	Substrate solution, 1 ml.

The reaction buffer is added to the flasks and then equilibrated with atmospheric oxygen before use. After the substrate solution has been added, the flask is thoroughly shaken up. The enzyme solution is then added from a microburette with a capillary attachment, the volume of crude or purified enzyme solution used being about 0.05 to 0.30 ml. The flask is rotated during the addition and also during the time the reaction is proceeding. As soon as the enzyme is added, a stop watch is started and after exactly one minute the sodium hydroxide/potassium cyanide solution is added to stop the reaction.

The optical density of the solution is measured in the spectrophotometer against the blank solution prepared as above.

APPENDIX II

MEASUREMENT OF HAEMOGLOBIN DESTRUCTION IN THE LIPOXIDASE-LINOLEATE SYSTEM

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MEASUREMENT OF HAEMOGLOBIN DESTRUCTION IN THE LIPOXIDASE-LINOLEATE SYSTEM

Reagents and Apparatus.

Reaction buffers, pH 7.6 to pH 9:- pH 7.6: 100 ml. 0.4M potassium dihydrogen phosphate and 40 ml. 0.2M sodium hydroxide, diluted to 2,800 ml. with distilled water. pH 9: 100 ml. 0.4M potassium dihydrogen phosphate and 50 ml. 0.2M sodium hydroxide, diluted to 3,000 ml. with distilled water.

Lipoxidase/haemoglobin mixture:- A 2 mg. per ml. solution of purified lipoxidase containing 5 x 10^{-3}M pure haemoglobin is used.

Sodium linoleate solution:- A substrate solution containing the equivalent of 2 mg. per ml. linoleic acid is used.

Butanol/diethyl ether/glacial acetic acid:- n-Butanol, diethyl ether and glacial acetic acid mixed together in the volume ratio 10:15:1.

Hydroxylamine hydrochloride:- A 10% solution in distilled water is used.

o-Phenanthroline:- A 0.75% solution in ethyl alcohol is used.

Potassium ferricyanide:- A 1% aqueous solution is employed to oxidise the haemoglobin present in the reaction system to methaemoglobin.

Colorimetric measurements:- For the measurement of acid methaemoglobin, the Hilger Uvispek spectrophotometer is used with wavelength 498mμ, slit width 5/ and 1 cm. cells. For the estimation of inorganic iron, the Hilger Spekker absorptiometer is employed with Ilford spectrum blue-green filters (No. 604) and 40 mm. cells.

Reaction flasks:- The reaction is carried out in 200 ml. conical flasks. All glassware must be scrupulously cleaned; the method described in Appendix I may be used.

Method.

0.1 ml. of the lipoxidase-haemoglobin mixture is introduced into the reaction flask containing 50 ml. buffer and 1 ml. sodium linoleate solution. The reaction is allowed to proceed for a suitable time. The reaction is then stopped by the addition of 1 ml. of hydroxylamine hydrochloride solution to the flask contents. One ml. of alcoholic o-phenanthroline and 2.5 ml. of the solution of potassium ferricyanide are immediately added to the flask, followed by 10 ml. of the butanol/ether/acetic acid mixture. The contents of the flask are shaken and then transferred to a separating funnel. After equilibration the two phases are separated. The aqueous phase is transferred to a 100 ml. graduated flask and the organic phase to a 10 ml. graduated cylinder. The volume of the organic phase in the cylinder is adjusted to 5 ml. with butanol/ether/acetic acid.

The colour of the ferrous-o-phenanthroline complex in the graduated flask is developed using dilute ammonia solution. The solution is made up to 100 ml. with glass-distilled water and mixed well. The coloured solution is read against a blank solution prepared in the same way as the test solutions except that distilled water is substituted for the lipoxidase and linoleate solutions.

The concentration of inorganic iron in the test solution is obtained by reference to a standard graph constructed from data obtained by use of a standard iron solution, prepared from reagent iron wire. From this figure, the haemoglobin destroyed in the lipoxidase-linoleate system within a certain time may be expressed as a percentage of the iron originally present in the intact haemoglobin. This latter figure is determined by wet oxidation of the pure haemoglobin solution using conc. sulphuric and nitric acids, followed by the colorimetric measurement of the iron by the method described above.

The optical density of the acid methaemoglobin fraction in the organic phase is measured in the spectrophotometer at 498 mμ against a blank solution of butanol/ether/acetic acid mixture. From this reading the haemoglobin destroyed in the particular time interval may be expressed as a percentage of the original acid methaemoglobin value of the system.