A STUDY OF UNSATURATED FAT OXIDASE SYSTEMS AND THEIR INHIBITION

BY

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A Study of Unsaturated Fat Oxidase Systems and their Inhibition.

Unsaturated fats readily oxidise, giving rise to hydroperoxides, which, in turn, oxidise with formation of a complex mixture of monomeric and polymeric oxy-compounds. Fatty acids, such as linoleic, linolenic and arachidonic, and their derivatives, contain methylene-interrupted unsaturated centres which on oxidation form initially conjugated diene hydroperoxide products. The oxidation of these acids may occur autoxidatively or may be catalysed by trace metals, Haematin compounds or by the enzyme lipoxidase. The two former catalysts are non-specific in that they catalyse the oxidation of unsaturated fats in general, but the action of lipoxidase is specific for compounds containing methylene-interrupted unsaturated centres.

Concurrent with unsaturated fat oxidation, many other substances undergo degradative oxidation. Among such compounds are carotene and vitamin A. The loss of such compounds from stored foods is undesirable, as is unsaturated fat oxidation which produces the unacceptable odours and tastes associated with rancidity.

The work described in this thesis is largely concerned with lipoxidase catalysed oxidation of systems consisting of linoleate linoleate-carotene, or linoleate-vitamin A. Some observations on the autoxidation and haematin catalysis of some of these

systems are also included. The systems used were biphasic emulsions of fat in aqueous media.

Initial studies, in which linoleic acid was enzymically oxidised by lipoxidase normally present in crude aqueous extracts defatted soya, confirmed the presence of a lipoperoxidase or hydroperoxide breakdown factor. This factor caused the loss of hydroperoxide content without corresponding loss of diene content from the initial conjugated linoleic acid hydroperoxide product. This factor was shown to act similarly with methyl linoleate hydroperoxide provided that a sufficiently high initial reaction rate could be induced to cause exhaustion of dissolved oxygen in reaction mixtures.

Under anaerobic conditions, the lipoperoxidase factor was shown to break down both linoleic acid hydroperoxide and methyl linoleate hydroperoxide, Which had been produced by lipoxidase oxidation of the respective substrates, but, it could not cause t break down of pure trans-trans methyl linoleate hydroperoxide.

Haemoglobin caused loss of peroxide and diene contents with both the lipoxidase produced linoleate hydroperoxide and the pure trans-trans linoleate hydroperoxide.

Studies of the inhibition of lipoxidase activity were carrie out. A series of unsaturated substances were examined and sever shown to inhibit the enzymic activity. The structure of these inhibitors closely resembled that of the lipoxidase substrates in that they were long chain methylene-interrupted unsaturated compounds. The unsaturation was acetylenic rather than the ethylenic found in the lipoxidase substrates. These inhibitors

are considered to be the most efficient competitive inhibitors of lipoxidase that have been so far demonstrated by anyone.

Further inhibitory studies included polyphenolic antioxidants which are considered to be the best inhibitors of lipoxidase. These compounds were shown to be largely ineffective under the reaction conditions employed. These conditions were low antioxidant levels in the presence of low substrate concentrations and the oxidation of the substrate was relatively rapid. A notable exception to this was the antioxidant, nordihydroguaiaretic acid (NDGA) which completely inhibited the enzymic reaction until it was itself oxidised.

An assay was developed to study the effect of antioxidants on the rate of bleaching of carotene in the absence and presence of fats. The carotene, antioxidants and fats were suspended in agar gels to produce systems which would remain stable over a period of days. The assay was used to study the effect of fats, both saturated and unsaturated, on the rate of autoxidative bleaching of carotene and also to study the efficacy of antioxidants in preventing such bleaching in the absence and presence of the various fatty substrates. This assay was also applied to studies incorporating the unsaturated fat oxidases, lipoxidase and haemoglobin. Inhibitory studies were carried out in the presence of these catalysts.

Finally, the effect of antioxidants on the coupled linoleate-vitamin A reaction catalysed by lipoxidase was studied. It was found possible with several antioxidants to inhibit vitamin A degradation without inhibiting primary linoleate oxidation, and,

with NDGA, both primary and secondary oxidation of linoleate and vitamin A were inhibited.

A C K N O W L E D G M E N T S

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C O N T E N T S

C O N T E N T S

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SECTION IV: (Contd.)

PREFACE

PREFACE

This thesis forms part of a general study being carried out at the Royal College of Science and Technology, Glasgow, to investigate factors affecting the oxidation of unsaturated fats. In particular, interest is focussed on the biological catalysts of this reaction viz., lipoxidase and the haematins. Lipoxidase is an enzyme found in various vegetable materials, notably the legumes, and catalyses the oxidation of unsaturated fats containing methylene-interrupted double bonds as in the so called essential fatty acids, linoleic, linolenic and arachidonic. The haematins——this term indicates iron-porphyrin compounds in general——are less specific, catalysing the oxidation of unsaturated fats.

The general study is being undertaken to further the understanding of the mechanism of the catalysts' action and to find means of controlling or inhibiting completely their action to investigate the conditions under which they may be expected to function and to establish their relative importance in producing unsaturated fat oxidation in biological systems; to study and if possible control the loss of substances which are known to be oxidised concurrently with fat oxidation.

This thesis deals with the inhibition of lipoxidase and where this is shown the mode of inhibition is investigated. It is also concerned with the development of an assay suited to the screening of inhibitors of the oxidation of coupled fats

and carotene where oxidation is produced autoxidatively or catalytically. Finally, it investigates the lipoxidase catalysed coupled oxidation of linoleate and vitamin A and the role of inhibitors in protecting either or both of these substances.

In many biological systems unsaturated fats and the catalytic haematins are known to occur in close association forming a system which is potentially very unstable. Various speculations have been made on the possibility of a metabolic role for such a system although there is no evidence, as yet, that this is the case. In stored foods, where it is recognised that unsaturated fats undergo oxidation causing rancidity and the loss of other essential nutrients, there is little known as to the relative importance of catalytic factors in producing this oxidation.

Studies of the nature of the general programme and of this particular thesis may increase the understanding of the processes of fat oxidation and it is hoped may ease the problems encountered in the applied field.

I N T R O D U C T I O N

INTRODUCTION

Lipoxidase is an enzyme found notably in legumes and is known to catalyse the oxidation of unsaturated fats. In the main this thesis is concerned with this enzyme system, but it is felt that this system may be more easily discussed, if the autoxidative process is considered in the first instance. This consideration will be restricted to the fats containing the methylene interrupted unsaturated system (-CH=CH.CH_2.CH=CH- for which the lipoxidase system is specific. These fats are the so called "essential fats" and include linoleic, linolenic and arachidonic acids and their derivatives.

The primary product of both autoxidation and enzymic oxidation of fats is the fat hydroperoxide. These hydroperoxides undergo secondary reactions giving rise to mixtures of ketones, aldehydes, acids and other oxy-compounds of monomeric and polymeric nature. These secondary changes are not relevant to the present work, and discussion, with one exception, will be restricted to the oxidation of the fat to the hydroperoxide.

History of Fat Oxidation.

Oxidative rancidity in fatty foods has been a problem to man from the earliest times. In 1827 Berzelius (1) noted many of the salient features of this process, viz., induction period, oxygen uptake, CO_2 formation and polymerisation. By

1897, enough was known for Bach (2) to propose a theory of autoxidation based on the formation of a cyclic peroxide by addition of oxygen across the double bond.

$$-CH=CH- + O_2 \longrightarrow -\underset{\displaystyle O-O}{CH-CH}-$$

No chemical evidence of such a cyclic peroxide was ever obtained but this theory was accepted since it adequately explained the known facts that the initial product was peroxidic and that there was a decrease in unsaturation.

This theory has been replaced since 1940 when Criegee et al.(3) and Farmer and his coworkers (4,5) showed the primary product of unsaturated fat oxidation to be a hydroperoxide. Farmer et al.(4) postulated a substitution reaction of a free radical chain nature. This was as shown, where the unsaturated fat is represented by RH.

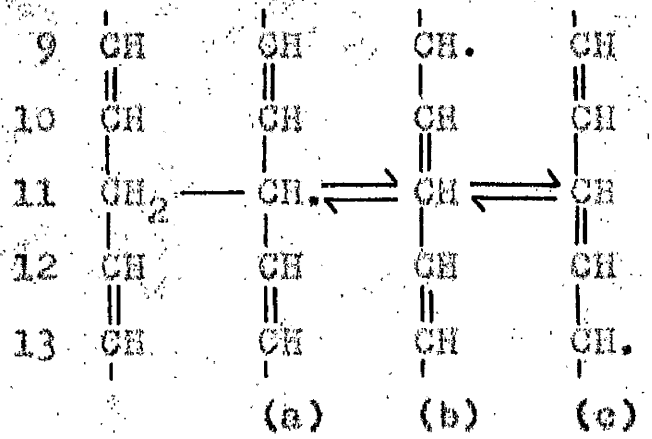
Initiation	$RH \longrightarrow R\cdot + H\cdot$	
Propagation	$R\cdot + O_2 \longrightarrow RO_2\cdot$	
	$RO_2\cdot + RH \longrightarrow RO_2H + R\cdot$	
Termination	$R\cdot + R\cdot$	$\longrightarrow$ inactive products
	$R\cdot + RO_2\cdot$	$\longrightarrow$ inactive products
	$RO_2\cdot + RO_2\cdot$	$\longrightarrow$ inactive products

This involves removal of a hydrogen atom from a methylene carbon adjacent to a double bond. The fat free radical (R.) produced, adds an oxygen molecule forming a peroxy radical ($RO_2\cdot$). This peroxy radical abstracts a

hydrogen atom from another fat molecule (RH) forming the hydroperoxide product ($ROOH$) and a fat free radical which can go through the same chain of events as the initial radical. The number of cycles of this chain depends on the physical conditions attendant on the reaction. This mechanism explains the main features of autoxidation of fats. Until the initial radical(s) are produced no oxidation occurs, thereafter oxidation progresses rapidly. Hence an induction period is observed followed by rapid oxidation.

Mechanism of Linoleate Autoxidation.

Linoleate autoxidises by a similar free radical chain mechanism with one added feature. It was noted by several workers (6,7,8) that during the oxidation of linoleate an increase in spectral absorption at 2340Å occurred. This could be correlated to the extent of oxidation and to account for it Bolland and Koch (9) postulated the mechanism shown



Here the methylene group at the centre of the pentadiene group is doubly activated by the adjacent double bonds and this group loses a hydrogen atom to give the linoleate free

radical (a) which may form the conjugated radicals (b) and (c). These radicals take up oxygen to form the corresponding peroxy radicals and subsequently the hydroperoxides by hydrogen atom uptake. Spectral absorption is due to conjugation of the double bonds.

The expected primary products of linoleate oxidation, using the carbon chain numbering shown, will be the 9-, 11-, 13-hydroperoxides. Thus, if each isomer is formed in equal amounts, two-thirds of the product should be conjugated. Such conjugation should give rise to light absorption of a known magnitude in the range 2300 - 2350Å. The absorption observed (9) corresponded to 70% of the theoretical value for a completely conjugated product, if it is assumed that each mole of oxygen taken up gives rise to a mole of hydroperoxide. This was close to the expected 66.6% if each of the three radicals was present in equal amounts. However, against this Bolland and Orr (10) considered that formation of the conjugated products was favoured thermodynamically and only the 9- and 13- hydroxystearates have been identified (11,12) after hydrogenation of the hydroperoxides.

Most of the early estimations of the extent of conjugation were based on the assumption that trans-trans 10, 12 - octadecadienoic acid was the primary hydroperoxide product and this had a molar extinction coefficient of 32,000. More recent work (13) with infra-red techniques points to the fact that products are the cis-trans isomers which have extinction

value of 28,000. On this basis these workers estimate at least 90% of the products are conjugated in autoxidations carried out at 0°C. Appreciable amounts of trans-trans isomers are formed in oxidations at 24°C. A mixture of cis-trans and trans-trans isomers whose extinction value was 26,400 was obtained by Banks et al. (14) in preparing methyl linoleate hydroperoxide. They isolated the trans-trans isomer (extinction value 29,000) by low temperature crystallisation.

Factors Affecting the Rate of Autoxidation.

Many factors influence the rate of autoxidation. Among these are degree of unsaturation, form of substrate, i.e. free acid or ester, state of dilution, oxygen pressure and temperature. These are discussed in a review by Holman (15) and need not be discussed here beyond noting that for the esters of linoleic, linolenic and arachidonic acids the rate of oxidation increased with the degree of unsaturation (16). The other factors are physical and would be common to most chemical reactions. Of special importance in chain mechanisms are factors which increase or retard the production of the initial free radicals. Such factors in this case are termed pro-oxidative or antioxidative respectively.

(1) Pro-oxidants.

Among the pro-oxidants of fat autoxidation may be listed further physical factors such as light, ultraviolet radiation and x-rays. These merely raise the energy of the substrate

molecules to a level at which chemical reaction may occur.

Addition of known free radical chain initiators, such as benzoyl peroxide, possibly is a more certain means of accelerating autoxidation. These compounds, known to be unstable, break down to give free radicals which produce the chain process in the linoleate and thereby reduce or eliminate the customary induction period (17).

Trace amounts of heavy metal ions are also excellent pro-oxidant factors. They produce free radicals by break down of peroxides present in trace amounts in the fat. Indeed, it is held by some that all autoxidative processes are catalyzed by trace metals (18). It is also noteworthy that this is the accepted mode of action of the hexanins viz., production of free radicals by breakdown of preformed peroxides (19,20, 21).

(11) Antioxidants.

These are compounds ——usually polyphenols or aromatic amines —— which when present in very small amounts effect a significant delay in oxidation of unsaturated fats. Olyce and Eckstrom (22) postulated that they acted as chain breakers This view is generally held at present although the antioxidants are known to produce other reactions. The point or mode of action of these compounds is not readily defined but the theory of Michaelis (23) certainly gives an insight int their radical chain breaking action.

$$ROO\cdot + AH_2 \longrightarrow ROOH + AH\cdot$$

$$AH\cdot + AH\cdot \longrightarrow AH_2 + A$$

Here the fat peroxy radical ($ROO\cdot$), which normally would remove a hydrogen atom from another fat molecule giving the chain mechanism, removes a hydrogen from the antioxidant (AH_2). The resulting antioxidant radical $AH\cdot$ reacts with another antioxidant radical forming a molecule of original antioxidant and a molecule of oxidised antioxidant. The normal chain mechanism in the fat is therefore cut short with consequent diminution of fat oxidation. An essential requirement of the antioxidant is that the intermediary radical $AH\cdot$ is less reactive or more stable than the fat free radical so that it does not give rise to a chain reaction. Antioxidants of the polyphenolic type are thought to obtain this inherent stability from their semiquinone radical.

Antioxidants often have optimal concentrations above which they exhibit a certain pro-oxidative effect. This is considered to be due to their causing production of radicals by destruction of formed peroxides. Privett (24) recently made some observations on this accelerated peroxide destruction by antioxidants.

There is a group of compounds termed synergists which reinforce the effect of antioxidants while themselves being poor or non-antioxidants. The best known of these are phosphoric, citric and ascorbic acids. Again it is not possible to give a general explanation of how these compounds

function. Columbic (25) thought that the synergist regenerated the primary antioxidant at its own expense. Uri (26) recently presented a theory similar in many ways to this while Privett and Quackenbush(27) have questioned it. They suggest that the synergist inhibits the antioxidants' ability to breakdown formed peroxides and in this way leaves the antioxidant to function in its role of oxidation inhibitor.

Phosphoric and citric acids are known to complex with metals and it may be that they function by complexing the pro-oxidative trace metals. Such action cannot be considered synergistic.

There are several reviews of this subject (26,28,29).

Before leaving this section on autoxidation it should be pointed out that most of the work discussed was carried out on fats as single phase substrates. It is doubtful whether it is justifiable to apply such theories of fat oxidation in entirety to aqueous or emulsion systems of fats without considerable reservations. It is in such systems that the enzyme lipoxidase is active.

Measurement of Oxidation.

The oxidation of fats may be followed in many ways. In general, the methods fall into four classes:- (a) oxygen uptake, (b) peroxide determination, (c) secondary product determination, (d) spectrophotometric.

Oxygen uptake is followed by means of standard Warburg

manometric techniques (30).

Peroxide determinations have been carried out using iodometric (31), ferrous ion (32,33), stannous chloride (34) and polarographic (35) assay techniques.

There are a number of assays of secondary oxidation products. Among these are the Kries test (36) and the thiobarbituric acid (T.B.A.) test (37).

Finally, spectrophotometric techniques may be applied. Infra-red (38) and ultraviolet techniques are both of value, with the latter being used more extensively.

Link and Formo (39) recently reviewed the many methods applied in this field.

Lipoxidase.

This enzyme, first discovered in legume seeds, was recognised by its carotene bleaching activity by Haas and Bohn (40) in 1927 and Hauge and Aitkenhead (41) in 1931 noted a similar loss of vitamin A activity of the provitamin carotene in dried alfalfa. This latter activity was likewise considered to be due to the presence of an enzyme system. Similar losses of carotene or vitamin A activity were noted during the 1930's and as a result this enzyme system was known as "carotene oxidase".

Contemporary with this, a fat oxidising enzyme system in soya beans was identified by Andre and Hou (42) and was termed lipoxidase. Craig (43) described a similar system in

white beans.

In 1940, Sumner and Sumner (44) and Tauber (45) separately recognised the fact that the carotene oxidase and lipoxidase systems of soya beans were identical. They showed that carotene bleaching only occurred subsequent to fat peroxidation and in the absence of the primary substrate the secondary reaction with carotene did not occur.

Since this time there has been a considerable literature concerning this system, with several reviews of the subject (46,47,48,49). When dealing with the pre-1950 literature caution has to be exercised because till this time a certain confusion existed between lipoxidase and haematin-catalysed oxidation of unsaturated fats.

A wide variety of systems have been used in studies dealing with this enzyme because of the difficulties encountered in the handling of the substrate. The substrate, being a fat, is insoluble in the aqueous phase and means have to be devised to make it available to the enzyme. The methods used are

1. emulsification of the substrate without emulsifier,
2. emulsification of the substrate with emulsifier,
3. use of the substrate as a soap.

Objections may be raised to all three methods. The first method involves the difficulty of producing reproducible and stable emulsions. The second may be objected to on the grounds that it introduces an unknown factor viz., the emulsifying agent, and the third that the substrate is a soap.

the effect of the emulsifying agent or the soap on the enzyme protein cannot be readily determined.

Another difficulty encountered is that most studies have been carried out using crude or partly purified extracts of the enzyme. Such extracts often contain several enzyme systems, e.g. urease and peroxidase are both present in soya extracts, the effect of which on the lipoxidase system is unknown. Recently, there has been presented evidence of the existence of more than one lipoxidase (50, 51) and also an enzyme system capable of causing hydroperoxide breakdown (52,53). There is also extraneous protein in these extracts and the role played by this in making the substrate available to the enzyme has not been elucidated. Therefore, it would be desirable to have a means of obtaining the crystalline enzyme by a less involved technique than that used by Theorell and his co-workers (54).

The most active source of lipoxidase is soya beans. Other legumes, e.g. peas, lentils, etc., cereals and a few other vegetable materials are alternative sources.

Further discussion of this enzyme system will be considered under appropriate headings.

Substrate Specificity

The enzyme is specific for the so called "essential" fatty acids or their derivatives, viz., linoleic, linolenic and arachidonic acids (55,56). These compounds contain methylene interrupted double bonds and the enzyme is specific

for the cis-configuration of these double bonds. Conjugated double bonds, trans-double bond and long double bond systems are not attacked. Holman and Elmer (16) report that the rate of enzymic oxidation of the active substrates is similar. This is in contrast to their findings for autoxidation where the oxidation rate increases with degree of unsaturation.

These substrate acids——linoleic (octa-deca-di-9,12-enoic linolenic (octa-deca-tri-9,12,15-enoic),and arachidonic (eicosa-tetra-5,8,11,14- enoic)——have the pentadiene (-CH=CH-CH_2-CH=CH-) grouping in common but the positioning of this with respect to the terminal carboxyl groups is variable. Esterification of the carboxyl group or its presence in the ionic form as a soap do not affect the enzyme specificity although such changes considerably alter the solubility and hence the availability of the fat to the enzyme. It appears that the methylene interrupted poly-unsaturated system with the double bonds in the cis-configuration is the sole structural group of consequence.

pH Optimum and Substrate Availability.

The pH optimum found for soya bean lipoxidase varies according to the form of the substrate being used. Thus when dealing with the methyl ester of linoleic acid which may be assumed to be little affected by pH changes an optimal pH value of approximately 6.5 has been observed (57,58). A similar value was obtained using polyoxyethylene linoleate (58) where

it would be expected that the ester group would merely help make the substrate more soluble.

When dealing with linoleic acid or more often sodium linolenate a pH optimum of 9.0 or over has been obtained (58,59). Smith (57) interpreted these high values as being due entirely to the increasing solubility of the substrate increasing its availability to the enzyme and therefore not the true optimum of the enzyme.

Other factors could alter the substrate availability, notably the extraneous protein present in the crude enzyme extracts used. It is such an explanation that is usually put forward to explain the findings of Balls et al.(55) and Theorell et al.(60) who showed the presence of an enzyme activator in soya beans. These workers, using partly purified enzyme extracts on emulsified substrates, found that the rate of oxidation could be accelerated by a heat stable protein extract of defatted soya beans. It is considered that this activator merely made the substrate more readily available to the enzyme. The effect of pH or of substrate changes on this extraneous protein role of making the substrate available to the enzyme is not known and hence the defining of an optimum pH value for the enzyme is made more difficult.

A similar explanation possibly could be applied to recent findings of Koch and his co-workers (50,51). They found that a partly purified soya extract acted on linoleic acid at pH 8.1 and not at pH 5.3. It did not act on

trilinoleate at either pH. Another partly purified extract acted on trilinoleate almost equally at both pH values, while being considerably less active with linoleic acid at these pH values. They postulate from this that there are at least two lipoxidase systems in soya one being "acid" specific and the other "triglyceride" specific. They did not consider the effect of their purification on the role of the extraneous protein.

Products of Lipoxidase Catalysed Linoleate Oxidation.

The products of linoleate oxidation catalysed by the enzyme lipoxidase were recognised to be similar to those of autoxidative processes by Holman and Burr (56) and by Bergstrom (61) i.e. conjugated hydroperoxides. They showed that parallel with oxygen uptake there was increase in light absorption at 234mμ and increase in peroxide value. Bergstrom extracted the hydroperoxides, reduced them and identified 9- and 13-hydroxystearic acids as he had done for autoxidation (11).

Calculation of the molecular extinction coefficient of the products of the catalysed reaction has been carried out and the highest value obtained under optimal conditions is 31,400 (59,62) which corresponds to complete conjugation of the double bonds. This was for oxidation of sodium linoleate at 0°C using the pure enzyme. The extinction value was lower for experiments performed at higher temperatures e.g. 23,000

at 37°C. In the main, all calculations of molecular extinction coefficient give higher values for the catalysed reaction in comparison to the autoxidation reaction.

Privett et al. (63) made a thorough investigation of the products of the oxidation of sodium linoleate at pH 9.0 using a crude enzyme. They showed the product to be cis-trans conjugated hydroperoxides which would be expected to have a molecular extinction coefficient of approximately 28,000. The higher value mentioned above is therefore somewhat difficult to explain unless the pure enzyme forms trans-trans conjugated products which would have extinction value of 32,000. However this seems unlikely even although Privett et al. showed the production of some trans-trans isomers under conditions of higher enzyme concentration, higher temperature or higher oxidation values. Such conditions allow secondary reactions to proceed and the change from cis-trans to trans-trans conjugation is considered to be such a reaction.

Probably of more significance was the observation by Privett et al. that the reduced and esterified product of the primary hydroperoxide exhibited optical activity as did some polymer formed concurrently with the primary product. The product of the autoxidation reaction exhibited no optical activity. The significance of this finding will be discussed in a subsequent section.

Inhibition of Lipoxidase.

Many studies (59,64,65,66,67,68) related to the

Inhibition of lipoxidase have been carried out. In the main the results of these studies are confusing, probably because of the wide diversity of enzyme preparations and substrates used. Polyphenols of the antioxidant type, however, are considered to be the most successful inhibitors of the enzymic action.

Holman (59) found pyrophosphate, fluoride, cyanide, azide, mercury ions, p-chloromercuribenzoate and diethyldithiocarbamic acid to be ineffective. He concluded that the active centres of soya lipoxidase were neither heavy metals nor sulphydryl groups. It should be noted, however, that the lipoxidase obtained from ard beans does give evidence of there being sulphydryl groups present at its active centres (69).

Holman (59) showed that lipoxidase could be competitively inhibited by non-substrate fatty acids, notably the unsaturated elaidolinolenic, conjugated linoleic, oleic and octanoic acids. They observed a 50% inhibition of the enzyme when present in the approximate ratio of 1.1, 1.3, 10 and 100 moles inhibitor/mole linoleate respectively.

The polyphenolic antioxidants are recognised as the best inhibitors of lipoxidase and evidence of such inhibition has been used in characterising an enzyme system as being a lipoxidase (69,70).

Of these polyphenols the most efficient inhibitor is nordihydroguaiaretic acid (NDGA) which has been claimed to function as a competitive inhibitor of soya bean lipoxidase (69 Earlier, Zeppol and his co-workers (71) had shown that under

stated conditions it was possible for NDGA to be oxidised without oxidation of substrate linoleate being observed.

Of the other antioxidants, possibly the most effective are propyl gallate, α-naphthol, α-tocopherol and hydroquinone. Certainly these have been subjected to more thorough investigation than other less common antioxidants. To be effective much higher concentrations (often 10, 100, 1000 fold) of these than of NDGA have to be used. It is often extremely difficult to obtain any true picture of the quantitative efficiency of these compounds since in most cases the inhibition is expressed as a percentage reduction of the initial rate of oxidation of the uninhibited reaction, this latter rate not being quoted. However, from two studies in which this information is provided (68,72) it seems safe to assume that in all the reports the initial rates of oxidation are low. Thus, in Kunkel's study (72) the oxidation rate as measured by diene formation (assuming molar extinction of 28,000) is 1.1×10^{-5} moles/minute, and α-tocopherol (6.1×10^{-5} M) prevents formation of approximately 90% of diene for about six minutes. The total diene formation inhibited is, therefore, approximately equimolar with the α-tocopherol present. In the study of Tappel et al. (68) the initial oxidation rate was 2.6×10^{-4} moles/minute as measured by oxygen uptake and α-tocopherol (2×10^{-4} M) exerted at most 10% inhibition of this rate. In the same publication an inhibition of 39% is quoted for the same concentration of

α-tocopherol, the only notable difference being the pH (7 instead of 9). Since for sodium linoleate, lipoxidase is more active at higher pH it seems reasonable to assume that the initial rate of reaction at pH 7.0 will be considerably lower than the value quoted for pH 9.0. The difference in initial rates may produce the difference in extent of inhibition or the inhibitor may be less efficient at the higher pH. From the data given it is impossible to decide.

Apart from pH change producing differences in inhibitor efficiency, other factors are known to cause variations. Temperature has been shown to play an important role the more common antioxidants being more efficient at 30°C than at 0°C (68). Lea and Kwiatny (73) also note that the tocopherols are more efficient if they are incorporated into the fatty phase prior to emulsification than if they are added after emulsification of the fat. The method of assay may also produce an apparent difference in efficiency. Thus, oxygen absorption studies show lower inhibition than direct spectrophotometric assays. This is most easily explained by assuming that oxygen is absorbed by the antioxidants as these compounds are known to be oxidised in the course of the reaction.

Mechanism of Lipoxidase Oxidation of Linoleate.

The mechanism of lipoxidase action has produced debate. Some investigators have postulated that the enzyme functions as a free radical chain initiator, while others consider that

the enzyme combines with and oxidises each individual substrate molecule. These views will be discussed separately.

(a) Chain Mechanism

Holman and Bergstrom (46,47) postulated the chain mechanism on the grounds that the products of the enzymic oxidation and autoxidation of linoleate were similar, viz., conjugated diene hydroperoxides. The autoxidation process was a known radical chain reaction. Since only the polyphenolic antioxidants served as inhibitors of the enzyme and since they seemed to function in a non-specific manner it suggested that the catalysed reaction also had a chain mechanism.

The enzyme was considered to function by removing an "active" hydrogen from the methylene group interrupting the double bonds of the substrate, giving rise to a free radical which, after isomerisation of the double bonds to give a conjugated radical, took up an oxygen molecule and then a hydrogen radical from a further linoleate molecule. This would produce a linoleate free radical capable of autoxidising in the accepted free radical chain mechanism. The overall picture would be of an enzymically controlled free radical reaction. Holman and Bergstrom (47) suggested that in emulsion systems the enzyme could produce free radicals at the surface of micelles with the chain mechanism proceeding within them.

The antioxidants would function as chain terminators but

unless present in considerable amounts they could only be hoped to slow the reaction rather than stop it completely as the initial free radicals produced by the enzyme would appear too quickly to be efficiently absorbed. This corresponds with observations made on the autoxidative reaction where, once the reaction has proceeded beyond the induction period, addition of antioxidants fails to completely inhibit the reaction rate (74). Nevertheless, it would be expected that the rate would be cut severely, until the antioxidant had been used up. The molar loss in conjugated hydroperoxide formed would be expected to be considerably greater than the molar quantity of antioxidant present. Thus, if an antioxidant reduced a normal chain reaction from a chain of 10 links to one of 2 link the rate of oxidation in the presence of antioxidant would be 1/5 that in its absence. Loss of one molecule of antioxidant would cause the loss of eight molecules of hydroperoxide. In the study by Kunkel (72) already discussed, where α-tocopherol inhibited the oxidation of methyl linoleate the initial rate wa cut to 10% but the total diene hydroperoxide lost at the termination of the "pseudo induction" period was equimolar with the α-tocopherol added.

Kinetic support for the chain mechanism was obtained by Kunkel (72) who showed that for the coupled oxidation of bixin (a dicarboxylic acid carotenoid) and linoleate the reaction velocity was proportional to the root of the enzyme

concentration. This is a chain reaction feature. With this knowledge and from deductions made on the basis of his tocopherol inhibition study he suggested that a chain length of at least twelve occurred. However, Tappel and his co-workers (71) question these deductions as does the author of this thesis.

More recently Haluing and Axelrod (75) showed the existence of a slight induction period for sodium linoleate oxidation catalysed by lipoxidase, if stringent measures were taken to eliminate trace amounts of hydroperoxide. This induction period could be reduced or eliminated by addition of linoleate hydroperoxide in catalytic amounts or by surface active agents in relatively large amounts. This is suggestive of there being a chain mechanism, although it is difficult to explain the action of the surfactants. This mechanism would be similar in character to both the autoxidative and haematin catalysed unsaturated fat oxidation which show induction periods in the absence of some preformed peroxide or initiator, which can breakdown to give the initial free radicals. The main difference was found in the fact that only the hydroperoxides of linoleate and linolenate would reduce the induction period in the lipoxidase oxidation whereas other peroxides of a non-specific nature catalyse the autoxidative or haematin catalysed reaction. This specificity in the lipoxidase reaction could be due to the presence of a

specific enzyme of the lipoperoxidase type. Such an enzyme has been demonstrated in soya extracts (52,53). Similar induction periods have been noted for sodium linoleate oxidation by other workers (51,76).

(b) Enzymic Mechanism.

Tappel and his co-workers consider that the oxidation of linoleate catalysed by lipoxidase is controlled completely by the enzyme. This confirmed an earlier speculation made by Lundberg (77) based on the difference in extinction values of the enzymic and autoxidative reactions. The former is generally higher.

Tappel et al. (71) repeated Kunkel's work and in the presence of surfactant showed the reaction velocity to be proportional to the enzyme concentration. In inhibitory studies they failed to observe many fold reductions of oxygen uptake. Indeed, in some studies with α-tocopherol, the loss of one mole of inhibitor caused the loss of only 0.3-0.4 moles of linoleate hydroperoxide while in another study, the antioxidant NDGA completely inhibited the reaction. If NDGA was functioning merely as a chain breaker they thought that there should have been at least some hydroperoxide product obtained from the chain initiating radicals produced by the enzyme.

They rejected the chain mechanism on the above grounds and proposed the following stepwise mechanism:-

(1) Complex of lipoxidase, linoleate and oxygen formed.

(2) An electron and hydrogen ion from the linoleate transfers to the oxygen forming an HOO. radical. The linoleate radical isomerises to the conjugated form.

(3) The HOO. radical adds to the conjugated linoleate radical forming linoleate hydroperoxide.

(4) The hydroperoxide dissociates from the enzyme which will then complex with further linoleate and oxygen molecules.

The antioxidants (AH$_2$) are considered to donate a hydrogen ion and electron to the linoleate radical forming the radical AH. and conjugated linoleate. The radical AH. then donates a further electron and hydrogen ion to the HOO. radical forming hydrogen peroxide. To account for the non-formation of conjugated products it is postulated that the conjugated linoleate and enzyme do not measurably dissociate and the lipoxidase withdraws a hydrogen ion and electron each time the cycle of events is performed until the antioxidant is consumed. Thus linoleate may be considered as a co-enzyme for the oxidation of secondary substrates with readily abstractable hydrogen atoms such as the antioxidants.

Two objections to this mechanism present themselves. Firstly, no-one has ever detected hydrogen peroxide during inhibitory studies and secondly, of all the antioxidants only NDGA has been shown to oxidise without concurrent linoleate

oxidation.

Since this enzymic mechanism was postulated Privett et al. (63) have shown the products of lipoxidase catalysed linoleate oxidation to be the cis-trans conjugated hydroperoxides and these products exhibited optical activity. This finding strongly suggests that a steric factor, presumably the enzyme, is influencing the product formation. The autoxidative products did not show this optical activity.

Subsequently, Siddiqui and Tappel (69) have postulated another enzymic mechanism incorporating the formation of an optically active product containing the cis-trans configuration of the double bonds. This mechanism may also be described in a stepwise manner:-

(i) A complex of lipoxidase, linoleate and oxygen is formed, with the linoleate and oxygen attached specifically on the enzymic surface.

(ii) The lipoxidase acts as an electron sink abstracting an electron from a hydrogen attached at the methylene group and the resulting hydrogen ion is released into the medium, leaving a linoleate free radical attached to the protein.

(iii) The free radical isomerises specifically to the conjugated cis-trans isomeric radical. Concurrently the oxygen attaches itself to the lone electron of the linoleate radical forming a

peroxy radical.

(iv) The product hydroperoxide forms when the oxygen of the peroxy radical approaches the site on the enzyme where the electron is held. This electron is picked up along with a hydrogen ion.

To account for antioxidants inhibiting lipoxidase they suggest that the linoleate radical abstracts a hydrogen from the polyphenol (and other oxidisable substances) preventing formation of linoleate hydroperoxide. In the same paper (69), NDGA was shown to competitively inhibit the enzyme and to account for this it was suggested that the enzyme abstracted an electron from the inhibitor thus inactivating some of its active sites.

In both publications (69,71) in which an enzymic mechanism is postulated, the possibility of some chain reaction occurring is not ruled out. In the earlier mechanism (71), the chain mechanism is considered to be a side reaction. It is used to explain lower extinction coefficients obtained at higher temperatures and also an anomalous increase in initial oxidation rate at linoleate concentrations above the concentration at which the enzyme is completely saturated. In the later paper (69), the chain mechanism is postulated as an alternative to step (iv). The peroxy radical is considered to be able to pick up a hydrogen atom from an adjacent linoleate molecule instead of an electron from the

enzyme and a hydrogen ion from the medium. The author states that if this alternative reaction occurred, "This would lead to a lipoxidase-modulated and controlled chain reaction".

In a more recent brief review of lipoxidase (78), Tappel has postulated a mechanism of a similar nature to his earlier one involving formation of linoleate and hydroperoxy free radicals which combine to give the linoleate hydroperoxide product.

Lipoperoxidase

The existence of such an enzyme system in crude soya extracts was first postulated by Blain and Styles (79). They found that defatted soya flour, extracted with distilled water and acetate buffer separately, gave rise to enzyme preparations which differed in their activity. The former produced diene conjugation of sodium linoleate and secondary bleaching of carotene both in the absence and presence of preformed diene. The buffer extract did likewise to a much lesser degree in the absence of preformed diene but in its presence the bleaching of carotene was much enhanced without corresponding enhancement of diene formation. They considered there was an enzyme present in the buffer extract which decomposed the conjugated peroxide with concurrent bleaching of carotene. This action is similar to that known for haematins.

More recently, Blain and Barr (52) and Koch et al. (53,80) have demonstrated the loss of linoleic acid hydroperoxide in the presence of relatively high concentrations of soya extracts.

The former workers (52) using a thiocyanate assay showed that after a brief rapid formation of hydroperoxide there followed a rapid breakdown of the hydroperoxide, without corresponding loss of diene. They postulated that the rapid change from formation to decomposition of hydroperoxide was due to exhaustion of the dissolved oxygen in the reaction mixture. Gini and Koch (53) using the same assay similarly demonstrated linoleic acid hydroperoxide breakdown with comparatively large quantities of crude soya extracts. They carried out heat inactivation experiments and preferentially destroyed the lipoperoxidase. They also preferentially inhibited this enzyme with potassium cyanide and demonstrated the existence of a peroxidase enzyme system using standard qualitative assays for this type of enzyme.

The products of lipoxidase oxidation of linoleate have been shown to be optically active and this has been used as evidence for an enzymic mechanism. If the lipoperoxidase enzyme was specific for one isomer of a mesomeric mixture of linoleate hydroperoxides produced by chain mechanism an optically active product would result.

Assay of Lipoxidase.

Various methods of assaying lipoxidase activity are used but no universally satisfactory assay technique applicable to all aspects of lipoxidase work has been devised. The techniques used may be classified under four headings:-

1. oxygen uptake.
2. peroxide determination.
3. spectrophotometric.
4. oxidation of secondary products.

The first three types are common to fat autoxidation studies but the fourth is more peculiar to lipoxidase assays.

Oxygen uptake studies follow the standard Warburg manometric techniques developed for tissue respiration studies. Several studies (61,62,65,66,71,81) of lipoxidase catalysed oxidation of fat have been made using this method. For such assays the advantage is that the substrate state is not important. Thus soaps and emulsions may be used and as a result pH changes over a wide range are possible. Disadvantages are that for accuracy fairly long reaction times are desirable and this increases the possibility of error produced by changes in substrate dispersion. Other oxidations may also be inadvertently assayed and this is especially true where oxidisable additives such as inhibitors are present.

Estimation of fat peroxides is another assay used. In the main, iodometric assays have not been used in lipoxidase studies. However, many studies (50,55,82,83) have estimated peroxides by the method based on the conversion of ferrous to ferric iron which can be estimated colorimetrically as its thiocyanate. This technique has been subjected to numerous modifications to accord with conditions of use. It is a good

assay in so far as it requires very little fat, it is rapid and is reasonably reproducible under standard conditions. In general, it tends to give proportionately higher peroxide values than other peroxide estimations and this is observed particularly under aerobic conditions.

Spectrophotometric assays of conjugated diene are considered to be the most accurate means of estimating fat oxidation. An assay devised by Theorell et al. (83) used homogeneous linolenate in oxygenated buffer at pH 9.0. A reaction of two minutes was allowed before stopping the reaction with ethanol. This ethanolic mixture was diluted with 60% ethanol and this solution used to determine optical density at 234mμ in a spectrophotometer. Tappel et al. (68,71) further modified this assay by carrying out the reaction in a cell in a thermostatically controlled compartment of a spectrophotometer making direct measurements of optical density at 15 second intervals.

These assays are extremely reliable and proportionality between conjugated diene, enzyme concentration and time have been demonstrated. The advantage is that the enzyme is acting in a single phase which eliminates the uncertainty of the biphasic systems. However, since the biphasic system is more often present where practical problems concerning the enzyme are involved the above assays are somewhat idealised.

Spectrophotometric assays have been used for emulsion

systems of fats. Smith (57), using emulsified methyl linoleate in aqueous ethanol, extracted the substrate with cyclohexane for spectrophotometric assay. Another assay, using samples of reaction mixture in 60% ethanol, has also been used to determine diene contents.

Spectrophotometric assays are applicable to inhibitory studies and have been used for such (71). This is probably the best technique for such studies although there is the possibility that the inhibitors or oxidation products of them may absorb in the conjugated diene range 230-235 mμ. This could influence results considerably and care must be exercised to ensure that any such absorption is allowed for before estimating inhibition.

Assays involving the coupled oxidation of secondary substrates in the presence of unsaturated fat and lipoxidase are numerous. These assays usually involve the bleaching of carotenoids although one study (55) made observations on the oxidation of the redox indicator leuco-o-chlorophenolindophenol. Many workers (55,67,76,84,85,86,87,88) using various modifications of the basic system have employed the oxidation of β-carotene as their lipoxidase assay. The advantage of this is that it gives a direct colorimetric assay and it has been shown that, for a standard reaction, carotene destruction and diene formation are proportional to enzyme concentration and to time. However, the limits within which this is the

case are often very narrow. Another disadvantage is that of reproducing reaction systems. Carotene is insoluble in aqueous medium and it has to be added in alcohol-acetone solution in order to obtain a stable suspension. Evaporation of solvent can upset this dispersion. It also has been found necessary to follow meticulously a standard procedure in order to reproduce reaction mixtures.

Other carotenes have been examined and Sumner and Smith (89) consider the dicarboxylic acid carotenoid, bixin, to be superior for assay purposes on account of its more intense colouration for equivalent amounts of material.

Secondary Oxidations Coupled with Fat Oxidation.

In the course of fat oxidation (autoxidative and catalysed oxidations) other substances can be readily oxidised. These secondary or coupled oxidations have been studied and among the many substances found to be co-oxidised with fat are biotin (90), chlorophyll (67), hemin (91) vitamin-A (92), carotene (93), ascorbic acid (vitamin-C) (27), vitamin-D(94), vitamin-E (27), glutathione (95) and dyes (67).

The mode of degradation of these secondary substrates in the course of fat oxidation is thought to be by removal of hydrogen atom(s) from the secondary substance by the fat free radical intermediates. This gives rise to an unstable free radical of the secondary substrate which undergoes oxidative degradation.

Probably the most widely studied of these coupled reactions are vitamin-A and carotene degradations and since studies of these reactions were undertaken in the course of work for this thesis, it seems pertinent to discuss such coupled reactions from the stand point of these secondary substrates.

Destruction of Vitamin-A and Carotene.

Vitamin-A and carotene are both highly unsaturated compounds. As a result they are labile molecules and readily undergo oxidative degradation. Light, even of a diffuse nature, may catalyse their breakdown (96). Protection of these compounds may be enhanced by the presence of antioxidants but direct sunlight or ultraviolet light has been shown to nullify this protection (96).

Vitamin-A and carotene are both fat soluble and it is in association with lipid material that their destruction has been most widely studied. Much of the early work was concerned with losses of vitamin-A from fish oils in which it occurred naturally. Olsen et al. (97) have recently reviewed much of this work.

Over the last fifteen years Bickoff and his co-workers have studied carotene loss in model systems (98,99,100,101). They suspended the carotene in refined mineral oil in the presence of antioxidants and studied the stability of the carotene. A similar study has been made by Budowski and Bondi(102) who studied both carotene and vitamin-A losses.

In the former series of studies, Bickoff demonstrated many highly effective antioxidants. For example, some substituted pyrogallols were capable of a 200 fold protection of carotene. However, most of these compounds were almost entirely useless in protecting carotene present in alfalfa meal. Even one of the most effective antioxidants, 2,2,4-trimethyl-6-ethoxy-1,2-dihydroquinoline(santoquin), in the oil phase failed to enhance greatly the protection of carotene in the alfalfa meal. Possible reasons for this are that there is a lack of contact of the antioxidant and carotene and that fat oxidation catalysed by lipoxidase in the meal is occurring. Alfalfa is a known source of the enzyme (103,104).

The question of contact of antioxidant and carotene in polyphasic systems such as alfalfa meal is of fundamental importance. Thompson (105) suggested that this was the reason for water soluble antioxidants such as pyrogallol being poor carotene protectors in water containing media. Bickoff and his co-workers (98,101,106) emphasise this point that to be efficient the antioxidant and carotene must be soluble in the same phase. This is of fundamental importance in all polyphasic fields where the use of antioxidants is necessary. Stadler et al. (107) found that the addition of fat to poultry feeds decreased carotene loss. The fat may be considered to assist in bringing the carotene and natural antioxidants into better contact.

In contrast to this, addition of unsaturated fats increased

the loss of both vitamin A palmitate and carotene in the study of Budowski and Bondi (102). In this case, the vitamin was present in an oil phase and addition of fat could in no way enhance the vitamin's dispersion or antioxidant contact and only the pro-oxidative character of the added fat was observed.

Vitamin A and Carotene Destruction Coupled with Unsaturated Fat Oxidation.

That unsaturated fat oxidation causes vitamin A losses has been suspected since 1920 (108,109). The observed losses of vitamin in fish oils were certainly largely due to this. These oils are extremely rich sources of highly unsaturated fats which themselves are capable of readily oxidising and which, in turn, are capable of causing coupled vitamin A oxidation.

More recently, Holman (92,93) studied losses of carotene and vitamin A in autoxidising linoleate and found that these compounds were largely destroyed before 10% of the linoleate had oxidised. It also appeared that vitamin destruction had little effect on the course of linoleate oxidation.

Lohmar and Tookey (110) studied carotene-linoleate oxidations in aqueous medium and found that, below the critical micelle concentration of sodium linoleate, the oxidation of carotene was not proportional to linoleate oxidation. Above i the rate of carotene loss increased with increasing levels of carotene or linoleate.

In a similar study of lipoxidase catalysed coupled oxidati

of linoleate and carotene (76) the rate of carotene loss again increased with increasing levels of carotene. Carotene concentration had little effect on the rate of linoleate oxidatio while variation of linoleate concentration produced an anomalous maximum in linoleate oxidation. This maximum was shown to correspond to the critical micelle concentration of the linoleate. Below this concentration the linoleate oxidation rate decreased sharply and above it there was a notable decrease, followed by an increase in the rate at higher concentrations. These rate changes occurred both in the presence and absence of carotene. The rate of carotene loss was proportional to the rate of linoleate oxidation throughout.

In an earlier study (46,111) Holman showed that carotene destruction and linoleate oxidation were proportional to enzyme concentration and to time. Like Tookey et al. (76) he showed that carotene destruction was proportional to its concentration. In contrast, however, he found that increasing concentration of carotenoid, both β-carotene and bixin, caused a decrease in linoleate conjugation. He calculated that 1 mole of β-carotene and 1 mole of bixin prevented formation of 43 moles and 26 moles of conjugated linoleate respectively. He surmised that the carotenoids were acting as chain breakers.

The fact that carotene bleaching is proportional to enzyme concentration, as is linoleate oxidation, justifies

the use of carotene bleaching assays for lipoxidase estimations. These are numerous and within their own prescribed conditions they will give reasonable results.

In conclusion, therefore, it is considered that the biologically important vitamin A and carotene are labile compounds whose lability is due to oxidative degradation thought to be brought about *via* a chain mechanism. This is supported by the fact that the degradation is reasonably rapid and may be inhibited by chain breaking antioxidants. This lability is increased when the vitamin is in contact with unsaturated fats in which the vitamin is soluble. It has been suggested that this increased lability is produced by the vitamin acting as a breaker of the fat oxidation chains.

Vitamin A and its provitamin, carotene, are important dietary factors for animals and at the present time there is an increasing tendency to add synthetic vitamin to animal feeds. This is pointless, as well as costly, if the vitamin is largely destroyed before the feeds are consumed. Study of both fundamental and practical aspects of vitamin destruction would appear to be worthwhile and it is hoped that part of the work to be described here may, in some little way, fulfil this purpose.

S E C T I O N I

ASSAYS OF LIPOXIDASE AND A STUDY OF THE LIPOPEROXIDASE FACTOR.

SECTION I

ASSAYS OF LIPOXIDASE AND A STUDY OF THE LIPOPEROXIDASE FACTOR

Initially it was considered desirable to establish assay methods and conditions suitable for the study of inhibition of a lipoxidase system. Certain requirements were demanded of the system and these are listed.

1. A physiological pH was desired, although it was realised that a biphasic emulsion system would result. Since it was reported that lipoxidase had optimal activity at pH 6.5 this pH was chosen. The buffer selected was McIlvaines phosphate-citrate buffer since this buffer is capable of giving a fairly wide pH range (3.0-8.0) if for any reason this is required and since the constituents are known to complex with metal ions. This would minimise trace metal catalysis.
2. A stable emulsion was wanted so that sampling of the reaction mixture could be performed over a reasonably long period of time.
3. The system had to be flexible with regard to substrate and to solvents in which substrates and antioxidants might be added.

The system evolved is given in Appendix I. This conformed to the conditions stated although methyl linoleate

tended to come out of solution. It was found possible to overcome this by producing a finer emulsion of the ester by use of a top drive homogeniser. This was only used on occasions when it was considered to be necessary.

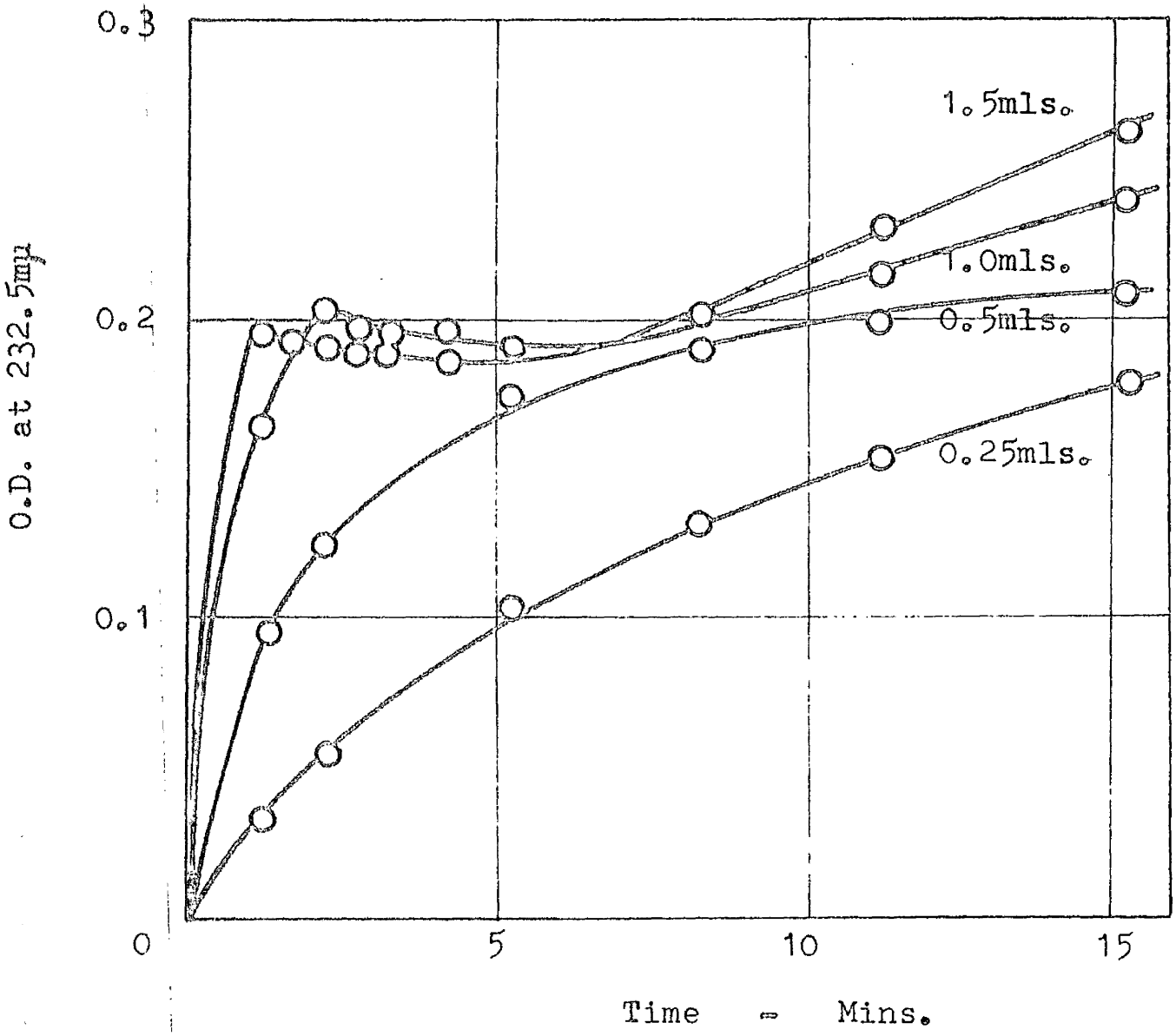
For inhibitory studies it was found necessary to conduct control (non-inhibited) reactions concurrent with inhibited reaction, since aqueous extracts of defatted soya flour, which were used as a source of lipoxidase and which were prepared daily lost activity in the course of the day. This occurred even if the extracts were kept in the refrigerator.

It was found to be most convenient to prepare these soya extracts in the same way each time. This preparation was termed the "Standard Soya Extract" and is described in Appendix I.

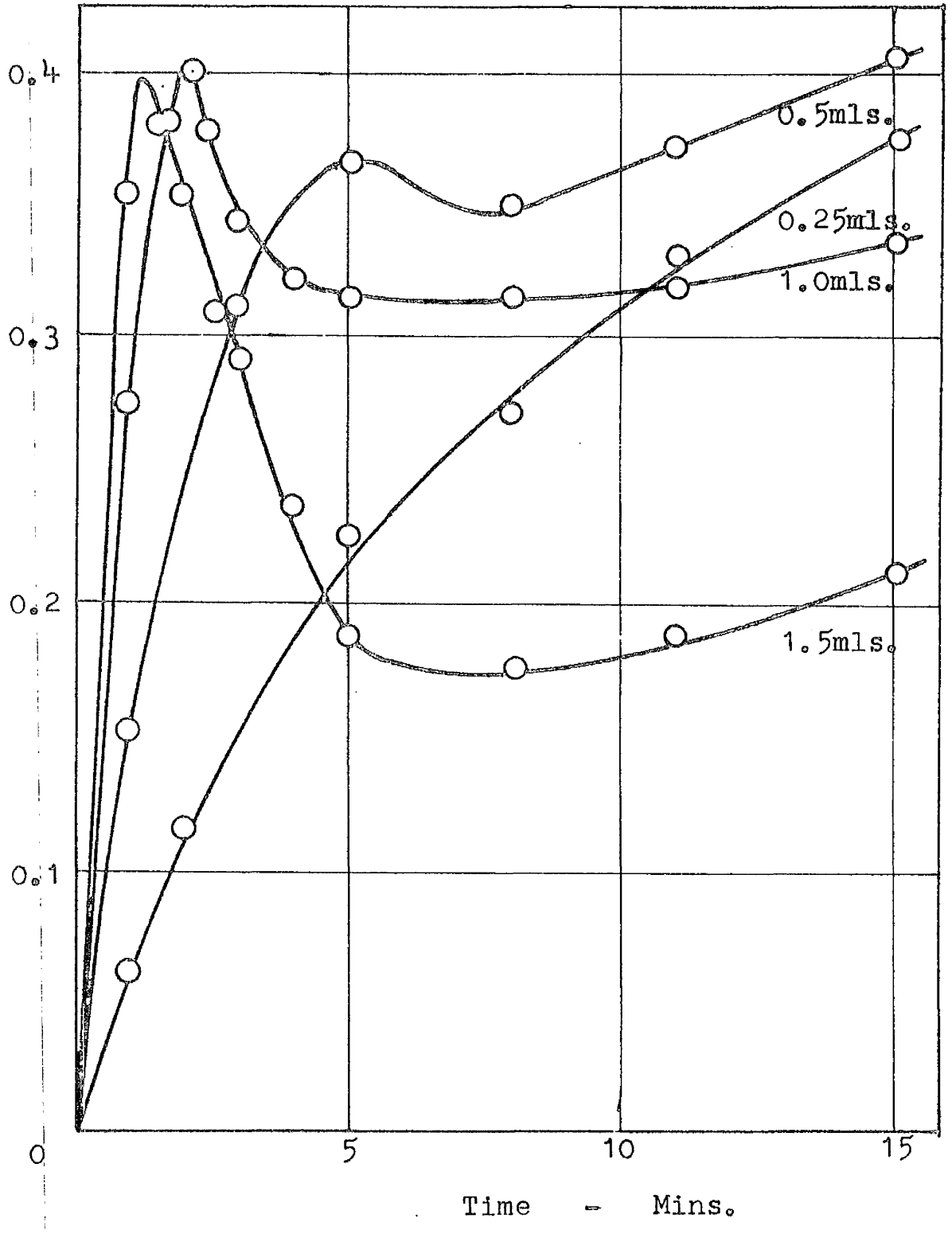
Initially, variation of conditions of the standard system was studied. Because of the biphasic nature of the emulsion system being used, it was possible to use only limited amounts of substrate. This, in turn, limited the quantities of enzyme which could be used and also made desirable the use of assays which were sensitive enough to detect the oxidation of the small quantities of substrate being used.

The assays investigated were such as to allow the course of oxidation of individual reaction mixtures to be followed over varied periods of time. This was considered to be a more satisfactory means of studying the effects of inhibitors than

Fig. 1 Effect of Enzyme Concentration on the Rate of Conjugated Diene Formation in the Course of Linoleic Acid Oxidation.



g.2 Effect of Enzyme Concentration on the Rate of Hydroperoxide Formation in the Course of Linoleic Acid Oxidation.



estimating the extent of oxidation after a given time or comparing the initial rates of an inhibited and uninhibited reaction.

Most of this preliminary background work is not described here. However, in the course of this work it was found possible to make further limited studies concerning the lipoperoxidase system which had been shown to be present in soya extracts by earlier work in this laboratory (52). A report of these studies is given in the first section.

Effect of Enzyme Concentration on Linoleic Acid Oxidation.

With linoleic acid (6.6×10^{-4} M) in the standard assay, the effect of the addition of various volumes of crude soya extracts was studied. Linoleic acid oxidation was followed by measuremen of conjugated diene and hydroperoxide formation at varied time intervals (These assays are described in Appendix I). The results are shown in Figures 1 and 2.

For low enzyme (0.25mls.) additions, diene and peroxide values increase in parallel with time. For higher additions ($\geqslant$ 0.5mls.) there is a notable discrepancy between these values. The diene value (Fig.1) increases rapidly till an optical density of approximately 0.2 is attained when there is a slight decline in this value followed by a subsequent slow rise due to further conjugated diene hydroperoxide formation. The peroxide value (Fig.2) increases rapidly then a sharp

Fig.3 Effect of Stirring on Rate of Conjugated Diene Formation in the Course of Linoleic Acid Oxidation.

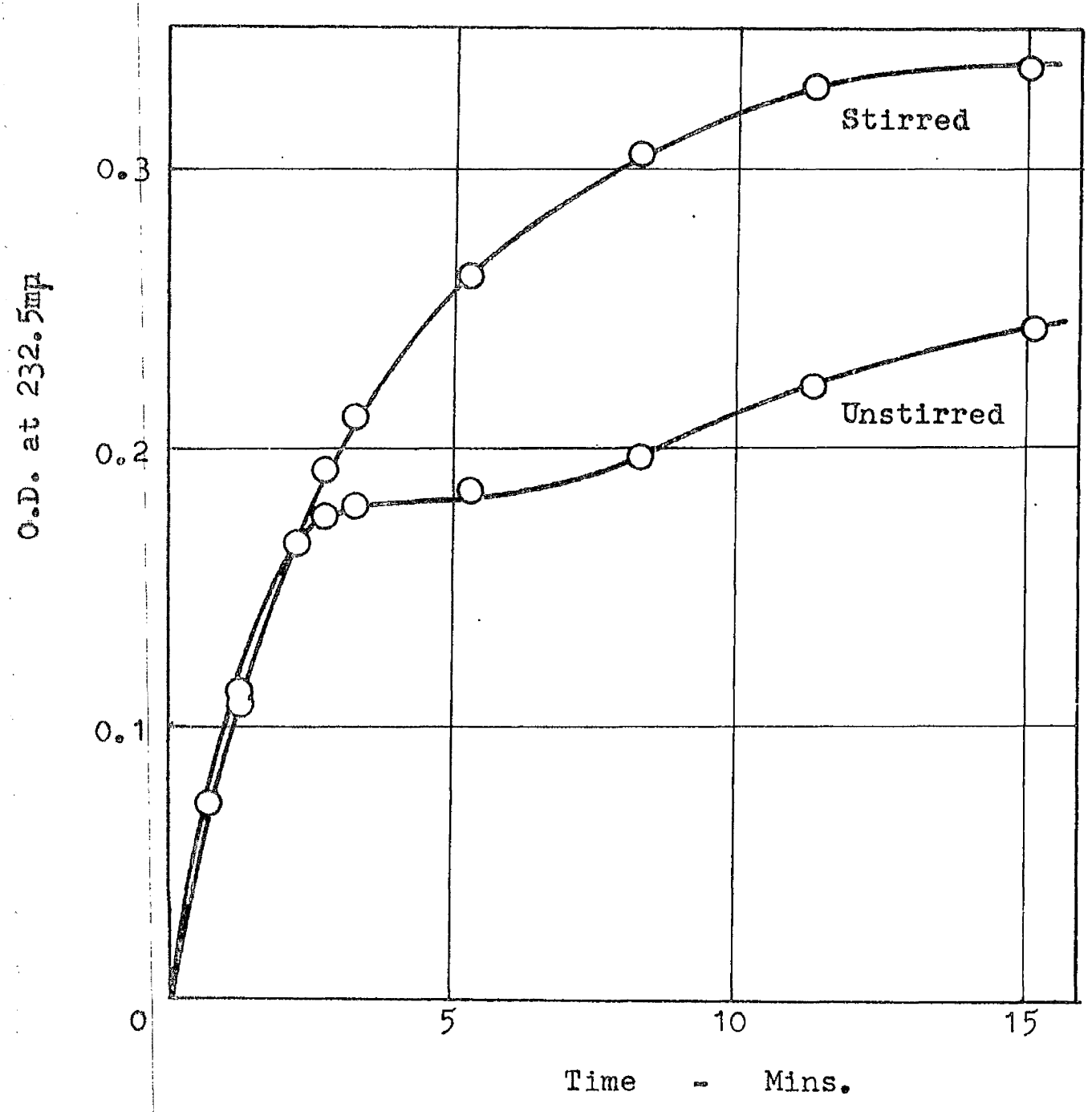
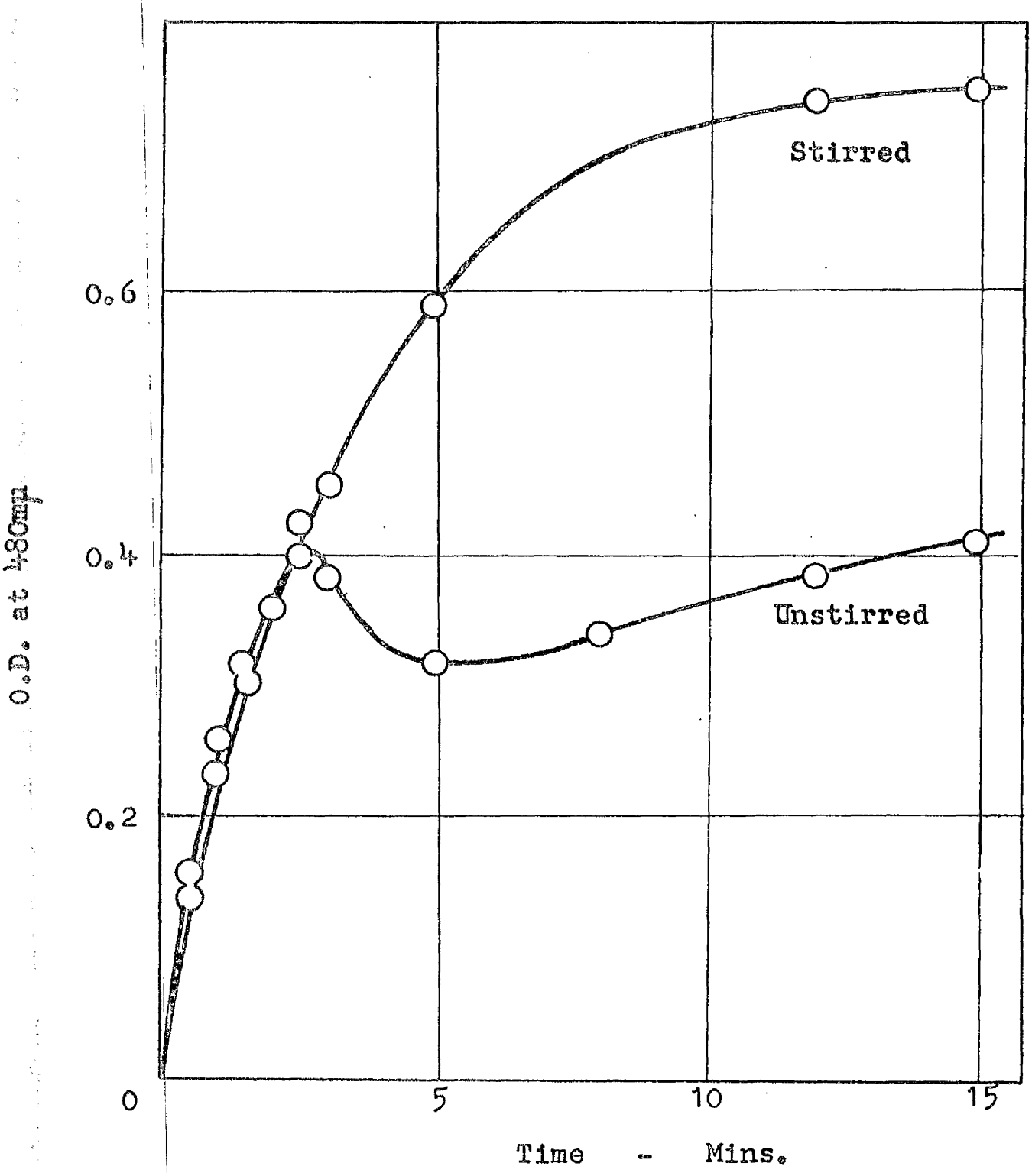


Fig. 4 Effect of Stirring on Rate of Hydroperoxide Formation in the Course of Linoleic Acid Oxidation.



decline is observed to be followed by a slight slow increase in this value. The decline in diene and peroxide values are in no way comperable, the former constituting a few percent and the latter up to 50% of the total product formed.

Similar results were obtained by Blain and Barr (52) and Gini and Koch (53) who suggested that the loss was due to the presence of a lipoperoxidase enzyme system. The former workers suggested that the sudden change from hydroperoxide formation to destruction could be attributed to oxygen exhaustion, and evidence of the destructive factor was obscured by increasing the oxygen tension of the reaction mixture by pre-oxygenation of the buffer solution.

It was found here that evidence of the destructive factor could also be obscured by stirring the reaction mixture. In this case magnetic stirrers were used. Presumably this made sufficient oxygen available to the system for the lipoperoxidase activity to be unobservable. Typical results are shown in Figures 3 and 4 for a reaction mixture of 6.6×10^{-4} M linoleic acid and 1.0ml. of standard soya extract. It is noteworthy that little difference in the initial rates of formation of conjugated diene hydroperoxide is observable until the point of oxygen exhaustion.

Fig. 5 Effect of Enzyme Concentration on Conjugated Diene Formation in the Course of Methyl Linoleate Oxidation.

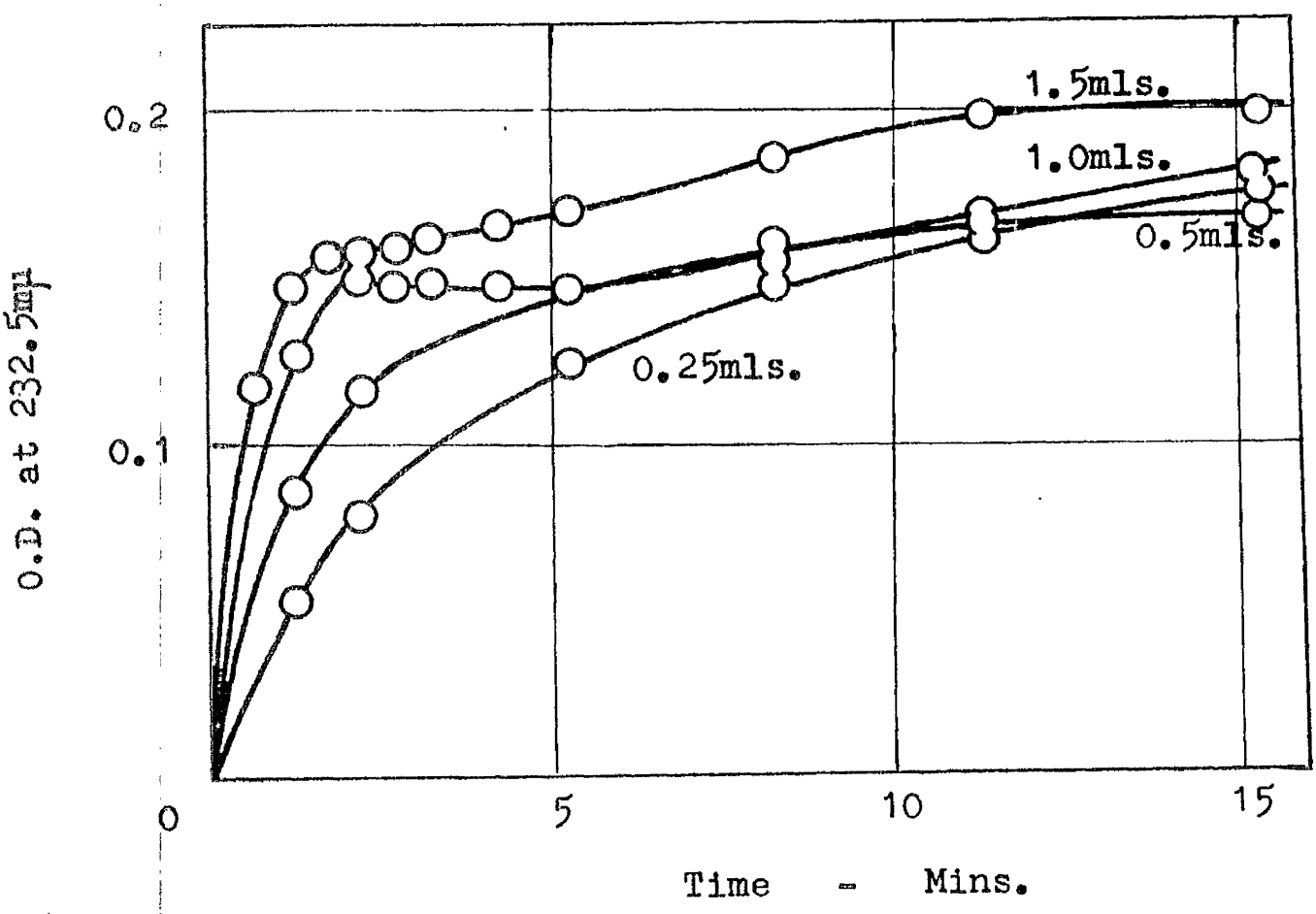
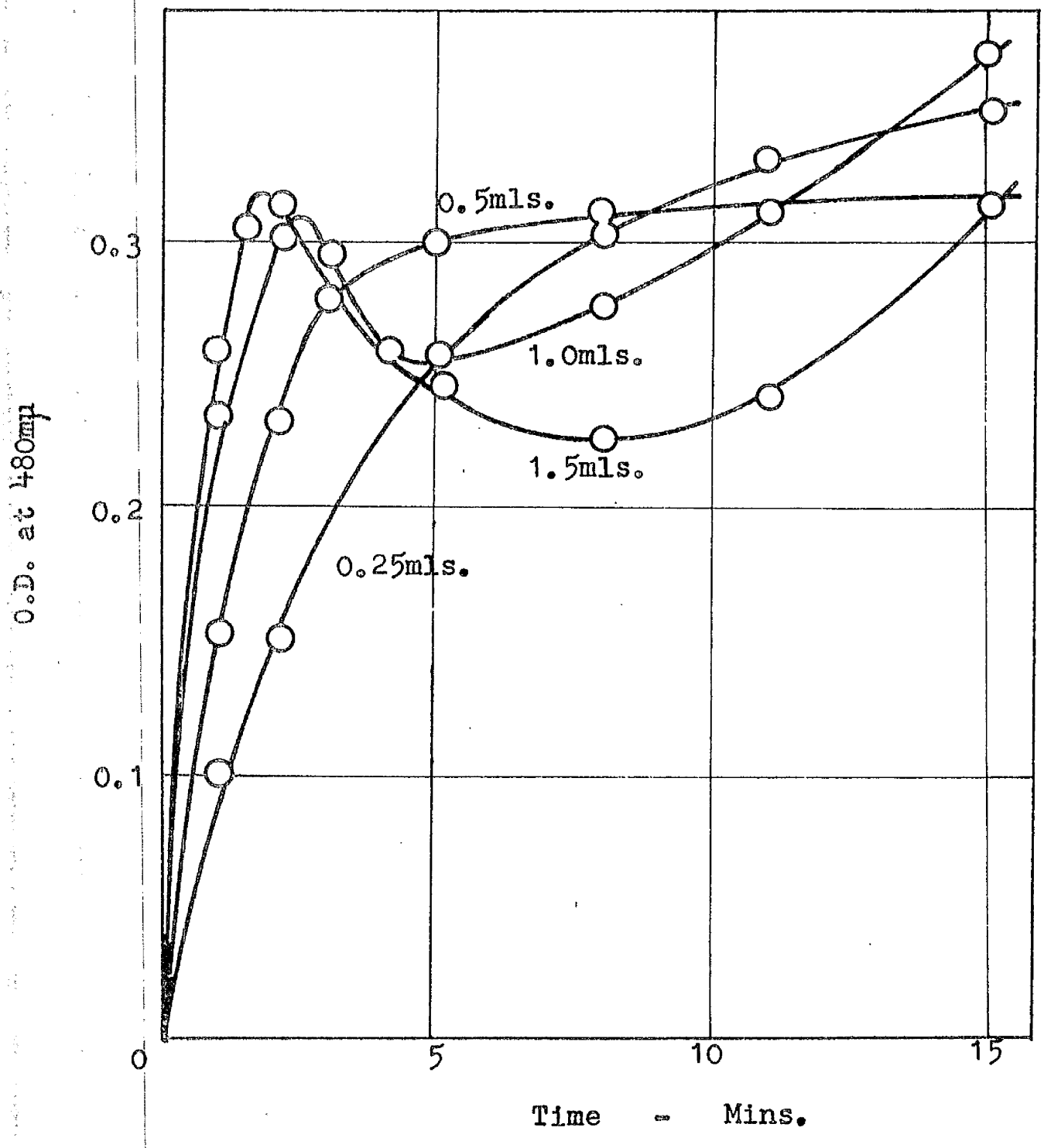


Fig.6 Effect of Enzyme Concentration on the Rate of Hydroperoxide Formation in the Course of Methyl Linoleate Oxidation.



Effect of Enzyme Concentration on Methyl Linoleate Oxidation.

Methyl linoleate was substituted for linoleic acid in a similar series of experiments. It was found impossible to show evidence of the hydroperoxide breakdown even at high enzyme levels (2.0mls.). This was because the initial rate of oxidation could not be raised to a level where dissolved oxygen could be exhausted. It was considered that this could be due to a lack of substrate being available to the enzyme. A finer emulsion of methyl linoleate was prepared by use of a high speed top-drive homogeniser. When high levels of enzyme were added to this system it was possible to show hydroperoxide breakdown. The effect of a range of enzyme concentrations on the rate of diene and hydroperoxide formations are shown in Figures 5 and 6. These are similar to previous curves for linoleic acid. Stirring again removed evidence of the breakdown factor.

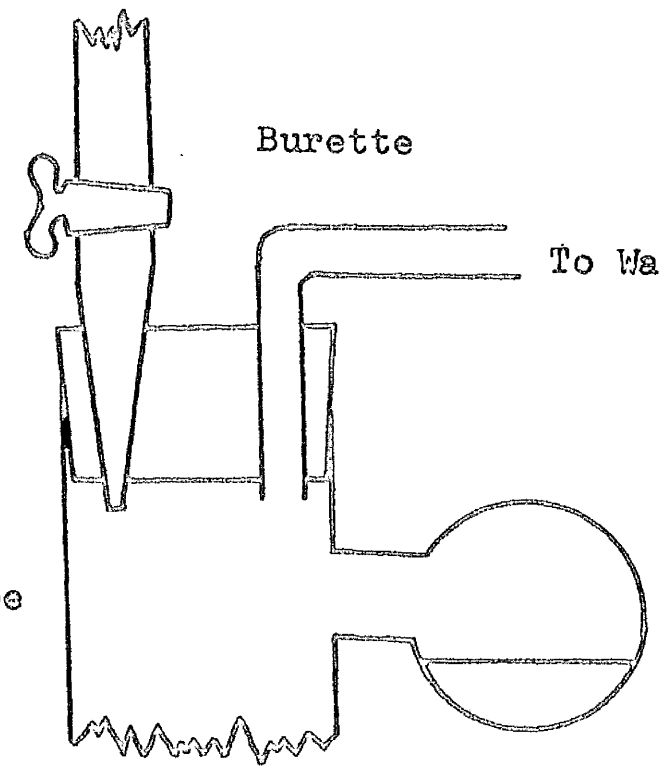
It should be noted that homogenising of linoleic acid emulsions had little or no effect on the initial rate of oxidation.

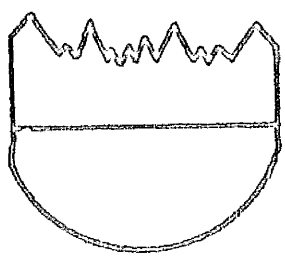
Hydroperoxide Breakdown in Vacuo.

Since it was considered that the linoleate hydroperoxides were destroyed enzymically under anaerobic conditions, it was decided to study this further using prepared hydroperoxides in an evacuated system.

The hydroperoxides of linoleic acid and methyl linoleate

Fig.7 Apparatus Used to Study the Anaerobic Breakdown of Linoleate Hydroperoxide Catalysed by Soya Extract.





Reaction Mixture:-

Buffer + Linoleate Hydro-peroxide

were prepared by enzymic oxidation using crude soya extracts (see Appendix II). The hydroperoxides were not pure samples but ethanolic solutions of them were prepared containing 5mgs. lipid/ml. ethanol. A sample of pure trans-trans methyl linolenate hydroperoxide (kindly provided by Dr. Hardy, The Torry Research Station, Aberdeen) was similarly prepared at a concentration of 5mgs./ml. ethanol. These solutions were used in the experiment described below.

Hydroperoxide solution (0.4mls.) was added to phosphate-citrate buffer pH 6.5 (10 mls.). A portion (5mls.) of this was pipetted into absolute ethanol (10mls.) and samples (1ml.) of this latter ethanolic mixture were pipetted into absolute or 60% aqueous ethanol (12.5mls.). The peroxide content of the sample in absolute ethanol was determined using a scaled down version of the Thiocyanate assay of Koch et al.(50). The diene content of the sample in 60% ethanol was determined using a spectrophotometer.

A second portion (5mls.) of the original emulsion was pipetted into a Thunberg tube. A sample (0.25ml.) of standard soya extract was added to the side arm of this tube and a rubber stopper, through which a burette and a glass tube were passed, was fitted to the Thunberg tube (see Fig.7).

The glass tube was attached to a water pump to evacuate the Thunberg tube and then the soya extract was tipped from the side arm into the reaction mixture. After the times shown

Table I. Catalysed Linoleate Hydroperoxide Destruction, in Vacuo.

Substrate	Catalyst	Reaction Time	Diene Value Before	Diene Value After	Peroxide Value Before	Peroxide Value After
Linoleic acid hydroperoxide (soya prep.)	Soya extract	1min.	0.171	0.200	0.414	0.477
			0.171	0.180	0.424	0.464
		2min.	0.167	0.188	0.391	0.284
			0.191	0.150	0.384	0.209
		4min.	0.159	0.162	0.350	0.249
			0.181	0.165	0.350	0.213
		6min.	0.182	0.122	0.377	0.188
			0.192	0.130	0.357	0.175
		8min.	0.175	0.210	0.400	0.144
			0.184	0.152	0.385	0.106
Methyl Linoleate hydroperoxide (soya prep.)	Soya extract	1min.	0.065	0.048	0.223	0.179
			0.050	0.048	0.196	0.170
		2min.	0.062	0.045	0.196	0.110
			0.058	0.044	0.162	0.120
		4min.	0.102	0.055	0.209	0.121
			0.070	0.060	0.198	0.114
		6min.	0.075	0.053	0.214	0.124
			0.065	0.057	0.208	0.118
		8min.	0.078	0.059	0.212	0.134
			0.080	0.062	0.208	0.141

Table I (contd.)

Substrate	Catalyst	Reaction Time	Diene Value Before	Diene Value After	Peroxide Value Before	Peroxide Value After
Methyl Linoleate hydroperoxide (pure trans-trans)	Soya extract	1min.	0.295	0.268	0.685	0.650
			0.304	0.292	0.668	0.658
		3min.	0.325	0.291	0.720	0.702
			0.333	0.316	0.702	0.715
		4min.	0.332	0.271	0.673	0.648
			0.337	0.276	0.685	0.640
		6min.	0.354	0.260	0.700	0.670
			0.359	0.272	0.650	0.662
		8min.	0.301	0.248	0.550	0.620
			0.311	0.258	0.560	0.628
Methyl Linoleate hydroperoxide (pure trans-trans)	Haemoglobin	1min.	0.289	0.150	0.680	0.420
			0.297	0.167	0.628	0.399
		2min.	0.320	0.175	0.723	0.385
			0.316	0.174	0.718	0.411
		4min.	0.314	0.167	0.718	0.385
			0.322	0.177	0.729	0.367
		8min.	0.303	0.200	0.700	0.337
			0.303	0.243	0.702	0.345

Diene blank = 0.160

Peroxide blank = 0.040

in Table I, ethanol (10mls.) was run into the reaction mixture from the burette: this stopped the reaction. Samples (1ml.) were taken into absolute and 60% ethanol (12.5mls.) for the determination of peroxide and diene values respectively.

The results with appropriate corrections for blanks in both assays are shown in Table I. Duplicates of all samples were taken. A certain lack of reproducibility of individual reaction values was encountered but this does not affect the conclusions to be drawn.

These results show that, in vacuo, a soya extract can cause destruction of the hydroperoxides of both linoleic acid and its methyl ester when these are the product of lipoxidase catalysed oxidation. The destruction of peroxide is not complete although it is marked. There is no correspondingly large loss of diene although there is a slight decrease in this value. These findings are similar to those obtained in the aerobic system after oxygen exhaustion.

With pure trans-trans methyl linolenate hydroperoxide the soya extract had little effect. There was a slight loss of both peroxide and diene value in the course of the reaction but this was not comparable with the losses produced with the soya produced hydroperoxides.

Haematins are known to cause hydroperoxide destruction (52,112). Using haemoglobin (4.5×10^{-6} M) in this vacuum system destruction of the pure trans-trans methyl linolenate hydroperoxide was shown. In this case there was a concurrent loss of diene.

Oxygen Uptake Measurement and Extinction Coefficient of Catalysed Linoleic Acid Oxidation.

Measurements of oxygen uptake were carried out using a standard Warburg manometric apparatus. The reaction mixtures (3.85mls.) used consisted of linoleic acid (3.3×10^{-3}M) suspended in phosphate-citrate pH 6.5 buffer and a standard soya extract (0.15mls.) or dilutions of this. Oxygen uptakes were followed in the usual manner.

At intervals, reaction flasks were removed and samples (1ml.) of the reaction mixtures were pipetted into 60% ethanol (25mls.) so that a diene determination could be made. From this value and the oxygen uptake the apparent molar extinction coefficient of conjugated linoleic acid hydroperoxide was calculated, assuming that each mole of oxygen absorbed gave rise to one mole of conjugated linoleic acid hydroperoxide.

This apparent extinction coefficient varied considerably depending on the conditions attendant on the reaction. In the main the highest values were obtained for very low oxygen uptakes (< 0.1 mole O_2 per mole linoleic acid) and this decreased for higher oxygen uptakes. Also effective in producing high extinction values was the use of dilute soya extracts and the use of an oxygen atmosphere. Typical results are shown in Table II.

Table II Effect of Various Factors on the Extinction Coefficient of Linoleic Acid Hydroperoxide.

Soya extract	Atmos.	O_2 uptake	Diene Value	Apparent Ext.Coeff.
1/3 standard	O_2	12.4µl.	0.156	29,300
1/3 standard	O_2	42.2µl.	0.427	23,600
standard	O_2	32.0µl.	0.386	28,050
standard	air	61.0µl.	0.538	25,500
standard	air	108.3µl.	0.779	16,700

These results accord with those of other workers who found that extinction values were close to the theoretical (ε=28,000 for cis-trans conjugated linoleate hydroperoxide) at a low state of oxidation and decreased for higher states of oxidation. Here extinction values were found to decrease to about 50% of the theoretical at oxygen uptakes of approximately 0.4 mole O_2/mole substrate and above. This corresponds to results obtained throughout these studies where the diene value obtained when oxidation was permitted to the fullest extent never exceeded approximately 50% of the theoretical.

This clearly shows that it is wrong to assume a molar formation of conjugated diene hydroperoxide per mole of oxygen taken up under all conditions, especially so for higher oxygen uptakes. It is also wrong to calculate extinction coefficients for conjugated linoleate hydroperoxide on the basis of this

assumption thereby obtaining values considerably less than 28,000 which is the true and only value for this product. In Table II where extinction values are shown the term "apparent extinction coefficients" is used to avoid this difficulty. Obviously in the course of linoleate oxidation either oxygen is taken up by linoleate without producing conjugated diene or, more likely, that initial conjugated diene hydroperoxide takes up further oxygen and breaks down to other conjugated and non-conjugated products.

Conclusions and Discussion.

Evidence of a factor in soya extracts capable of causing the breakdown of formed linoleate hydroperoxide is presented, confirming earlier findings (52,53). Blain and Barr (52) suggested that this breakdown factor was only observed under conditions where all dissolved oxygen has been consumed. This theory is supported here since stirring of the reaction mixture obscured this peroxide loss. This stirring of the reaction mixture would appear to have had little effect other than aeration since similar initial rates of conjugated linoleate hydroperoxide formation (Figures 3 and 4) are obtained in the unstirred and stirred reactions. For observation of hydroperoxide destruction it appears necessary to consume the dissolved oxygen more quickly than oxygen from the atmosphere can diffuse into the reaction mixture. This explains why, under

normal atmospheric oxygen tension, relatively large amounts of enzyme are required to observe this breakdown. In the case of methyl linoleate, with which the hydroperoxide breakdown had not been previously observed, the formation of a finer emulsion was also necessary to obtain the required rate of oxidation to cause oxygen depletion.

This latter finding is of some significance with regard to the suggestion of Koch et al. (50,51) that there are at least two lipoxidase enzymes one being "acid" specific and the other "triglyceride" specific. These workers used for their experiments, very similar reaction conditions to the present one and with partly purified soya extracts showed a difference in activity with trilinoleate and linoleic acid as substrates. Similarly, here, there would be considerable difference in activity between the reactions with linoleic acid and methyl linoleate as substrate but for this homogenising of methyl linoleate reaction mixture. Thus an apparent difference in enzyme activity was due to the physical condition of the substrate. Koch et al. have not investigated this possibility of a physical effect nor have they considered the effect of differences in proteins present in their reactions as a result of their enzyme purifications. There are features of their work which suggest that their conclusions are justified but it would seem desirable for some further experimentation in the light of the present results.

The study of this breakdown or so called lipoperoxidase

factor, in vacuo, produced some interesting results. Here activity was shown with enzymically produced linoleic acid and methyl linoleate hydroperoxides, but not with pure trans-trans methyl linoleate hydroperoxide. Privett et al. (59) showed that the primary product of soya catalysed linoleate oxidation was cis-trans linoleate hydroperoxide. It would seem that the lipoperoxidase factor in soya was specific for this cis-trans isomer. In contrast to this, under similar conditions haemoglobin caused destruction of the trans-trans methyl linoleate hydroperoxide with loss of peroxide and diene value. The lipoperoxidase factor caused only loss of peroxide value. These results seem to offer a possible qualitative means of distinguishing between the cis-trans and the trans-trans linolenate hydroperoxide.

A question which cannot be answered from the present work is whether the hydroperoxide breakdown factor is active only under anaerobic conditions. It could well be that this factor is active under aerobic conditions also and that the observed rate of formation of hydroperoxide is merely the difference of the actual rate of formation and the rate of destruction of the hydroperoxide. Certain available evidence points to this factor being active only under anaerobic conditions. Thus, from Figure 3, if it is assumed that the highest diene value in the stirred reaction corresponds to complete oxidation of the 6.6×10^{-4}M linoleic acid, the apparent extinction coefficient would be approximately 13,000.

This is less than 50% of the theoretical but nevertheless corresponds to the calculated values for high states of oxidation in the oxygen uptake studies. If the breakdown factor is active under aerobic conditions a loss of peroxide value should be observed at this point. No such loss is observed (Fig.4).

If, indeed, the breakdown factor is only active under anaerobic conditions, an interesting possibility would be that the lipoxidase enzyme system itself functioned as the lipoperoxidase factor under such conditions. Further work, preferably with pure hydroperoxide samples and pure enzyme systems, would be necessary to confirm or refute these speculations.

S E C T I O N II

THE INHIBITION OF LIPOXIDASE.

SECTION II

THE INHIBITION OF LIPOXIDASE

These inhibitory studies were performed at pH 6.5 using the reasonably stable emulsion systems examined previously. These systems were found to be stable over a period of several hours but to ensure that no physical effect due to the breaking of the emulsions influenced the course of the reaction the assays were allowed to progress for one hour at the most. The preparation of these emulsions necessitated the use of relatively low concentrations of substrate and, for the purpose of measurement of the course of oxidation, the fat was allowed to oxidise to a relatively great extent.

Of the assays examined in the first section, the thiocyanate and spectrophotometric assays showed considerable sensitivity. This sensitivity in both cases, could be increased either by increasing the volume of the sample taken from the reaction mixture or by decreasing the volume of ethanol into which the sample was put. The oxygen uptake method was less sensitive despite being the only assay of the three which allowed direct measurement of the extent of oxidation. This was because the volume of the reaction mixture was restricted. Consequently the assays used for these studies were the thiocyanate and spectrophotometric methods. (Appendix I)

The inhibitors were used at a low concentration in comparison to the substrate. It was considered that if they were acting as true enzyme inhibitors they would exert their effect even at such a low level. Thus in initial studies the inhibitors were added to the reaction mixtures at a concentration approximately 5% that of the substrate.

The inhibitors chosen fell into two groups. The first group consists of unsaturated compounds. Since lipoxidase specificity appears to be confined to the methylene-interrupted unsaturated system found in its substrate fatty acids, it was considered possible that the enzyme action could be inhibited by use of compounds containing similar methylene-interrupted unsaturated sites. A series of such compounds as well as others(kindly supplied by Roche Products Ltd.) were examined as potential inhibitors of lipoxidase. These are listed in Table III.

The second group of compounds examined as potential inhibitors of lipoxidase were polyphenolic antioxidants, which are considered the best inhibitors of the enzyme. These are listed in Table VI.

The compounds of both groups were subjected to an initial general screening for inhibition of lipoxidase activity and where a compound gave evidence of such inhibition it was subjected to further examination.

Table III. Unsaturated Compounds as Potential Inhibitors of Lipoxidase.

Typical control reaction:-	Peroxide Content			Diene Content		
	1min.	2min.	5min.	1min.	2min.	5min.
	0.153	0.228	0.368	0.088	0.116	0.186

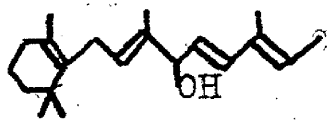
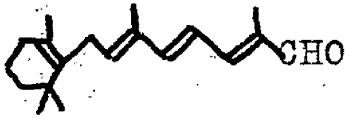
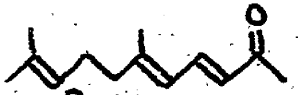
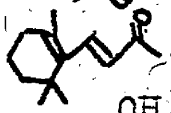
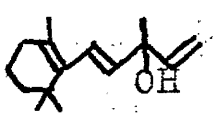
	Compound	Structure	Average % Inhibition
1.	1.6-dioxy-3.7-dimethyl-9-(2'.6'.6'-trimethyl-cyclo-hex-1-enyl)-nonatri-2.4.7-ene		0
2.	d.l-Linalool		0
3.	2.6-dimethyl-6-oxy-oct-2-en-7-yne (d.l-Dehydrolinalool)		0
4.	2.6.10.15.19.23-hexamethyl-tetra-cosa-hex-2.6.10.14.18.22-ene		0
5.	3-(2'.6'.3'-trimethyl-cyclo-hex-1-enyl)-2.6-dimethyl-octa-tri-2.4.6-en-1-al		0
6.	Pseudo-Ionone		0
7.	β-Ionone		0
8.	Isophytol		0
9.	Nonadeca-di-10.13-ynoic acid		30
10.	Undeca-di-2.5-yn-1-ol		0

Table III. (contd.)

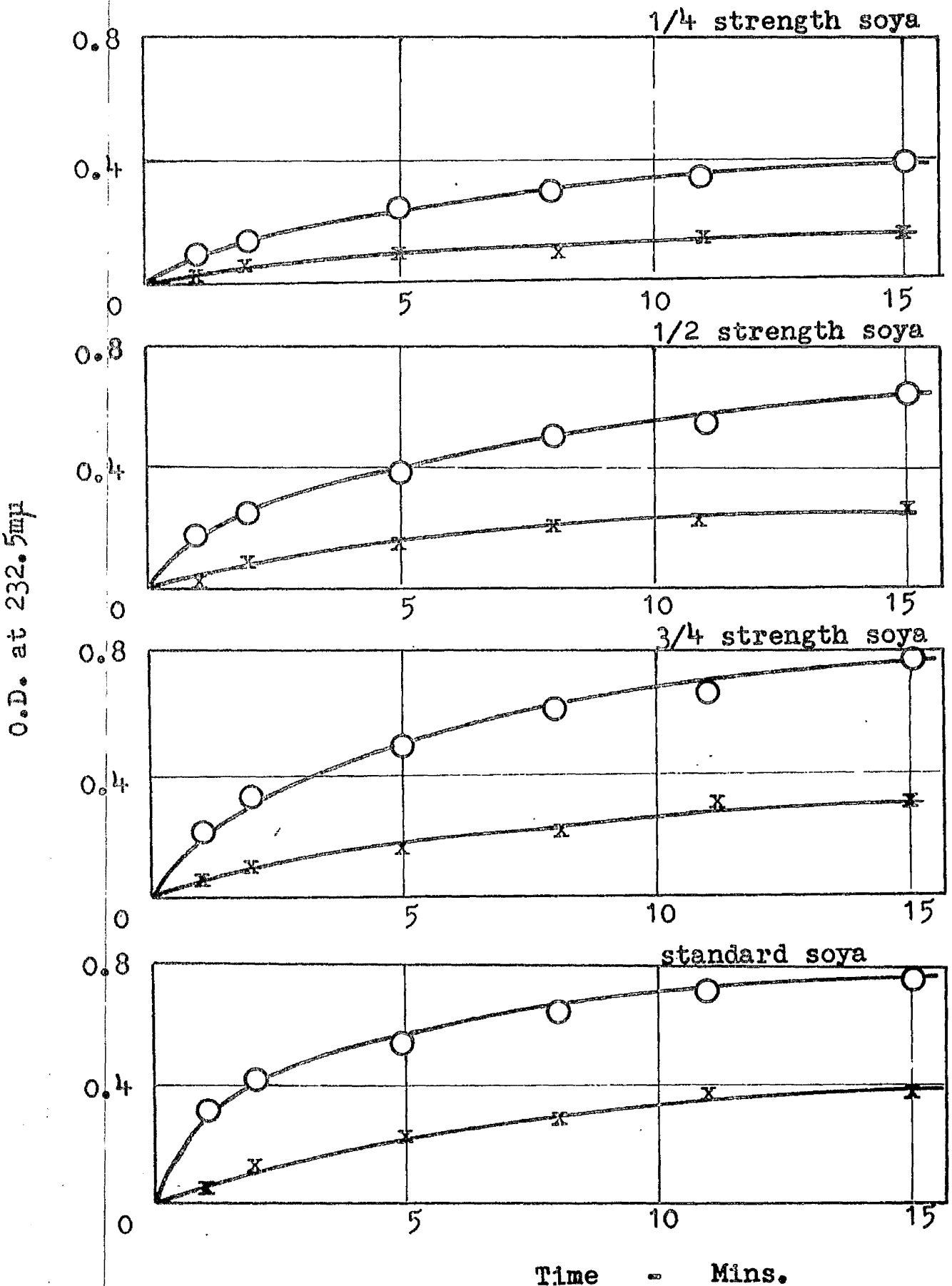
	Compound	Structure	Average % Inhibition
11.	Undeca-di-2.5-en-1-ol		0
12.	Eicosa-tetra-5.8.11.14-ynoic acid		68
13.	Octadeca-tri-6.9.12-ynoic acid		22
14.	Eicosa-tetra-5.8.11.14-yn-1-ol		84
15.	Octadeca-(cis)-12-en-9-ynoic acid		21
16.	1-Bromo-octadeca-di-9.12-ene (linoleyl bromide)		9
17.	1-Bromo-undeca-di-2.5-yne		0
18.	1-Amino-undeca-di-2.5-yne hydrochloride		0
19.	Vinyl-dehydro-β-ionol		0
20.	Ethynyl-β-ionol		0
21.	Octadeca-di-9.12-ynoic acid		17

General Screening of Unsaturated Compounds for Lipoxidase Inhibition.

The system used was similar to that described in Appendix I. Linoleic acid in ethanol (1ml.) was added to phosphate/citrate buffer pH 6.5 (25mls.) followed by the addition of the unsaturated compound in ethanol (0.25mls.). To this unstirred mixture was added soya extract (0.3mls.). At time intervals, samples (1ml.) were taken into ethanol or 60% ethanol (10mls) for determination of their peroxide and diene content respectively. The overall concentration of substrate was 3.3×10^{-4}M. Control reactions, identical but for the exclusion of the inhibitor, were performed concurrently with the inhibited reactions. This allowed a stricter comparison of the inhibitor efficiency to be made. Average figures for a control reaction are shown in Table III, where the effect of the compounds is expressed as a percentage inhibition of its own individual control assay.

Of the compounds screened for possible inhibition, some were successful to varying degrees and others were totally unsuccessful. The most notable feature of the unsuccessful compounds is that they have considerably shorter carbon chains than the successful compounds. They also do not contain the methylene interrupted unsaturated systems for which the enzyme is specific. The unsaturated centres which they do contain are either conjugated (compounds 1,5,7,etc.), or are separated by

Fig.8 Effect of Enzyme Concentration on Efficiency of Tetra-ynoic Acid Inhibition of Lipoxidase.



more than one methylene group (compounds 2,3,4,etc.), or are separated by substituted methylene groups (compounds 1,20,21).

The successful compounds are all long chained and they all contain the typical methylene-interrupted unsaturated system In this case the unsaturation is acetylenic in nature rather than the ethylenic of the lipoxidase substrates. The close similarity of the enzyme inhibitors and substrates suggested that the inhibition could be of a competitive nature and further studies using the most successful of the inhibitors viz., eicosatetra-5.8.11.14-ynoic acid, were made to confirm this.

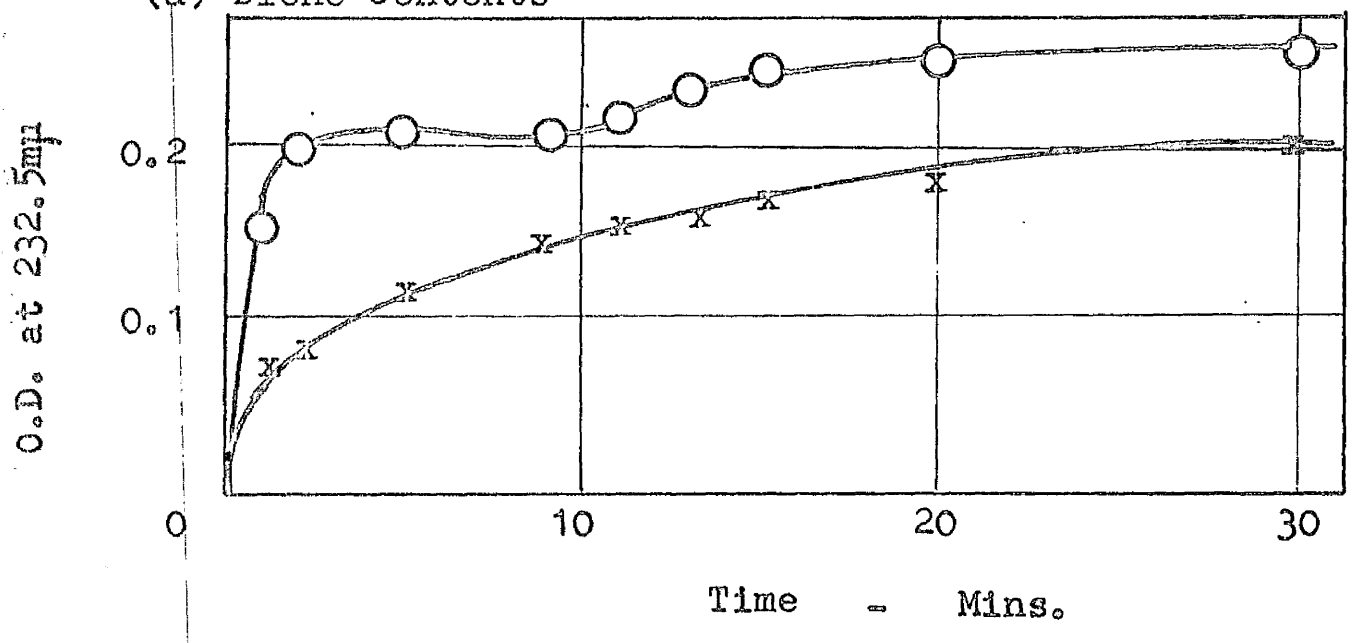
Effect of Enzyme Concentration on Efficiency of Inhibitor.

The assay used for this study consisted of linoleic acid (6.6×10^{-4}M) and tetra-ynoic acid(3.1×10^{-5}M) added in the same volumes of ethanol(1ml. and 0.25ml. respectively) to buffer(25mls.). Soya extracts(1ml.) were added to the stirred reaction mixture. The soya extracts used were the standard extract or dilutions of this. Peroxide and diene contents were determined at various time intervals by removing samples of reaction mixture(1ml.) into ethanol or 60% ethanol(10mls.).

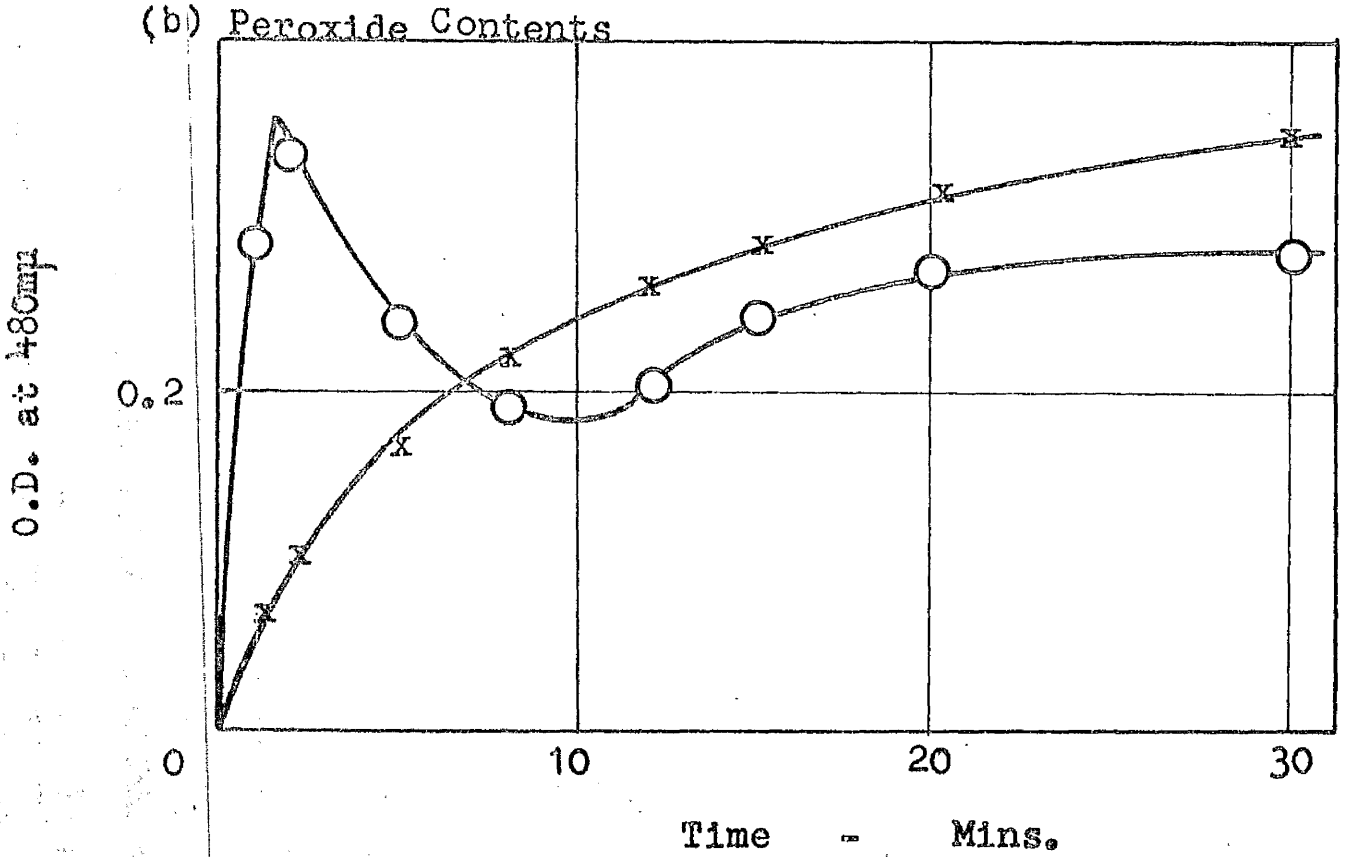
As may be seen from Figure 8, where diene content has been plotted against time, the efficiency of the inhibitor is unaffected by enzyme concentration. The average inhibition over the reaction period examined is of the order of 65%. Similar results were obtained from peroxide content determinations. This result is consistent with competitive

Fig.9 Effect of Tetra-ynoic Acid on Rate of Linoleic Acid Oxidation Produced by High Enzyme Concentration in Unstirred Reaction.

(a) Diene Contents



(b) Peroxide Contents



inhibition where both inhibitor and substrate compete for active sites on the enzyme. Thus, with the same ratio of inhibitor to substrate, variation of enzyme concentration merely alters the number of available active sites and does not alter the overall efficiency of the inhibitor.

Effect of Inhibitor in Unstirred Reaction Using High Enzyme Concentration.

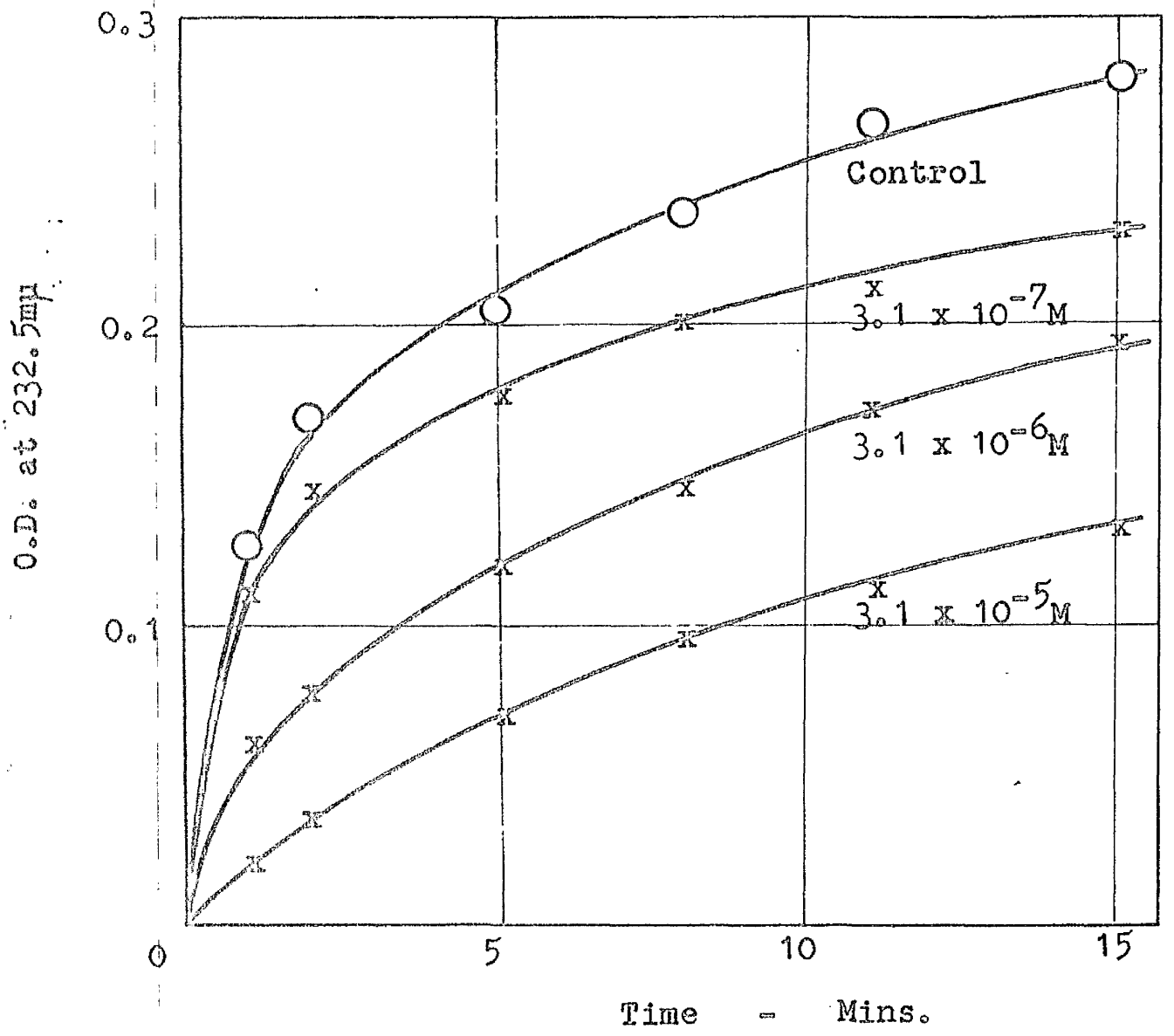
Having shown the tetra-ynoic acid inhibitor to be effective at high enzyme concentrations at which it has been shown to be possible to demonstrate enzymic destruction of peroxide in an unstirred reaction it was decided to look at the effect of the inhibitor under such conditions.

This was done to ensure that the tetra-ynoic acid was not appearing to be efficient by causing breakdown of the primary conjugated hydroperoxide product. While this seemed unlikely it was considered wise to check. If it was acting as a true inhibitor by slowing the primary reaction rate it was expected that oxygen exhaustion, with its consequent peroxide breakdown being obvious, would not be accomplished.

The same assay as in the previous work was used, the reaction not being stirred. For peroxide and diene content determinations, samples(1ml.) of reaction mixture were taken into ethanol or 60% ethanol(25mls.).

The results are shown in Figure 9 where it is obvious that

Fig. 10 Effect of Tetra-ynoic Acid Concentration on Rate of Linoleic Acid Oxidation.



the inhibitor reduces the initial rate of oxidation so that oxygen exhaustion is not accomplished. Consequently there is no typical rapid loss of peroxide content nor is there a period when diene content remains stationary. Both these features are observed in the uninhibited control reaction which was performed concurrently and which is shown in both diagrams.

Effect of Inhibitor Concentration on Lipoxidase Catalysed Linoleate Oxidation.

The assay system used for this study was similar to that in the immediately preceding study, except that the inhibitor concentration was varied and the reaction mixture was stirred. The concentrations of tetra-ynoic acid used were 3.1×10^{-5}, 3.1×10^{-6} and 3.1×10^{-7}M which represent approximately 5, 0.5 and 0.05% of the substrate concentration(6.6×10^{-4}M). Diene and peroxide contents were determined in the usual manner (1ml. into 25mls.).

The results are shown in Figure 10 for the peroxide content determinations which were similar to the diene content results. These results show that the tetra-acetylenic acid is a highly efficient inhibitor of lipoxidase since at these low percentage concentrations it still shows considerable inhibition. The average inhibitions are 65, 40 and 15% at 5, 0.5 and 0.05% of the substrate concentrations respectively.

Higher concentrations of inhibitor were used but with little improvement in the overall effectiveness. It is thought

Table IV. Effect of Substrate Concentration on Initial Rate of Linoleic Acid Oxidation in the Absence and Presence of Inhibitors.

		Substrate Conc. M x 10^{-3} (S)						
		3.2	2.6	1.95	1.6	1.3	1.0	0.66
	1/S	312.5	384.5	513	625	770	1000	1515
Diene contents at 8 minutes in:-								
Control	v	0.196	0.180	0.181	0.161	0.166	0.141	0.125
	1/v	5.1	5.55	5.52	6.21	6.02	7.09	8.0
Eicosatetraynoic Acid inhibited	v'	0.106	0.090	0.073	0.076	0.055	0.047	0.034
	1/v'	9.44	11.1	13.7	13.1	18.2	21.2	29.4
Octadecatriynoic Acid inhibited	v'	0.169	0.158	0.138	0.124	0.103	0.100	0.085
	1/v'	5.9	6.3	7.25	8.0	9.7	10.0	11.8
Eicosatetraynol inhibited	v'	0.185	0.158	0.159	0.147	0.126	0.116	0.091
	1/v'	5.4	6.3	6.3	6.8	7.9	8.6	11.0

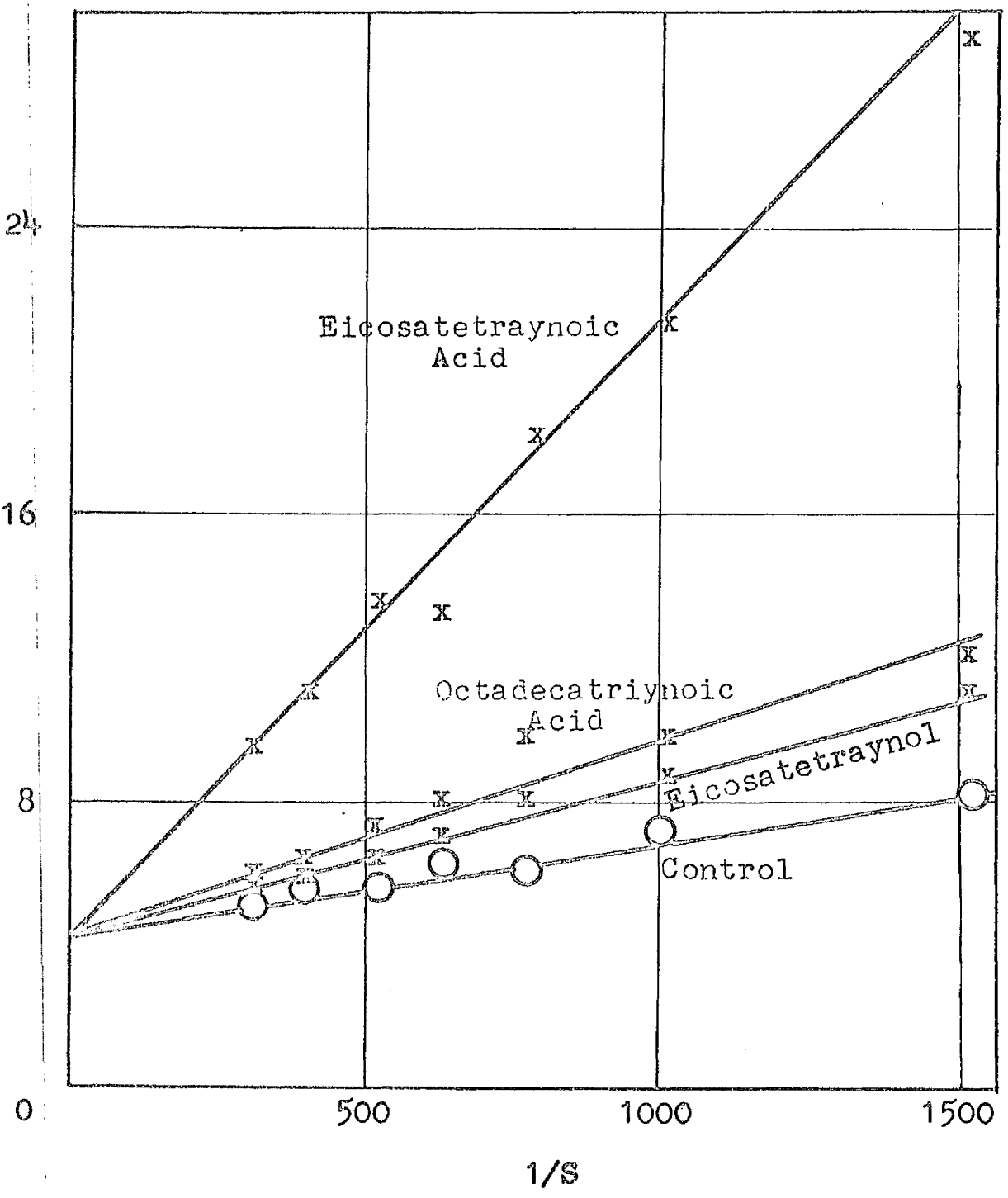
that this is due to a lack of solubility or availability of the inhibitor to the enzyme rather than to a diminution in its effect. The fact that, over the range of concentrations investigated, there is not a more marked variance of the inhibitory effect is also thought to be due to this lack of solubility or lack of availability of the inhibitor to the enzyme.

Effect of Substrate Concentration on Inhibitor Efficiency.

The effect of variation of the linoleic acid concentration in the reaction mixture containing the inhibitor (3.1×10^{-5}M) was studied. The reaction was catalysed by the addition of standard soya extract (1ml.). The reaction mixture was stirred and the diene content of the various reactions was determined after two minutes by taking a sample (1ml.) into 60% ethanol (25mls.). Control reactions without added inhibitor were performed.

The results are shown in Table IV for the inhibitors eicosa-tetra-5.8.11.14.-ynoic acid, octadeca-tri-6.9.12-ynoic acid and eicosa-tetra-5.8.11.14.yn-1-ol. As can be seen for all these inhibitors the percentage inhibition of the initial rate of linoleic acid oxidation increases with decreasing substrate concentration. This is to be expected of a competitive inhibitor since with decreasing substrate concentration, the ratio of inhibitor to substrate increases and hence so should the efficiency of the inhibitor.

Fig. 11 Lineweaver-Burk Treatment of Lipoxidase Inhibitors.



v = optical density at 232.5mμ after 2 minutes reaction.

S = moles linoleic acid in reaction mixture.

As a final confirmation of competitive inhibition these results were used for a Lineweaver and Burk (113) mathematical treatment. This involves plotting the reciprocal substrate concentration against the reciprocal of the initial rate of reaction which was taken here as the diene content at two minutes. These reciprocal values are shown in Table IV and are plotted in Figure 11. All the points lie on straight lines having a common origin. This is the desired result for demonstration of competitive inhibition. For such mathematical treatments of normal enzymic reactions the slopes of the lines correspond to K_m/V and the common origin to $1/V$ where K_m is the Michaelis constant and V is the maximal rate of reaction. The Michaelis constant is a characteristic constant of an enzyme catalysed reaction and it corresponds to the substrate concentration required to attain the half maximal rate of reaction (V/2). From the data available here the determination of the Michaelis constant for lipoxidase could be readily calculated. However, it is considered that the value so obtained would not be the true value on account of the biphasic nature of the system. It seems possible that the substrate concentration available to the enzyme will be different from the total concentration.

Study of Inhibitors as Possible Lipoxidase Substrates.

Since all the inhibitors contain methylene-interrupted unsaturated sites and are thought to be competing for the

enzyme's active sites it was considered possible that they might act as substrates for the enzyme. Accordingly, they were tested as such. The inhibitors (2.5mgs/ml.ethanol) were added to phosphate/citrate pH 6.5 buffer (25mls.). Standard soya extract (0.3mls.) was added and after five minutes reaction period a sample (1ml.) was taken into ethanol (10mls.) for peroxide content determination. After corrections for blank values, the results are shown in Table V.

Table V. Peroxide Contents of Inhibitors in Presence of Lipoxidase

Nonadecadi-10.13-ynoic acid	0.031
Eicosatetra-5.8.11.14.-ynoic acid	0.000
Octadecatri-6.9.12-ynoic acid	0.003
Eicosatetra-5.8.11.14-yn-1-ol	0.000
Octadeca (cis)-12.en-9-ynoic acid	0.000
1-Bromo-octadecadi9.12-one (linoleyl bromide)	0.211
Octadecadi-9.12-ynoic acid	0.029

As may be seen lipoxidase does not react with any of the inhibitors except linoleyl bromide. There is some slight activity with octadecadi-9.12-ynoic acid but this is negligible. Linoleyl bromide acts as a substrate for lipoxidase and also as a weak inhibitor of the enzyme. This

latter inhibition could be due either to the linoleyl bromide oxidation rate being somewhat slower than that of the acid or ester substrate or could be due to the presence of some trans isomers or even conjugated isomers which are acting in an inhibitory manner.

Discussions and Conclusions.

Of the compounds examined, seven have been shown to be inhibitory towards lipoxidase. These seven are all long chain unsaturated acids or their derivatives and are very similar structurally to the lipoxidase substrates. Indeed, some are identical structurally except for acetylenic unsaturated centres replacing the ethylenic ones of the substrates. Thus, eicosa-tetra-5.8.11.14-ynoic acid and octadecadi-9.12-ynoic acid differ only in this manner from arachidonic and linoleic acids respectively. The linolenic acid equivalent octadecatri-9.12.15-ynoic acid was not obtainable, but another tri-yne (the 6.9.12) was obtained for examination.

It seems reasonable to assume that these compounds all act as competitive inhibitors of lipoxidase. This has been demonstrated for the most successful of the inhibitors, eicosa-tetraynoic acid, in a variety of studies and for this compound and eicosatetraynol and octadecatriynoic acid by the Lineweaver Burk treatment of results.

That these compounds act as competitive inhibitors, at

a concentration of 5% of the substrate, makes them by far the most efficient competitive inhibitors of lipoxidase demonstrated to date. Previous competitive inhibitors were required in concentrations of at least 100% and higher in order to produce 50% inhibition (59). These were also long chain unsaturated fatty acids. NDGA has been claimed to competitively inhibit lipoxidase (60) but this seems doubtful and will be discussed later.

The order of efficiency of these inhibitors is difficult to explain. However, it appears notable that variation of the terminal group can cause a difference in the extent of inhibition. Thus, while the tetraynoic acid is the most outstanding of these inhibitors, the tetraynol is not nearly so effective, being only approximately 50% as efficient as the acid. It could be that the polarity of the terminal group alters the efficiency of the complexing of the inhibitor and enzyme and produces this result. On the other hand, since free acid, ester and now the bromide of linolenate, all with varied polar end groups, act as substrates of the enzyme, it may well be that the difference in efficiency of the tetraynol and tetraynoic acid results from the solubility of these substances and in no way involves the linkage of the terminal group and the enzyme.

The order of efficiency of the acids nonadecadi-10,13-enoic, octadeca (cis)-12en-9-ynoic and octadecadi-9,12-ynoic also seems

worthy of comment. They effect 30, 21 and 17% inhibition respectively. The acetylenic bond is slightly shorter than the ethylenic bond and it also confers a different shape to the molecule. For the acetylenic bond the α-carbon atoms are linear with the carbon atoms forming the bond, while, for the ethylenic bond, the α-carbon atoms are at an angle of 120° to the carbons of the bond with all the carbons held in the same plane. It seems possible that this shortening of the chain length and different configuration of the molecule around the acetylenic bond may be sufficient to produce the variation in the inhibitor efficiencies. When the acids mentioned above are compared it is readily seen that the distance between the terminal group and the second site of unsaturation is greatest for the nonadeca-10.13-diynoic acid, less for octadeca-(cis)-12-en-9-ynoic acid and shortest for octadeca-9.12-diynoic acid. The efficiency of these acids as inhibitors is the same. It may well be that this distance between the terminal group and the sites of unsaturation in both the substrates and the inhibitors are of consequence. Where there are more than two centres of unsaturation it is impossible to further this speculation.

General Screening of Antioxidants.

The assay system used for screening these compounds was similar to that used for the unsaturated compounds which were screened previously. The principal compounds of the assay were

Table VI. General Screening of Antioxidants for Inhibition of Lipoxidase.

Antioxidant	Diene Contents at				Comment
(3.2×10^{-5} M)	2 min.	8 min.	20 min.	45 min.	
None	0.155	0.319	0.417	0.504	
Catechol	0.151	0.332	0.444	0.544	All these antioxidants
3-Methyl catechol	0.159	0.300	0.413	0.513	have little or no
4-Methyl catechol	0.151	0.298	0.421	0.520	effect
None	0.152	0.279	0.430	0.583	
p-^{t}Butyl catechol	0.126	0.266	0.396	0.602	No inhibition
α-Naphthol	0.140	0.266	0.386	0.507	Little initial inhibition
β-Naphthol	0.150	0.269	0.376	0.551	but some in later stages.
None	0.123	0.264	0.427	0.532	
α-Tocopherol	0.158	0.282	0.429	0.577	All three tocopherols
β-Tocopherol	0.164	0.330	0.448	0.587	appear to be slightly
γ-Tocopherol	0.163	0.302	0.440	0.606	pro-oxidant.

Table VI. (contd.)

Antioxidant (3.2×10^{-5} M)	Diene Contents at 2 min.	5 min.	20 min.	45 min.	Comment
None	0.099	0.252	0.390	0.605	
NDGA	0.015	0.010	0.013	0.010	Almost 100% inhibitory
BHA	0.090	0.233	0.310	0.458	Slight inhibition initially increasing
Santoquin	0.089	0.259	0.369	0.562	Slight inhibition
None	0.097	0.248	0.422	0.592	
2.6-di-^{t}butyl-phenol	0.088	0.223	0.380	0.603	Slight inhibition initially
BHT	0.093	0.231	0.380	0.613	No inhibition
2.6-di- butyl-methoxy-cresol	0.100	0.247	0.407	0.593	No inhibition
None	0.125	0.424	0.702	0.743	
2.6-di-^{t}butyl-di-methylamino-cresol	0.121	0.370	0.735	0.775	No inhibition
4.4'-bis-di-^{t}butyl-phenol	0.125	0.405	0.746	0.770	No inhibition
4.4'-methylene-bis-2.6-di-^{t}butyl-phenol	0.119	0.426	0.715	0.764	No inhibition

Table VI. (contd.)

Antioxidant (3.2×10^{-5} M)	Diene Contents at 2 min.	8 min.	20 min.	45 min.	Comment
None	0.065	0.198	0.370	0.504	
4.4'-methylene-bis-6-tbutyl-cresol	0.067	0.187	0.319	0.541	Slight inhibition initially, increasing
Propyl Gallate	0.052	0.165	0.270	0.426	Inhibits throughout

linoleic acid (6.6×10^{-4}M), antioxidant (3.2×10^{-5}M) and standard soya extract (0.3mls.). The reaction was unstirred and only diene contents were determined by means of samples (1ml.), taken at various time intervals, into 60% ethanol (10mls.). Control reactions, without antioxidant, were performed concurrently with the reactions containing the polyphenols.

The antioxidants were used on an equimolar basis rather than on the equal weight basis of the previous screening studies. It was considered that a better comparison of their efficiencies would be obtained. The molarity of the antioxidants was approximately 5% of that of the substrate.

Some typical results are shown in Table VI, where it can be seen that there is considerable variation in the control reactions. It is for this reason that the inhibited reactions are listed with the control reaction with which they were performed. This variation in the control reactions is due to the lipoxidase activity varying over the period during which these experiments were performed. It has been found that defatted soya flour loses lipoxidase activity during storage. To ascertain the results obtained, replicates (often several) were performed.

With the exception of NDGA, no antioxidant exhibits outstanding inhibition of lipoxidase. This is probably due to the conditions of the assay in which there is a relatively high rate of oxidation in the presence of low concentration of antioxidants. However, NDGA, even under these conditions, exhibits

virtually 100% inhibition of the enzyme activity throughout the course of the assay. Of the other antioxidants only propyl gallate, santoquin and BHA exhibited a notable inhibition. Propyl gallate and santoquin both reduced the diene determinations of their respective control reactions uniformly throughout the course of reaction. This reduction was approximately 25% for propyl gallate and 6% for santoquin. BHA, on the other hand, initially reduced the diene determinations of its control reaction by some 7% and this reduction increased to approximately 25% for longer reaction times. The most surprising results were those with the tocopherols. They exhibited a certain pro-oxidant effect which cannot be explained satisfactorily.

Apart from the results with the tocopherols, the results obtained here accord reasonably with earlier results of other workers. Before notable inhibition was demonstrated, these other workers often had to use higher concentrations of antioxidant than are used here. A few experiments using higher levels of some of the better antioxidants still failed to demonstrate remarkable improvements in the extent of inhibition. Indeed, with higher concentrations of tocopherol difficulty through lack of solubility of the inhibitor was encountered. This suggested that under the conditions of this assay there might be insufficient fat present to bring the antioxidants effectively into solution at these higher concentrations.

In biphasic systems the partition of the antioxidant between the aqueous and lipid phase must be of major importance. It may be that in the assays performed, the antioxidants are either not suitably partitioned to exert an inhibitory effect or their addition immediately prior to initiation of reaction does not enable them to attain a suitable partition. Lea (114) notes that many antioxidants, especially those which are rather sparingly soluble in fat, may only very slowly attain their true partition. It is noteworthy that NDGA belongs to this class of antioxidant and it is the most successful inhibitor of lipoxidase known. It could be that it is successful because it is partitioned at the interface of the aqueous and lipid phases. Further studies of the inhibition of lipoxidase by this antioxidant were carried out.

Effect of NDGA Concentration on its Inhibition of Lipoxidase Activity.

NDGA was a highly effective antioxidant for inhibiting lipoxidase activity at a concentration of 5% of the substrate, linoleic acid. With the same assay conditions as for the general screening, the enzyme concentration was increased until oxidation of linoleate was observed. This occurred when an addition of 1ml. of standard soya extract was made. Under such conditions the effect of NDGA concentration on lipoxidase inhibition was studied.

The assay components were linoleic acid (6.6×10^{-4} M),

Fig.12 Effect of NDGA Concentration on Lipoxidase Activity

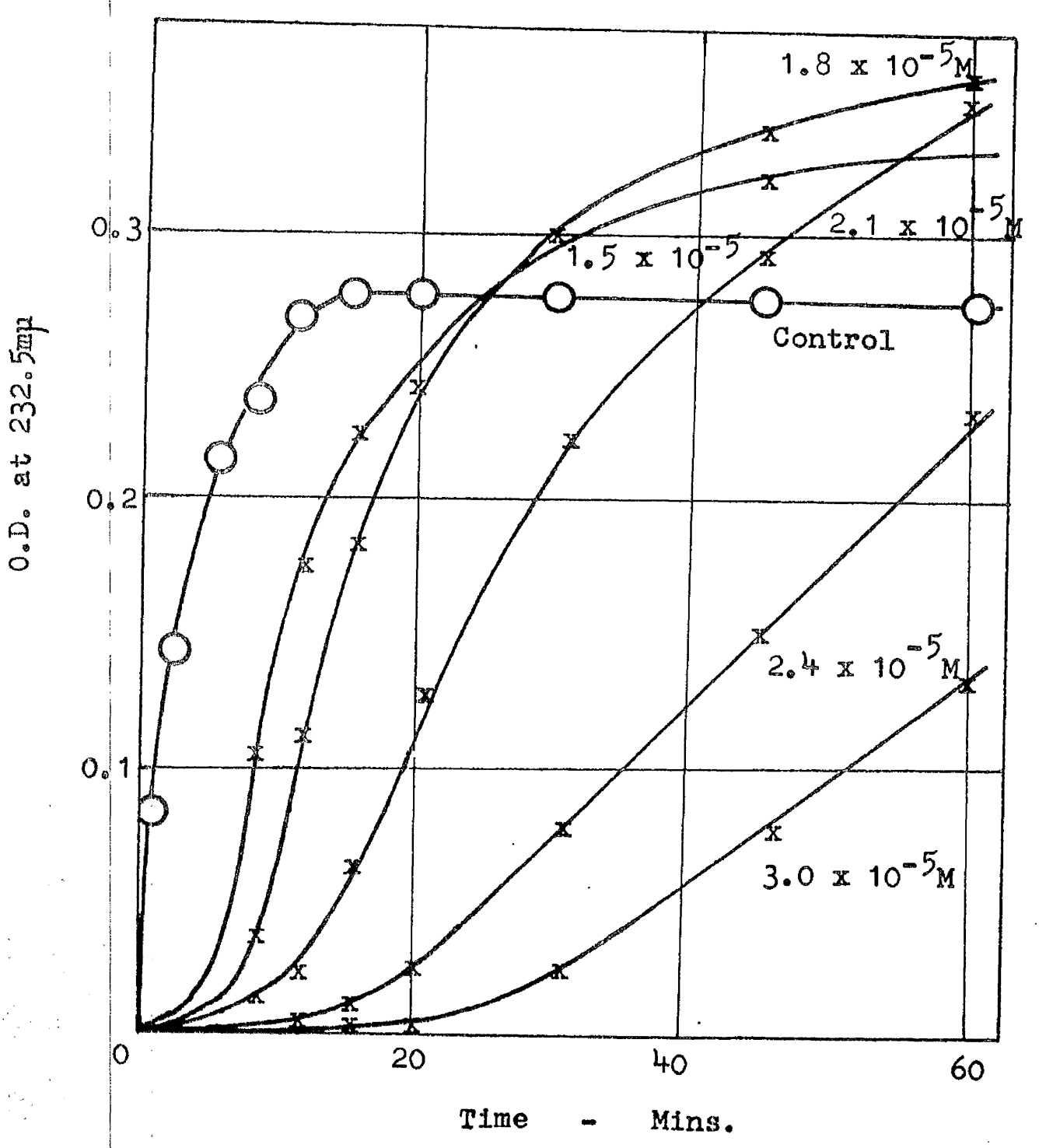
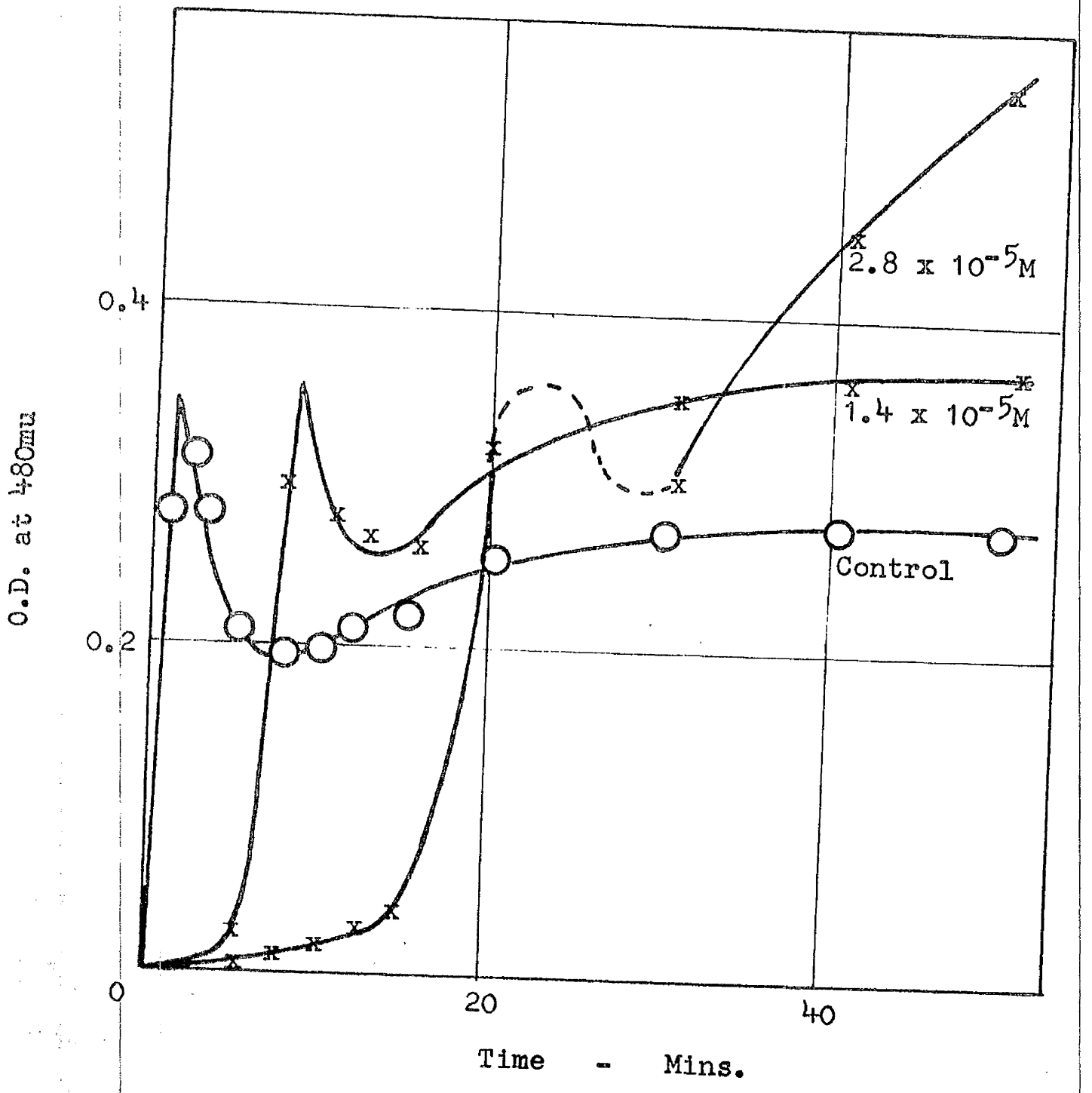


Fig. 13 Effect of NDGA Concentration on Lipoxidase Activity



NDGA (varied concentration) and standard soya extract (1ml.). The reaction was stirred and samples (1ml.) were taken at various time intervals into 60% ethanol for diene content determinations. The results are shown in Figure 12. This shows that increasing NDGA concentration has the following effects.

(a). There is an increasing induction period followed by a rapid oxidation of linoleic acid as measured by its diene content.

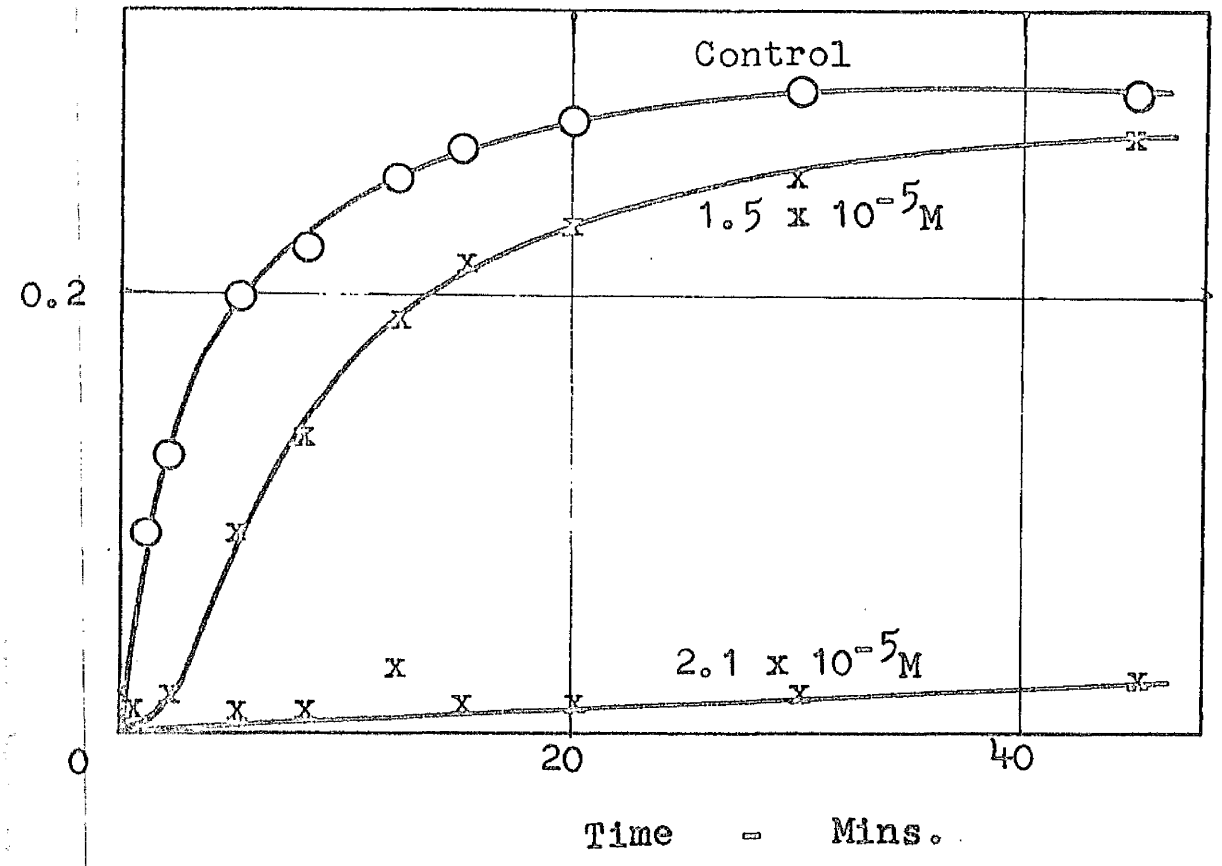
(b). The rate of oxidation of linoleic acid decreases with increasing inhibitor concentration.

(c). The final diene content attained increases with increasing inhibitor concentration.

Results of a similar experiment in which the reaction mixture was not stirred are shown in Figure 13. In this case peroxide contents were determined. Here, similar induction periods were observed followed by slightly lower rates of linoleic acid oxidation in comparison to the control reaction. The subsequent peroxide content loss appears less marked in the presence of NDGA and the final peroxide content observed is very much higher than for the control. The actual length of the induction period in this experiment does not correspond to that of the previous experiment but this is thought to be due to the use of a soya extract with a higher lipoxidase activity.

Olcott (115) recently observed that NDGA was a much less effective antioxidant in preventing the autoxidation of linoleic

Fig. 14 Effect of NDGA Concentration on Lipoxidase Activity with Methyl Linoleate as Substrate.



acid than it was for the autoxidation of linoleate. Methyl linoleate was substituted for linoleic acid in this assay in order to observe if the substrate made a difference to the inhibitor efficiency. The reaction mixture was homogenised before addition of soya extract (1ml.) and the reaction was stirred throughout. Diene contents were determined (1ml. into 25mls.). The results are shown in Figure 14.

At an NDGA concentration of 1.5×10^{-5}M there is a short induction period of two minutes followed by linolenate oxidation at a lower rate than in the control reaction. At 2.1×10^{-5}M, NDGA produces an induction period of more than one hour. The induction periods for comparable concentrations with linoleic acid are five and ten minutes respectively. It would appear that the antioxidant is more efficient at the higher concentration for the ester than for the free acid. This is as Olcott found in his autoxidation studies.

Discussion and Conclusions.

These results showing the effect of NDGA on lipoxidase catalysed oxidation of linoleate are of considerable importance. The production of an induction period during which no measurable oxidation of linoleate is observed suggests that the NDGA efficiently inhibits the linoleate oxidation until it is itself completely oxidised. These results while being original in so far as they clearly demonstrate the existence of an induction period followed by linoleate oxidation in the presence

of an antioxidant, may be correlated with earlier results of Tappel et al. (67). These workers used an oxygen absorption technique to follow their oxidation and they presumably allowed the reaction to progress for a set time. They then examined the products spectrophotometrically. At low concentrations of NDGA (5×10^{-5}M), linoleic acid oxidised along with NDGA; between 5×10^{-5}M and 3×10^{-4}M NDGA only the inhibitor oxidised and above the latter concentration little or no measurable oxidation occurred. From Figure 12, taking an arbitrary reaction period of 20 minutes, these same features can be observed. It is felt that a clearer insight into what is occurring is given here.

The second feature of these studies was the reduced rates of oxidation after the induction period. This suggests that either remaining NDGA or oxidised NDGA can exert an inhibitory effect on the rate of linoleate oxidation. This was noted in both stirred and unstirred reactions although it was not so obvious in the latter case. However, it may well be that this lowering of oxidation rate is due entirely to slight enzyme inactivation rather than to enzyme inhibition. This enzyme inactivation is a possibility, especially in the case of the stirred reactions, because it has been noted in preparing soya extracts that stirring for periods of more than twenty minutes can lower lipoxidase activity (116).

The final feature of increased diene and peroxide contents

In the later stages of the reaction in the presence of increasing concentration of antioxidant can only be accounted for by postulating protection of the conjugated diene hydroperoxide products from subsequent breakdown by the oxidised NDGA or by remaining trace amounts of unoxidised NDGA. If this feature of increased product content had been noted in the general screening it is likely that NDGA would have been described as pro-oxidant, just as the tocopherols were. It may be that this is how the tocopherols are functioning under the conditions of the general screening, viz., by inhibiting conjugated diene hydroperoxide breakdown.

From Figure 12, it is possible to readily calculate that during the respective induction period produced by 1.5×10^{-5}M and 2.1×10^{-5}M NDGA the formation of 1.95×10^{-4}M and 2.19×10^{-4}M conjugated linoleate hydroperoxide is prevented. (This is assuming an extinction co-efficient of 28,000). Thus one mole of NDGA prevents the formation of approximately ten moles of linoleate hydroperoxide. If, as would seem reasonable from work described previously, an extinction value of about 14,000 were used to make these calculations for what is relatively highly oxidised linoleate, one mole of NDGA would prevent the oxidation of about twenty moles of linoleic acid. This would suggest that lipoxidase was initiating a chain reaction with NDGA functioning as an efficient chain breaker. The chain length would be short, being at the most an average

length of twenty links. However, if each molecule of NDGA can donate the four hydrogen atoms attached to its hydroxyl groups, this chain length will be severely reduced. On the other hand, this assumes that the antioxident is 100% efficient and is itself completely used up. If this is not the case, the chain length could be considerably longer.

Siddiqui and Tappel (69) from inhibition studies measured by oxygen uptake, showed NDGA to be a competitive inhibitor of lipoxidase. To explain its action they postulated that it competed with lipoxidase substrate in donating a hydrogen atom to the enzyme's active sites. In view of the present results and the earlier results of Tappel et al. (71) it is difficult to understand this action of the inhibitor. For competitive inhibition it would be necessary for the initial rate of linoleate oxidation in the inhibited reaction to be reduced from that of the control reaction; here there is a distinct period during which there is no observable linoleate oxidation.

SECTION III

CAROTENE-AGAR PLATE ASSAY.

SECTION III

CAROTENE AGAR PLATE ASSAY

Destruction of carotene in biological systems may occur by means of photoxidation, autoxidation or enzymically (117). The enzymic process may be due to a specific "carotenase" enzyme although such an enzyme has yet to be identified. Carotene may be co-oxidised by oxidising unsaturated fats in which the carotene is soluble and with which it often occurs in close association in biological systems. The unsaturated fats may be oxidised autoxidatively or catalytically; this latter process may be produced by trace metals, haematin compounds or lipoxidase.

Protection of carotene against such oxidative degradation is best afforded by the use of antioxidants. Bickoff and his co-workers (98,99,100,101) and Budowski and Bondi (102) have dissolved carotene in mineral oils and studied the effect of antioxidants on the rate of loss of carotene. They have found many antioxidants which were highly efficient in these systems but, which on application to practical conditions, were found to be considerably less efficient. This is thought to be due to the polyphasic nature of the practical systems and to fat oxidising enzymes present in these systems. The development of an assay to study protection of carotene under such conditions seemed desirable.

The system described in Appendix III was designed to compare the carotene protecting effect of antioxidants against

autoxidation, lipoxidase and haematin catalysed oxidation in aqueous emulsion systems in which the carotene was dispersed with unsaturated and saturated fats. In order to stabilise physically these emulsion systems containing fat and carotene, (both of which are insoluble in aqueous media) over the period of time necessary, the studies were carried out in agar gels.

Outline of the Assay.

The procedure employed entailed standard additions of acetone solutions of fat, β-carotene and antioxidants to buffered agar solutions held at a temperature of 65°C. In the case of the catalysed reactions the aqueous solutions of the catalyst were also added. After thorough mixing of the reactants in a test tube, they were poured into a receptacle where the agar was allowed to set in a thin layer in the dark. After one hour in which time any major changes in gel opacity occurred the carotene colour was estimated by placing the receptacles containing the agar gels vertically in an absorptiometer cell compartment and reading the optical density at 450mμ against a blank identical but for the exclusion of carotene. The gels were then stored at 20°C in blackened desiccators, readings of residual carotene being taken at suitable time intervals. For the autoxidation studies it was found expedient to store the gels under an oxygen enriched atmosphere to increase the rate of bleach of carotene. The oxygen enrichment presumably increases the rate of diffusion of

oxygen into the gel and in this way accelerates carotene bleaching. However, even in an atmosphere of air, carotene bleaching progressed uniformly throughout the gel and this suggests that there is no lack of oxygen available for bleaching. The catalysed reactions were stored under a normal air atmosphere.

Initial studies were made as to the applicability of this system for both autoxidised and catalysed reactions and to determine suitable conditions for the general screening of antioxidants. The system for these initial studies used methyl linoleate, β-carotene and agar buffered with McIlvaine's phosphate-citrate buffer pH 6.5. The quantity of carotene employed (2.2×10^{-5}M) was such as to give an initial optical density of approximately 0.8.

It was found that not all agars were suitable and the agar used initially was Kobe (Hopkin and Williams) since it gave rapid bleaching together with reproducible results. All the initial studies and some general screening of antioxidants were carried out using this agar in the system described in Appendix IIIa. After some time an even more suitable agar (Ionagar No.2 Oxoid) was found. This gelled at lower concentrations giving rise to a much clearer gel than did Kobe agar. Carotene bleaching was more rapid and consequently it was decided to use this agar for subsequent work. At the same time, it was decided to alter slightly

the system being used to overcome minor objections to the earlier system. These objections were related to the receptacles in which the gels were stored. These were small plastic boxes which after considerable usage became somewhat opaque as a result of contact with the acetone solvent. It was also pointed out that there could be small quantities of antioxidant present in the plastic which might affect results (This latter point is considered to have had little or no noticeable effect on the results obtained.). These boxes were replaced by small Petri dishes made of optically clear glass. The surface area of these dishes was somewhat greater than that of the boxes and in order to obtain a gel of comparable thickness a greater volume of agar was used. This made it necessary to increase the carotene concentration in order to have as high an initial optical density as possible. However, the other reagents were added in the same quantities as before except for haemoglobin which was increased in concentration to maintain an overall concentration of 10^{-6}M (expressed as haematin) in the assay. The details of this assay system are given in Appendix IIIb.

The results obtained with both these systems were as would be expected. In autoxidation studies there was an induction period during which little or no carotene bleaching occurred followed by a period of rapid bleaching. This is typical of results of studies of unsaturated fat autoxidation.

Table VII. Effect of Methyl Linoleate Concentration on the Initial Optical Density of Linoleate-Carotene-Agar Plates.

Linoleate Conc. M x 10^{-4}	Opt.Dens. at 450mμ
0.1	0.59
0.25	0.685
0.5	0.79
0.75	0.83
1.0	0.83
2.5	0.85
5.0	0.86
7.5	0.82
10.0	0.84

where little oxidation is observed for a time until the chain reaction has developed when oxidation is rapid. In the catalysed reactions there was no induction period.

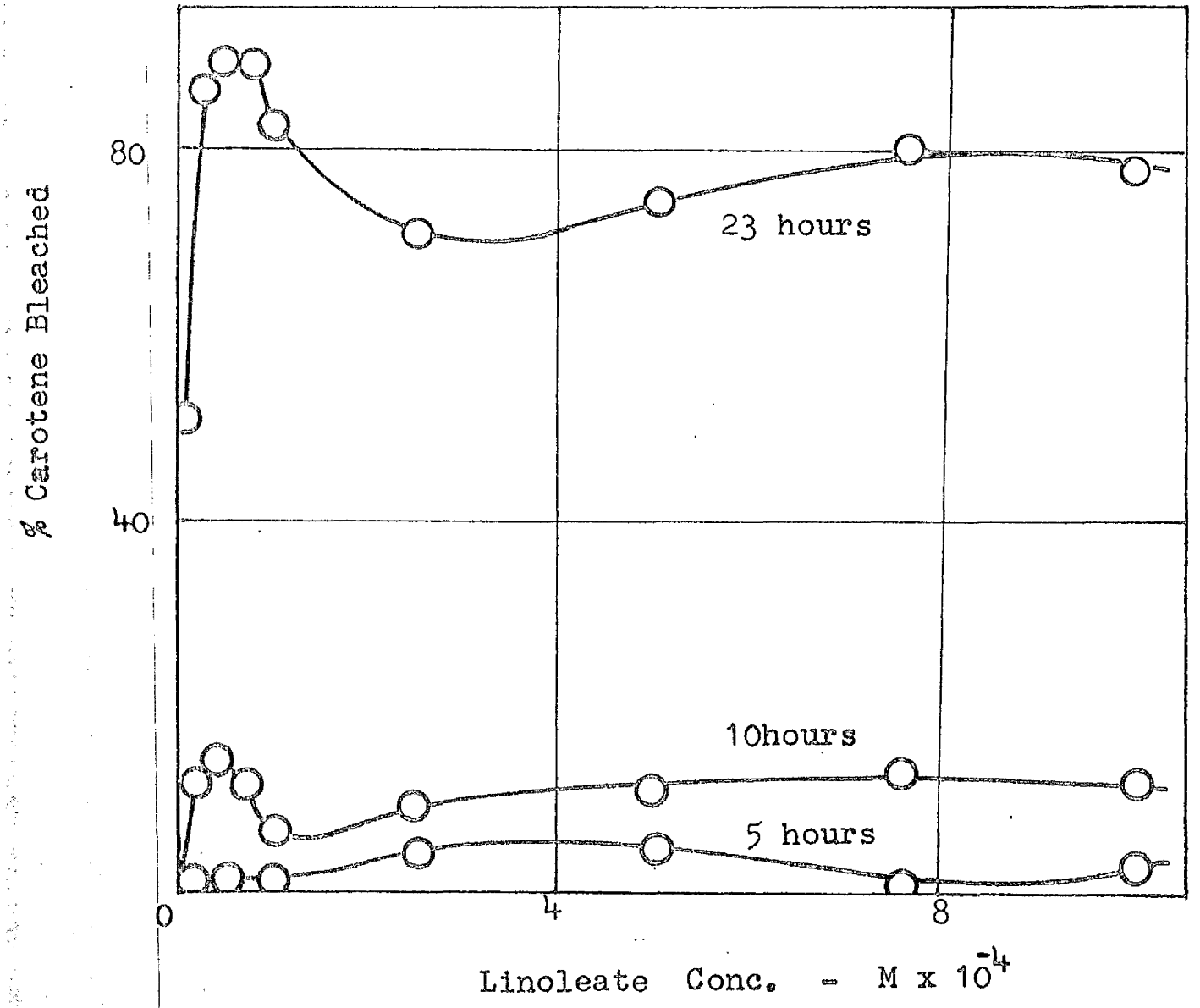
Further aspects of this assay system will be discussed under separate headings.

Effect of Substrate Concentration on the Rate of Bleach of Carotene

Various quantities of methyl linoleate were added to the assay system described in Appendix IIIa. It was noted that the substrate concentration had an effect on the initial optical density of the plate as measured at 450mμ. These initial optical densities are shown in Table VII where it is seen that initial optical density increases with linoleate concentration to 7.5×10^{-5}M. Above this value there is no notable increase in optical density. It is considered that the increasing linoleate concentration alters the dispersion of the carotene in the agar media, this dispersion reaching a maximum for methyl linoleate at a concentration of 7.5×10^{-5}M linoleate. Similar observations to these have been made for sodium linoleate in aqueous media (118).

The rate of bleach of carotene also varies with substrate concentration. The results of these studies are shown in Figure 15 where percentage carotene bleached at 5, 10 and 23 hours is plotted against methyl linoleate concentration. The

Fig.15 Effect of Linoleate Concentration on Rate of Bleach Of Carotene in Linoleate-Carotene-Agar Plates.



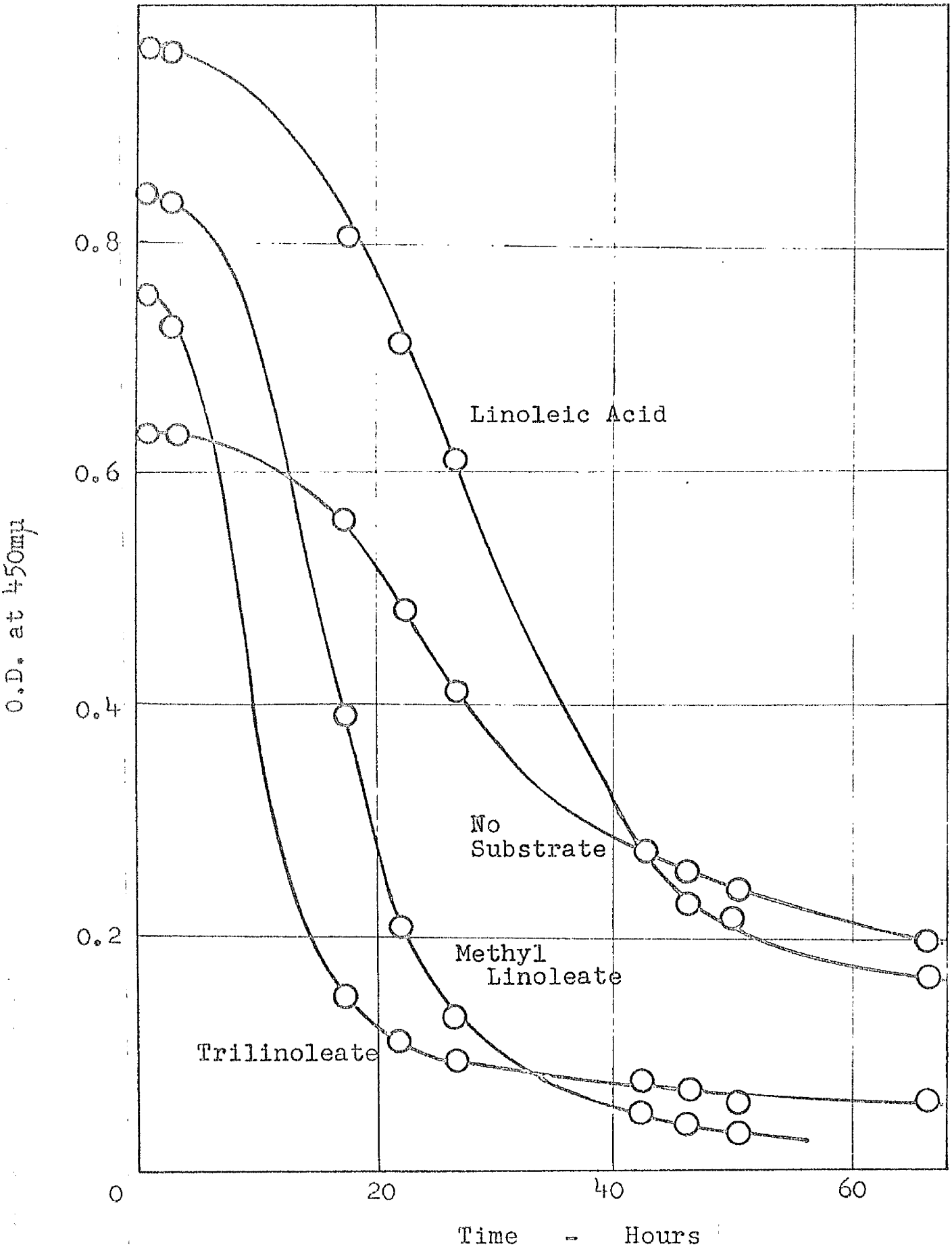
bleaching rate increases with increasing substrate concentration reaching a maximum value near 7.5×10^{-5} M linoleate. Above this concentration there is a notable decrease in carotene bleaching rate which is at a minimum value at approximately 2×10^{-4} M linoleate. Increasing linoleate concentration above this latter concentration has little effect on bleaching rate.

Lohser and Tookey (110), working with sodium linoleate and carotene at pH 9.0 in aqueous media noted a similar minimum in the rate of bleach at a concentration of 2.5×10^{-4} M linoleate. This concentration corresponded to the critical micelle concentration (CMC) of sodium linoleate. Above this concentration, carotene bleaching increased with increasing linoleate concentration. They suggested that below the CMC, carotene bleaching was independent of linoleate concentration while above it carotene was dependent on linoleate concentration.

In this case working with methyl linoleate at pH 6.5 there appears to be little chance of micelle formation occurring with increasing linoleate concentration. However, methyl linoleate at all concentrations is presumably present in small droplets similar in nature to micelles and as linoleate concentration increases so will the number and size of these droplets. At 7.5×10^{-6} M linoleate it would seem that there was just sufficient linoleate present to disperse the carotene as efficiently as methyl linoleate is able. Up to this concentration there is presumably increasing association of the

oxidisable linoleate and carotene and this readily explains the increasing rate of bleaching to 7.5×10^{-5}M linoleate. The decrease in bleaching rate above this concentration is less readily explained but a possibility is that the carotene becomes incorporated into the centre of the linoleate droplets as they grow in size and number and are further removed from the site of active oxidation at the surface of the droplet. Thus linoleate, depending on its concentration, may have a pro-oxidative or partial protecting effect on carotene. That fat can have some protective influence on carotene has been shown by Siedler et al. (107) and by subsequent work of this thesis. Above the linoleate concentration at which maximum dispersion of carotene occurs, the rate of oxidation will depend on the balance of this pro-oxidant and protective effect of the linoleate. Here the rate decreases and remains relatively constant above a concentration of 2×10^{-4}M linoleate, while in the study of Lohmar and Tookey (110) the rate increased with increasing linoleate concentration. The difference in these contrary findings could result from physical differences between sodium linoleate micelles and methyl linoleate droplets but a more acceptable explanation would be that in the work of Lohmar and Tookey there was an adequate supply of oxygen to aid the pro-oxidant factor while here there is a restricted supply of oxygen. This supply is restricted by the rate of diffusion of oxygen into the agar gels. However, this rate appears to be adequate to produce a uniform

Fig. 16 Effect of Different Substrates on Rate of Carotene Bleaching in Linoleate-Carotene-Agar Plates.



bleach throughout the gel.

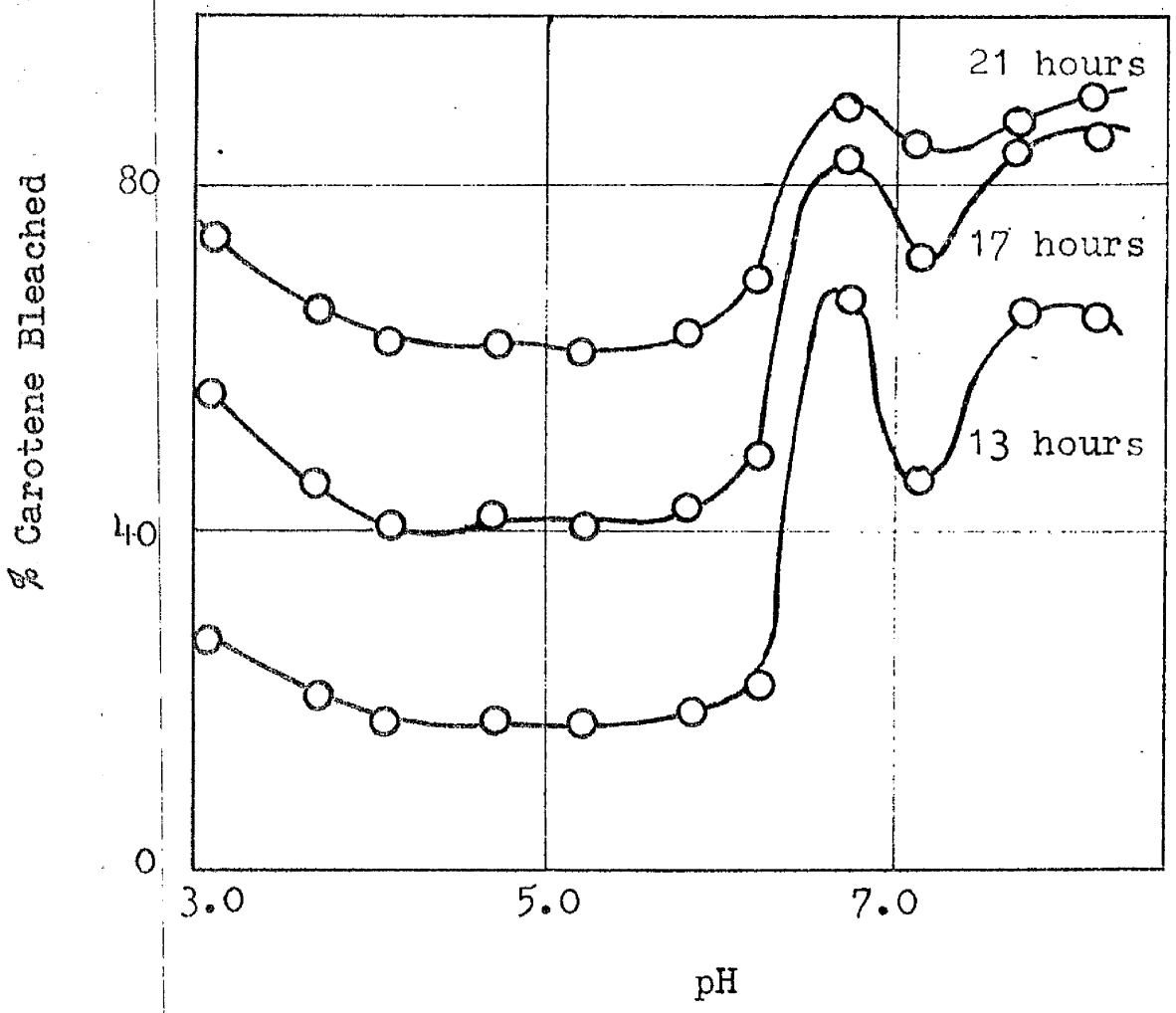
Effect of Different Substrates on the Rate of Bleach of Carotene in Carotene-Agar Plates.

Lee (119,120) and Olcott (115) have both noted that variation in the fatty substrate can alter the efficiency of an antioxidant in preventing oxidation. The substrates studied in this assay were methyl linoleate, trilinoleate and linoleic acid and also without any fatty substrate. The substrates were added on an equal weight basis, this giving approximately equal amounts of unsaturation in all three systems.

It was found that these different substrates produced different initial optical densities as measured at 450mμ. Thus with no substrate the value was ~0.6, with trilinoleate ~0.75, with methyl linoleate ~0.85 and with linoleic acid ~0.95. This variation is considered to be connected with the ability of the individual substrates to disperse the carotene in the agar gel just as varying concentrations of methyl linoleate altered the dispersion.

Typical curves for the bleaching of carotene in the presence of different substrates are shown in Figure 16. It is seen that the length of induction period and the rate of carotene bleaching varies with the substrate. Initially it was thought that this resulted from the variation in the nature of the substrate but subsequently doubt was cast on this when it was shown that varying quantities of trace peroxides were

Fig.17 Effect of pH on Rate of Carotene Bleaching in Linoleate-Carotene-Agar Plates.



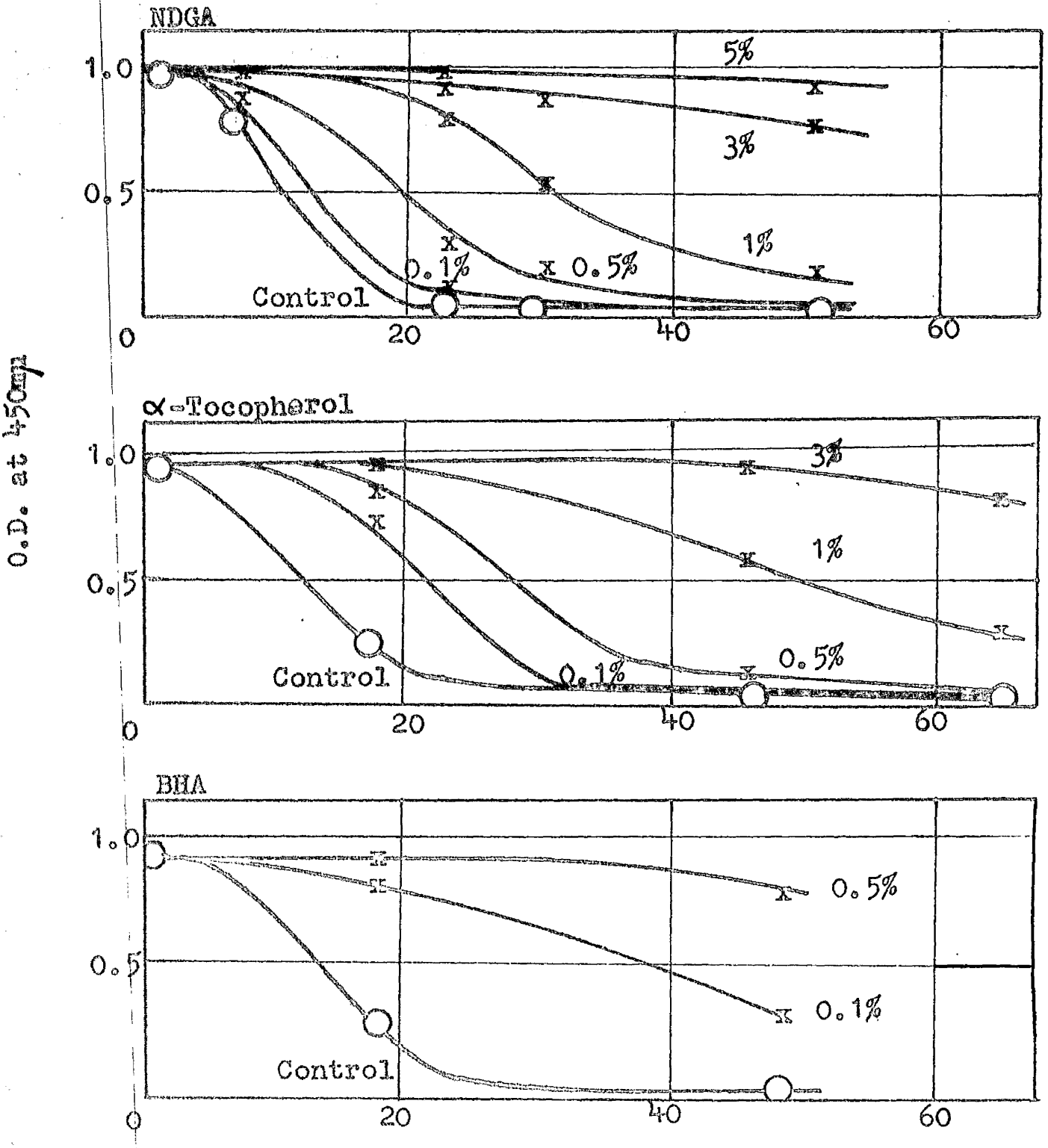
detectable in these substrates. It had been assumed that the substrates, having been supplied by the Kennel Institute and having been stored at -20° C in evacuated ampoules, would not have contained such peroxides. The presence of such peroxides ruled out any semiquantitative evaluation of antioxidant efficiencies being made for the different substrates. However, it was considered permissible to compare the antioxidant efficiencies quantitatively for a single substrate and qualitatively for different substrates. This was done and the results are given subsequently.

Effect of pH on the Rate of Bleach of Carotene in Methyl Linolenate-Carotene-Agar Plates.

The range of pH studied corresponds to the range of the McIlvaine's phosphate-citrate buffer, viz., pH3.0-8.0. The assay system was otherwise as described in Appendix IIIe, using methyl linolenate as substrate. The results are shown in Figure 17 where percentage carotene destroyed at 13,17 and 21 hours is plotted against pH. The variation of pH in no way influenced the dispersion of the carotene as measured by the initial optical density of the plates. This is largely as would be expected since methyl linolenate will be little influenced physically by change of pH.

The rate of bleach of carotene shows a maximum near pH 6.7 and a minimum value at pH 7.1. Below pH 6.7 there is a rapid decrease in bleaching rate. The use of the assay at pH 6.5

Fig. 16 Effect of Inhibitor Concentration on Rate of Carotene Bleaching in Linoleate-Carotene-Agar Plates.



affords a reasonably rapid bleaching rate although slight variations of pH about this value could cause considerable change in rate of bleach. Since all antioxidant studies were performed on a comparative basis and since this pH corresponded with the reported maximum for lipoxidase activity for which the assay was also used, it was decided to continue to use the assay at this pH value.

Effect of Inhibitors on the Rate of Bleach of Carotene in Methyl Linolenate-Carotene-Agar Plates.

This assay was devised in order to have a reliable means of studying the efficiency of antioxidants in preventing carotene destruction in a polyphasic system containing fats. Accordingly known polyphenolic antioxidants were added to the assay system and their efficiency measured. In Figure 13 is shown the effect of the antioxidants NDGA, α-tocopherol and BHA at low (between 0.1 and 5%) molar percentages of the fatty substrate. This clearly shows that the antioxidants are effective in lengthening the initial induction period, as well as the fact that the assay is sensitive to varied amounts of inhibitor.

Effect of Catalyst Concentration on Rate of Bleach of Carotene in Methyl Linolenate-Carotene-Agar Plates.

This assay was applied to carotene bleaching studies

Fig. 19 Effect of Soya Concentration on Rate of Carotene Bleaching in Linoleate-Carotene-Agar Plates.

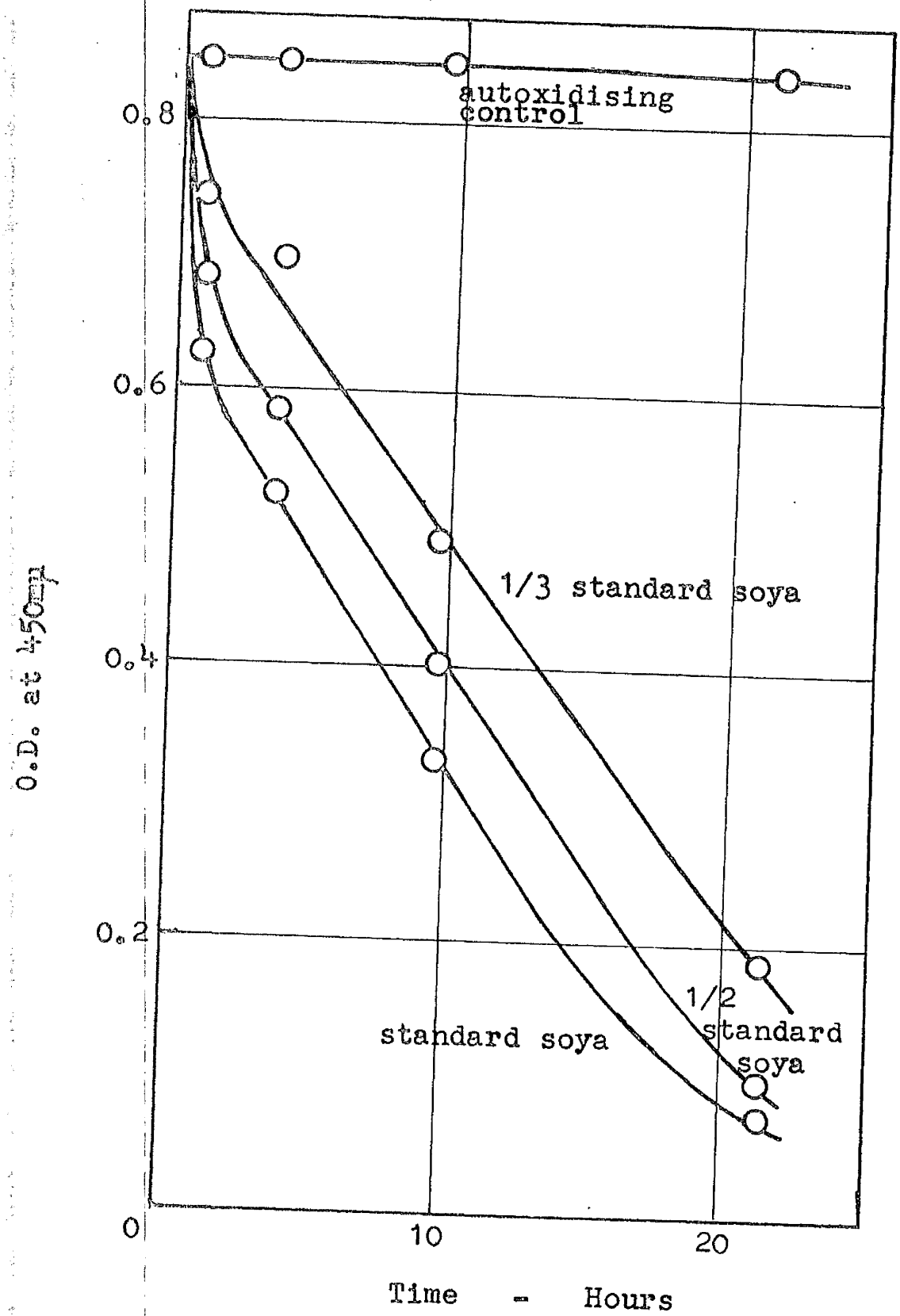
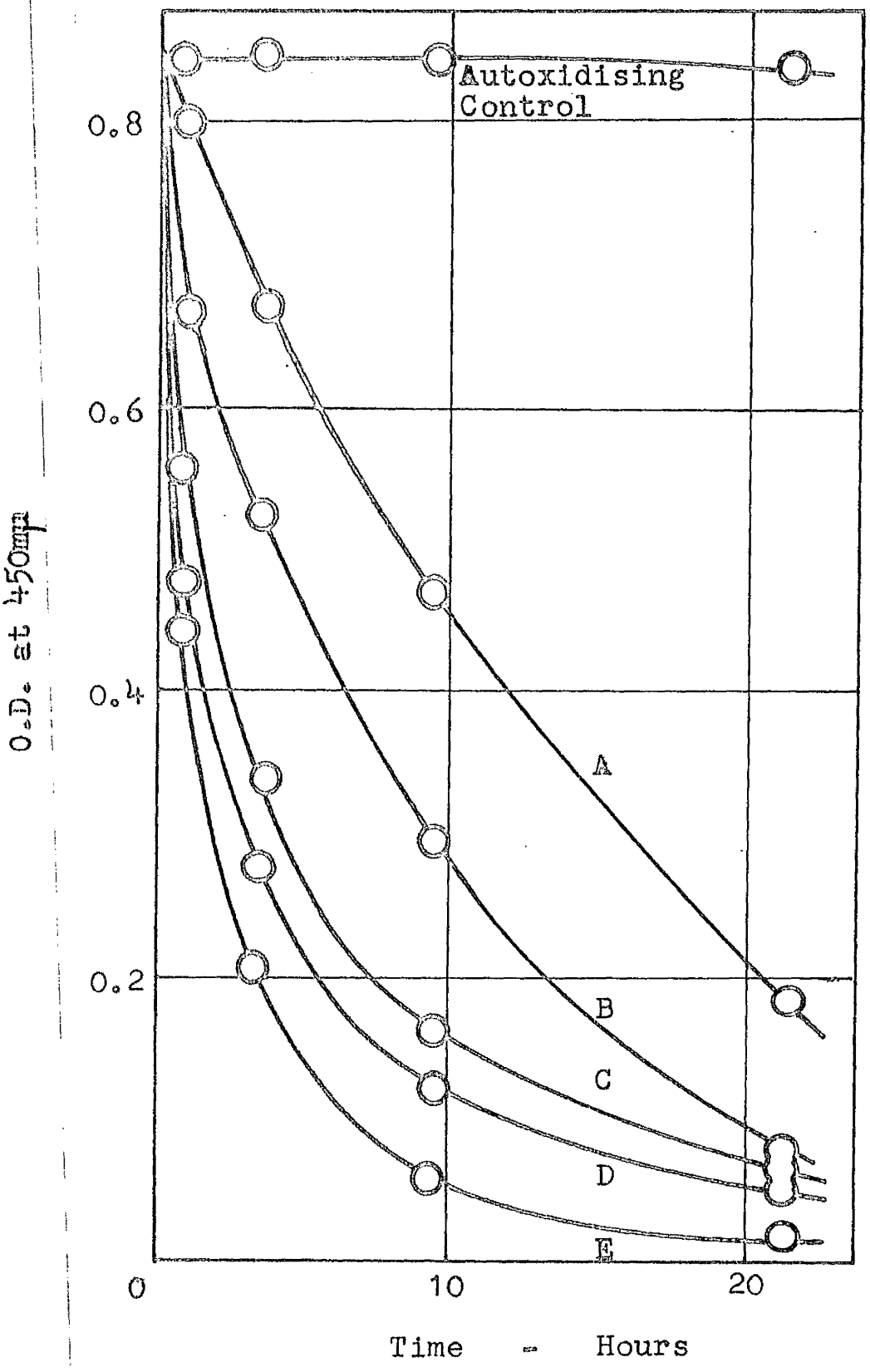


Fig. 20 Effect of Haemoglobin Concentration on Rate of Carotene Bleaching in Linoleate-Carotene-Agar Plates.



Haemoglobin concentrations expressed as haematin:-

A	10^{-7}M	C	5×10^{-7}M	E	10^{-6}M
B	2.5×10^{-7}M	D	7.5×10^{-7}M		

catalysed by fat oxidation catalysts. In this case, lipoxidase from soya extracts and haemoglobin were used as linolenate oxidation catalysts. The standard soya extract (0.5gm defatted soya meal in 40mls. water) and haemoglobin (bovine, Sigma), at a concentration of 2.8mgs/ml water or dilutions of these, were used to test the sensitivity of this assay system to catalyst concentration. The catalyst solutions (0.1ml.) were added to the standard assay at 65°C and were mixed in prior to pouring of agar gels. The reactions produced were rapid and it was found to be unnecessary to use an oxygen enriched atmosphere during dark storage. An autoxidising control was run concurrently with all catalysed systems. The difference in the length of the induction period with and without oxygen enrichment of the storage atmosphere may be readily compared.

The results are shown in Figures19 and 20 for lipoxidase and haemoglobin catalysis respectively. It is obvious that the assay is sensitive to catalyst concentration although this sensitivity does not appear to be directly proportional to catalyst concentration.

The lipoxidase catalysed curves are essentially different in character from those for haemoglobin catalysis. The latter curves tend to show rapid carotene bleaching with the rate decreasing as carotene concentration decreases. That there is no induction period suggests that initially there are present trace amounts of linolenate peroxides with which the

haemoglobin may react. The curves for lipoxidase catalysis show high carotene loss during the first hour of reaction, followed by a period during which the rate of bleach is lower but apparently linear. The rates of bleach during this period appear to be similar regardless of the concentration of soya present. It is considered that there is no catalysed oxidation occurring during this period merely autoxidation. The reason for inactivity of the enzyme after the initial high activity is difficult to understand. Suggested reasons may be:-

1. that the enzyme is being inactivated by heat almost immediately after addition to the hot agar solution. Catalytic bleaching would then occur in the short time prior to inactivation of the enzyme, the high temperature producing high enzyme activity or accelerated rate of carotene bleaching or both.
2. that the enzyme is being inactivated by association with agar. Enzymic bleaching would then occur either prior to gel setting or at least prior to association of agar and enzyme.
3. that the enzyme is being inactivated by contact with linolenate hydroperoxide.

Of the above reasons for enzyme inactivation under the present conditions the first reason seems least possible. Heat inactivation of lipoxidase usually requires higher temperatures (>80°C) for longer periods (several minutes) than

are used here.

Either or both the second or third reasons may be the cause of the inactivation. Many protein materials have been shown to strongly adsorb to agar (121,122). Such adsorption may well cause temporary enzyme inactivation. Peroxide inactivation of enzymes has been studied by Wills(123), who has demonstrated the inactivation of a range of enzymes as a result of their contact with fatty peroxides. Inactivation of lipoxidase has not been demonstrated by him but it seems possible. Support for such inactivation is found in the fact that throughout the course of the work for this thesis there has been little lipoxidase activity observable beyond one hour regardless of the initial levels of substrate or enzyme used.

Steps were taken in an effort to overcome this difficulty of a two phase reaction with lipoxidase. Use of Ionagar,as described in Appendix IIIb, certainly reduced evidence of this second phase but whether this resulted from a faster rate of bleach during the suspected subsequent autoxidative reaction is not known.

Effect of Antioxidants on the Autoxidative Rate of Bleach of Carotene in Agar Plate Assay.

These studies were carried out using the two assay systems described in Appendix IIIa and b. They will be reported in a

Fig. 21 Effect of Antioxidants on Rate of Carotene Bleaching in Methyl Linoleate-Carotene-Agar Plates.

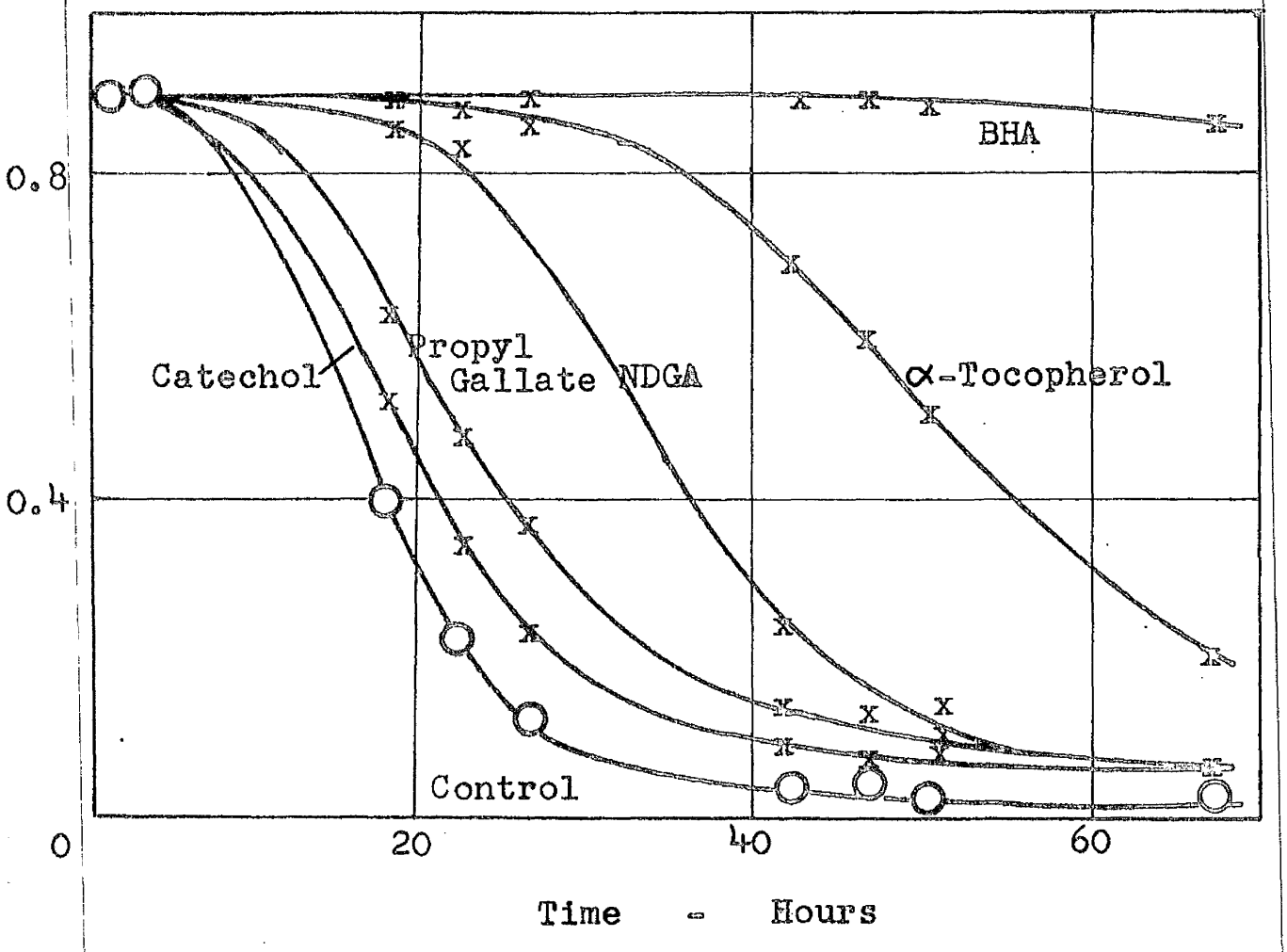


Table VIII. Effect of Antioxidants on the Rate of Bleach of Carotene in Carotene-Agar Plates with and without Fatty Substrates.

Antioxidants	Time in Hours to 5 and 50% Carotene Bleach.			
	No Substrate		Methyl Linolenate	
	5%	50%	5%	50%
None	15,15	35, 38	7, 8	17, 17
NDGA	18,19	41, 47	18, 21	34, 36
Propyl Gallate	15,15	37, 34	11, 11	24, 28
DMA	26,36	>115,>90	90,105	»140,>135
α-Tocopherol	21,40	>115,>90	29, 31	53, 51
Catechol	15,15	35, 35	10, 11	80, 83

	Linoleic Acid		Trilinolenate	
	5%	50%	5%	50%
None	11,11	32, 36	4, 5	9, 9
NDGA	30,28	68, 69	6, 6	12, 16
Propyl Gallate	38,37	96,>95	4, 4	8, 13
DMA	23,16	120, 83	16,21	52, 61
α-Tocopherol	35,21	62, 54	15,12	34, 31
Catechol	17,18	48, 54	4, 4	8, 12

like manner.

(a) Using the assay described in Appendix IIIa the effect of the antioxidants, NDGA, propyl gallate, α-tocopherol, BHA and catechol, on the rate of bleach of carotene was studied. The antioxidants were incorporated on an equimolar basis at a level of ~1% (9×10^{-6}M) of the methyl linoleate substrate (10^{-3}M). In Figure 21 are shown typical results of these studies. It is readily seen that the successful antioxidants prolong the induction period during which little or no carotene bleach occurs. The subsequent carotene bleaching may also be affected in the presence of some of the antioxidants, especially some of the more successful ones. In order to show both effects in tabular form it was considered that the time in hours for the initial 5% and for 50% bleaching should be shown. This was done and the results of typical replicate assays are shown in Table VIII.

Also shown in this Table are results of similar studies in which no fatty substrate, trilinolenate and linolenic acid were substituted for methyl linoleate. Linolenic acid was at a concentration of 10^{-3}M and trilinolenate at 3.3×10^{-4}M. in these assays. This gave equal degrees of unsaturation in all three assays.

(b) Using the improved assay system described in Appendix IIIb the effect of a further group of antioxidants on carotene bleaching rate was studied. The results are shown in Table IX.

Table IX Effect of Antioxidants on the Rate of Bleach of Carotene in Carotene-Agar Plates, with and without Fatty Substrates.

Antioxidants	Time in Hours to 5 and 50% Carotene Bleach					
	No Substrate		Pelargonic acid		Methyl Linoleate	
	5%	50%	5%	50%	5%	50%
None	6, 6	16, 18	18	45	6, 5	11, 14
NDGA	6, 7	19, 22	20	67	14, 13	60, -
BHA	8, 9	25, 30	18	48	23, 17	58, 57
^{t}Butyl-phenol(mixed)	7, 8	22, 25	29	75	17, 15	34, 26
2.6-di-^{t}butyl-phenol	6, 7	17, 20	26	66	16, 13	34, 31
BHT	10,11	27, 29	42	79	15, 13	40, 42
2.6-di-^{t}butyl-methoxy-cresol	8, 9	25, 26	38	140	15, 15	39, 55
2.6-di-^{t}butyl-dimethylamino-cresol	5, 6	16, 17	12	46	10, 7	16, 16
4.4'-bis-2.6-di-^{t}butyl-phenol	39,39	380,360	210	-	150,165	300, 300
4.4'-methylene-bis-2.6-di-^{t}butyl-phenol	18,19	73, 72	44	240	63, 47	173, 150
4.4'-methylene-bis-6-^{t}butyl-cresol	12,15	58, 55	40	220	88, 72	240, 270
Santoquin	9,11	40, 43	50	190	133, 70	300, 300

Table IX.(contd.)

Antioxidants	Time in Hours to 5 and 50% Carotene Bleach			
	Linoleic Acid		Trilinolenate	
	5%	50%	5%	50%
None	14, 12	28, 28	2, 3	7, 8
NDGA	30, 36	144, 138	4, 4	16, 18
BHA	19, 26	62, 64	9,10	31, 40
^{t}Butyl-phenol(mixed)	18, 19	36, 43	5, 5	18, 20
2.6-di-^{t}butyl-phenol	21, 22	46, 58	5, 6	21, 23
BHT	21, 26	49, 61	8, 8	31, 33
2.6-di-^{t}butyl-methoxy-cresol	17, 19	36, 50	6, 6	24, 25
2.6-di-^{t}butyl-dimethylamino-cresol	14, 17	23, 37	3, 4	5, 6
4.4'-bis-2.6-di-^{t}butyl-phenol	96,300	128, 350	14,15	260,300
4.4'-methylene-bis-2.6-di-^{t}butyl-phenol	25, 38	108, 200	23,38	68, 85
4;4'-methylene-bis-6-^{t}butyl-cresol	28, 40	104, 135	13,15	80,140
Santoquin	125,144	220,>300	18,19	118,175

In order to correlate, in some manner, the results obtained with this system with those of the earlier system the antioxidants NDGA and BHA were again used. The antioxidants were again equimolar (6.7×10^{-6} M) and at a level ~1% of the methyl linoleat substrate (7.4×10^{-4} M).

The systems studied included assays with no substrate and others with the fatty substrates, pelargonic acid, methyl linoleate, trilinolenate and linoleic acid. (Pelargonic acid was used as the highest available saturated fatty acid which was liquid at normal temperatures). In this assay system the different substrates did not have such a great effect on the dispersion of the carotene as measured by the initial optical densities. Thus the values were with no substrate ~0.8, with pelargonic acid ~0.85, with trilinolenate ~0.9, with methyl linolenate ~0.95 and with linoleic acid ~1.0. The nature of the curves obtained here were similar to previous curves although the induction periods were all shorter except with linoleic acid where it was slightly longer. All the subsequent rates of oxidation of carotene were higher than before.

Of the antioxidants screened there were several excellent carotene protectors, notably santoquin and the bis-phenols. These appear to be several fold better than BHA which was the best antioxidant of the previous group. Again it was noted that some antioxidants appear to have a carry through or

Fig. 22 Effect of Antioxidants on the Rate of Carotene Bleaching in Methyl Linoleate-Carotene-Agar Plates Catalysed by Soya Extract

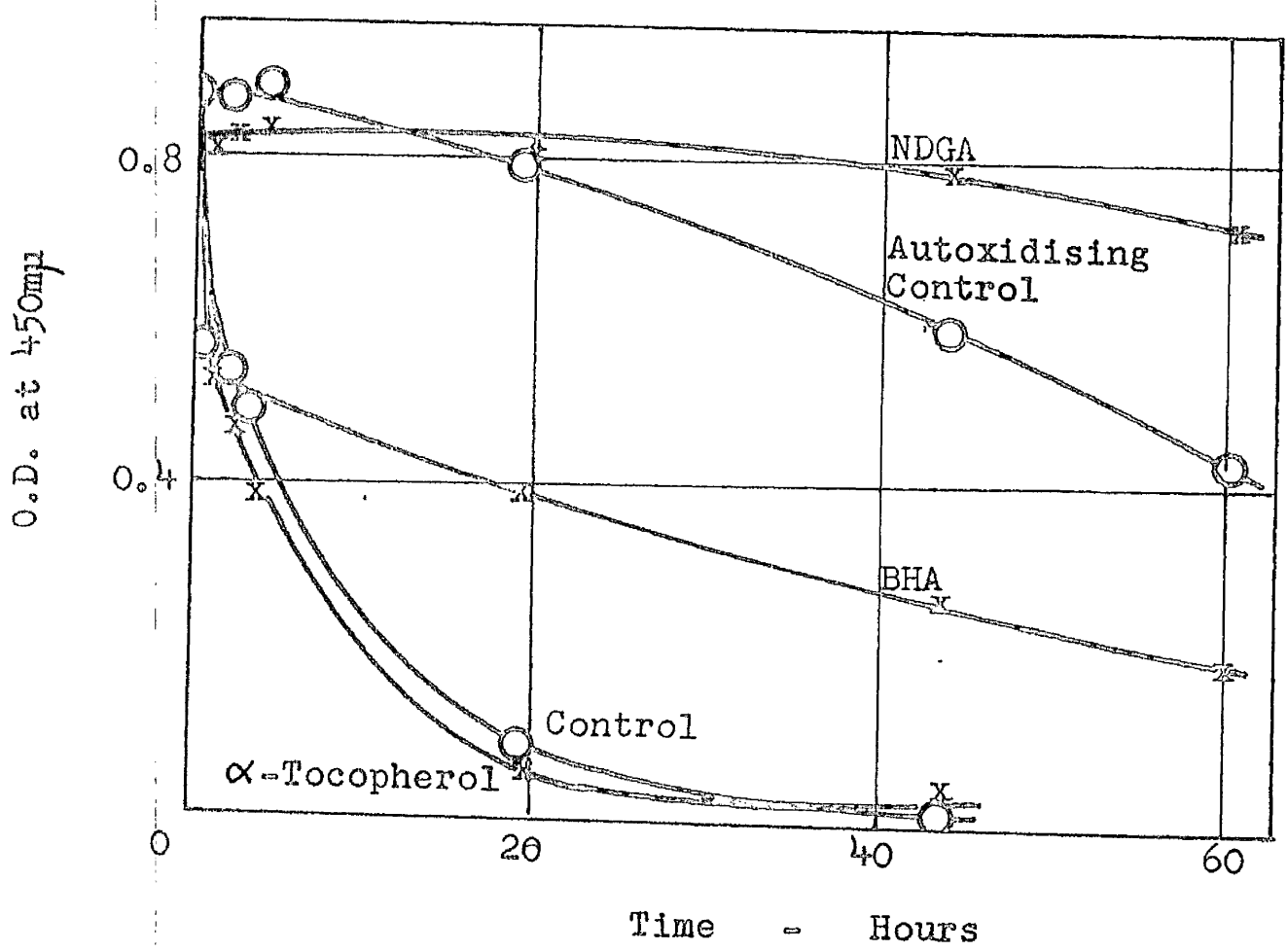
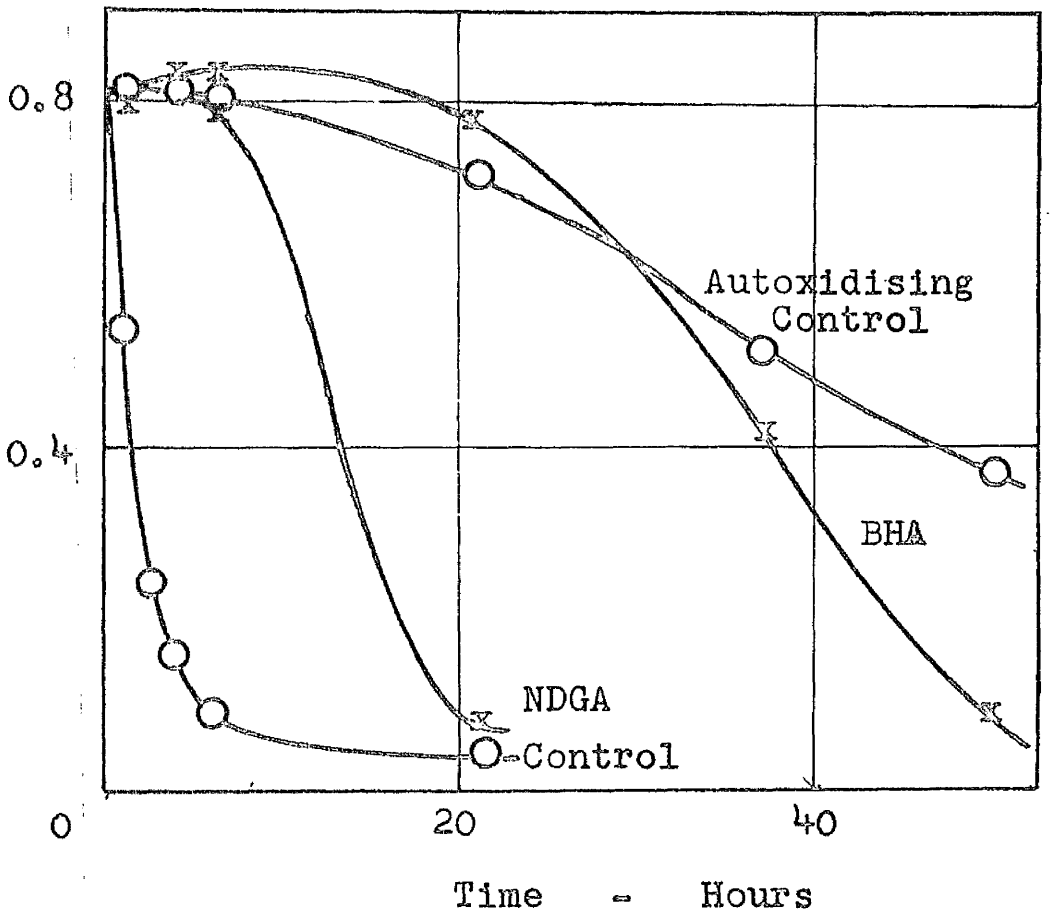


Fig.23 Effect of Antioxidants on Rate of Carotene Bleaching in Methyl Linoleate-Carotene-Agar Plates Catalysed by Haemoglobin.



slowing effect on the bleaching rate beyond the induction period. The results of Table IX accordingly show the time in hours for 5 and 50% destruction of carotene.

Effect of Antioxidants on the Rate of Bleach of Carotene in Methyl Linoleate-Carotene-Agar Plates Catalysed by Lipoxidase or Haemoglobin

As in the previous section, this work was carried out using the original and the improved assay techniques. Again the results will be reported separately.

(a) Using the original assay, described in Appendix IIIa, the effect of the antioxidants on the catalysed reaction was studied. The catalysts were added in aqueous solution (1 c.c.) to the assay mixes prior to gel setting. The concentration of catalyst used was the standard soya extract and haemoglobin (2.5 mgs/ml. this giving an overall concentration of 10^{-6}M haematin in the assay). These assays were carried out using an air atmosphere. The effect of the antioxidants, NDGA, propyl gallate, BHA, α-tocopherol and catechol was studied and typical results for some of the more successful of these antioxidants are shown. (Figs. 22 and 23).

For lipoxidase catalysis only NDGA succeeds in protecting carotene against the initial activity of the enzyme. This is as has been shown previously with other systems. Of the other antioxidants BHA inhibits carotene loss only after the initial

Table X. Effect of Antioxidants on the Rate of Bleach of Carotene in Lipoxidase or Haemoglobin Catalysed Methyl Linoleate-Carotene-Agar Plates.

Antioxidants	Time in Hours for 5 and 50% Carotene Bleach			
	Lipoxidase		Haemoglobin	
	5%	50%	5%	50%
None	<1	8, 12	<1	1, 1½
NDGA	>40, >40	-, -	10, 12	12, 14
Propyl Gallate	<1	6, 19	<1	1½, 2
BHA	<1	22, >50	30, 30	54, 66
α-Tocopherol	<1	4, 18	<1	1½, 2
Catechol	<1	8, 26	<1	1½, 2

bleach in the first hour of reaction. In this first hour there is, if anything, a slight increase in carotene bleaching in the presence of BHA in comparison to the control. The reason for the subsequent ability of BHA to inhibit further carotene loss is thought to be due to the antioxidant's ability to inhibit the autoxidative reaction. This result supports the suggestion made earlier that there is only a brief period during which the enzyme is active followed by a period when only autoxidation is occurring. With α-tocopherol the rate of bleaching is higher both during the initial and secondary periods. This is possibly because the α-tocopherol acts as a chain carrier under the conditions of the reaction rather than its normal role of chain breaker. However, as may be seen from Table X, where the times to 5 and 50% carotene loss are noted reproducibility of results of replicate assays is not great. Thus in one case α-tocopherol accelerates and in another slightly retards the time to 50% carotene loss in comparison with the control. This is considered to be due to variation in the activity of the soya extract used. With high activity the initial bleaching rate is high and the tocopherol tends to have a pro-oxidative effect during the secondary phase while with lower activity it can show a little antioxidative effect during this secondary phase.

The results with the other antioxidants show the same lack of reproducibility for the lipoxidase catalysed reaction (see Table X).

Table II Effect of Antioxidants on the Rate of Bleach of Carotene in Lipoxidase or Haemoglobin Catalysed Methyl Linoleate-Carotene-Agar Plates.

Antioxidants	Time in Hours to 5 and 50% Carotene Bleach			
	Lipoxidase		Haemoglobin	
	5%	50%	5%	50%
None	<1	5½, 5½	<1	3, 4
NDGA	3,3	38 , -	28,41	46,64
BHA	<1	2½, 4	22,33	60,70
^{t}Butyl-phenol(mixed)	<1	1½, 4	5, 6	11,12
2.6-di-^{t}butyl-phenol	<1	1 ,<1	6, 7	19,25
BHT	<1	<1 , 2	12,14	32,38
2.6-di-^{t}butyl-4-methoxy-p-cresol	<1	<1,, 1	11,12	54,58
2.6-di-^{t}butyl-dimethylamino-p-cresol	<1	1 ,<1	5, 5	7, 7
4.4'-bis-2.6-di-^{t}butyl-phenol	<1	9 ,39	<1	4, 5
4.4'-methylene-bis-2.6-di-^{t}butyl-phenol	<1	1 , 9	1, 2	4, 7
4.4'-methylene-bis-6-^{t}butyl-cresol	<1	1 , 3	15,20	47,81
Santoquin	<1	1 , 3	75,85	92, -
Autoxidising control	10, 9	28,29		

Despite this poor reproducibility it should be emphasised that this assay clearly indicates that, with the exception of NDGA, all the antioxidants are of little use as inhibitors of lipoxidase catalysed linoleate-carotene oxidation. This is largely similar to the results obtained from studies of the effect of these antioxidants on the lipoxidase catalysed linoleate oxidation reported in the previous section of this thesis.

The successful competitive inhibitor of lipoxidase, the tetraynoic acid, was of little use in the present assay.

For the haemoglobin catalysed reactions the results obtained were better in so far as they gave considerably better reproduction. In this case it is possible to add the same quantity of catalyst and it would seem that the catalyst is not inactivated in the course of the reaction. This latter point can be seen in Figure 23 where it is observed that the rate of oxidation of carotene is high even after long induction periods which are produced in the presence of the effective antioxidants. Here the successful antioxidants were NDGA and BHA with the other antioxidants being of little value as may be seen in Table X.

(b) Using the improved assay method, the effect of a further group of antioxidants on the catalysed reactions was studied. The antioxidants, and the results of their use, are shown in Table XI. The results obtained were very similar to those of the previous group.

Thus for the lipoxidase catalysed reaction the only inhibitor shown was NDGA. This again agrees with results of Section II of this thesis. The curve obtained for NDGA inhibition did not show a true induction period in this case but rather a slow loss of carotene. This slight loss of carotene in the initial stages would suggest that the enzyme activity was somewhat higher than in the previous assay system. This could occur as a result of a more loose or non-association of the enzyme protein with the Ionagar. The rate of loss decreased after a few hours presumably due to enzyme inactivation. Of the other antioxidants, only 4.4'bis-2:6-di-^{t}butyl-phenol was effective in prolonging the time for the initial 50% carotene loss. This antioxidant has been shown to be the most effective inhibitor of carotene loss in the presence of autoxidising methyl linoleate. All the other antioxidants, including BHA, tended to accelerate carotene bleaching.

For the haemoglobin catalysed reaction all the antioxidants were successful to some degree, some being poor while others were very good (notably santoquin.). They acted in a manner similar to that already shown, producing induction periods followed by carotene bleaching at variable rates depending on the antioxidant present.

Conclusions and Discussion.

In a recent review, Privett (124) makes plain that the use of antioxidants for carotene protection under practical

conditions has been on an empirical basis making use of antioxidants shown to be good for carotene protection in model systems which tend to be divorced from practical reality.

The present assay was developed as a model system which at least comes closer to practical conditions in that a biphasic system, to which desired constituents may be added, is used. Here, fats, mainly of an unsaturated nature, were added to determine their influence on carotene bleaching in the presence of antioxidants. This new assay has been shown to be applicable to autoxidative and catalysed reactions. The catalysts investigated were the unsaturated fat oxidases, haemoglobin and lipoxidase, which give rise to secondary bleaching of carotene.

For the autoxidative studies, the assay has been shown to be sensitive to the presence of efficient antioxidants by a prolonging of the initial induction period. Some antioxidants also show a "carry through" property in that even after completion of the induction period they manage to slow the rate of carotene loss in comparison to the control reactions. The assay has also been shown to be sensitive to variation of antioxidant concentration, inhibition increasing with antioxidant concentration.

The nature of the fat added to the system has a notable effect on the rate of carotene bleaching, apart from the actual effect which the fats have on the dispersion of the carotene. In Table IX it is seen that without any substrate bleaching of

carotene is fairly rapid, while in the presence of pelargonic acid this rate is markedly decreased. Presumably the association of the carotene and fat helps to protect the carotene from oxidative degradation. In the presence of the unsaturated fats, the time to 5 and 50% carotene bleach is much shorter than with pelargonic acid in the system. These fats have a pro-oxidant nature on account of their unsaturation and presumably the rate of carotene bleach is a balance of the antioxidative effect of the fat and the pro-oxidative nature of this fat. With the three unsaturated fats used here, it is impossible to speculate further on this balance since it has been shown that variable amounts of trace peroxides were present in these substrates.

The presence of fats and their nature also affects the efficiency of antioxidants in this assay. Taking santoquin as an example, from Table IX, it is seen that in the presence of no fatty substrate the initial induction period is approximately 10 hours, with pelargonic acid as substrate this has risen to 50 hours and with methyl linoleate or linoleic acid this value is of the order of 100 hours. Here obviously the presence of fat improves the contact or the orientation of the antioxidants with respect to the carotene. With santoquin the importance of the nature of the fat is shown by the fact that the induction period with the pro-oxidant unsaturated substrates is considerably greater than with the saturated

pelargonic acid.

While a longer induction period with the unsaturated fat than with the saturated fat, as mentioned above, is not exceptional neither is it the general rule because the reverse is the case with some of the antioxidants. Examples of both may be selected from Table IX. It is, however, more generally true that the presence of fat, be it saturated or unsaturated, improves the efficiency of all the antioxidants as carotene protectors in comparison to the system where no fat is present.

The addition of haemoglobin or soya extract to an assay system containing methyl linoleate accelerates the carotene bleaching rate. For both catalysts there is no observable induction period in the absence of antioxidant and the time of 50% bleaching is a few hours at the most. The assay has been shown to be sensitive to catalyst concentration although the increase in bleaching rate was not directly proportional to the increase in catalyst concentration.

In the assays with haemoglobin as catalyst the presence of an efficient antioxidant produced a notable induction period, at the end of which a high rate of bleaching was observed. This is indicative of the haemoglobin still being active as a catalyst of linoleate oxidation.

In the studies with antioxidants the results of the haemoglobin catalysed assays show reasonable replication with regard to the time to 5 and 50% carotene bleaching (Table X and XI

In contrast to the haemoglobin catalysed reaction, that with

lipoxidase is less satisfactory. As has been pointed out it is considered that the lipoxidase enzyme is inactivated in some way. Accordingly these assays give rise to two phases of reaction one during which the enzyme is active, the second during which it is inactive. In this latter phase the reaction is merely autoxidative using presumably a considerably peroxidised substrate.

Despite the fact that the lipoxidase catalysed assay seems to be somewhat unsatisfactory, it should be re-emphasised that of the antioxidants examined this assay showed NDGA to be the only satisfactory inhibitor of the enzymic activity. This is similar to the results shown in Section II of this thesis, where an aqueous system, which resembled the present one with regard to quantities of constituents, was used.

With the other antioxidants the replication of results, as shown in Table X and XI is not as good as for haemoglobin catalysed or autoxidative assays. This is considered to be due to variations in the enzyme activity of the soya extracts being used.

Consideration of the order of effectiveness of the antioxidants for autoxidative and haemoglobin catalysed assays, makes it possible to select some of the better antioxidants for particular conditions.

For autoxidation of carotene in the absence and the presence of both saturated and unsaturated fats the bis-phenols and

santoquin would appear to be the best antioxidants. Of the other antioxidants α-tocopherol, BHA, BHT and to a much lesser degree NDGA show reasonable, but considerably lower, efficiency in preventing autoxidation of carotene, Of the remaining antioxidants there are none which notably protect carotene with the exception of propyl gallate in the presence of linoleic acid as fatty substrate. With this substrate propyl gallate is the best antioxidant of those shown in Table VIII. This is surprising since, with the other substrates, propyl gallate was relatively ineffective.

On further examination of the order of effectiveness of the antioxidants with individual fatty substrates, NDGA was noted also to appear higher in the order of antioxidants with linoleic acid as substrate than with the other fatty substrates. It is not clear why NDGA and propyl gallate should appear more effective with linoleic acid than with the other substrates but it seems of some significance that of the antioxidants being examined these two contained the most polar groups and that linoleic acid was also the most polar of the substrates. It could well be that this increased polarity of antioxidant and substrate produced this effect.

With the haemoglobin catalysed reaction, the most effective antioxidant was santoquin. This antioxident is quite outstanding in comparison to other relatively efficient antioxidants such as NDGA, BHA, 4,4'-methylene-bis-6-^{t}butyl-cresol, BHT and 2,6-di-^{t}butyl-methoxy-p-cresol. Most of the other

antioxidants used gave some slight inhibition of carotene destruction although two of the bis-phenols which had shown exceptional inhibition in all the autoxidative assays were notably poor.

A disadvantage of the present assay system is that it is not possible to follow the course of oxidation of the unsaturated fatty substrates. It must be emphasised that the assay only determines the best antioxidants for protection of carotene and not for protection of fat oxidation. That an antioxidant may protect against the secondary oxidation and not the primary reaction will be demonstrated in the final section of this thesis. With such protection there possibly would be present undesirable fat rancidity although carotene had been protected. However, it is considered that, with a knowledge of the present results, the selection of an antioxidant, suitable for the protection of carotene in a biological system, could be made more readily. Thus, in such a biological system, if the presence of lipoxidase activity was suspected, the inclusion of NDGA would seem desirable; if the oxidative process was thought to be haematin catalysed or purely autoxidative, the use of santoquin would be suggested; and if the nature of the pro-oxidant was unknown, a mixture of NDGA and santoquin ought to be used.

It was with such an idea in view that the present assay was developed, and, if successful, this certainly would raise the use of

antioxidants above the empirical level at which they are at present employed. Whether in fact, under practical conditions, the selected antioxidants would function successfully is not known and it is realised that selection of even a suitable antioxidant for a particular oxidative process does not of itself mean success. There is the problem of incorporation of the antioxidant into the system at the site of oxidation but such a problem is outwith the scope of the author's field of work.

A more important problem, and one which to the best of the author's knowledge has not been widely studied, if at all, is that of elucidating the role of the various pro-oxidant factors in producing fat oxidation and the secondary degradations of other substances present in biological systems. Means of determining the relative importance of such pro-oxidant factors both individually and collectively would greatly aid the tackling of the major problem of fat rancidity and vitamin loss in stored foods.

S E C T I O N IV

A STUDY OF LIPOXIDASE CATALYSED LINOLEATE-VITAMIN A REACTION AND THE EFFECT OF ANTIOXIDANTS ON IT.

SECTION IV.

A STUDY OF LIPOXIDASE CATALYSED LINOLEATE-VITAMIN A REACTION AND THE EFFECT OF ANTIOXIDANTS ON IT.

From the foregoing results in aqueous media it appears obvious that under the present conditions only NDGA of the antioxidants is a completely successful inhibitor of the enzyme lipoxidase when the enzyme is acting as an unsaturated fat oxidising catalyst. In the agar plate assay NDGA again was shown to be the only successful inhibitor of carotene loss in the presence of unsaturated fat and lipoxidase. However, this latter study was concerned with long term study of antioxidant effect. It did not follow the course of carotene bleaching during the initial stages of reaction nor, as has been pointed out, did it allow determination of the extent of oxidation of the unsaturated fat.

Previous studies in which primary fat and secondary oxidations have been observed concurrently are limited. There have been several studies of coupled linoleate-carotene oxidations catalysed by lipoxidase, but they have been interested mainly in determining conditions for which carotene bleaching was proportional to enzyme activity so that a direct colorimetric assay of lipoxidase activity could be obtained. There are two studies only (82,106) known to the present author, in which both

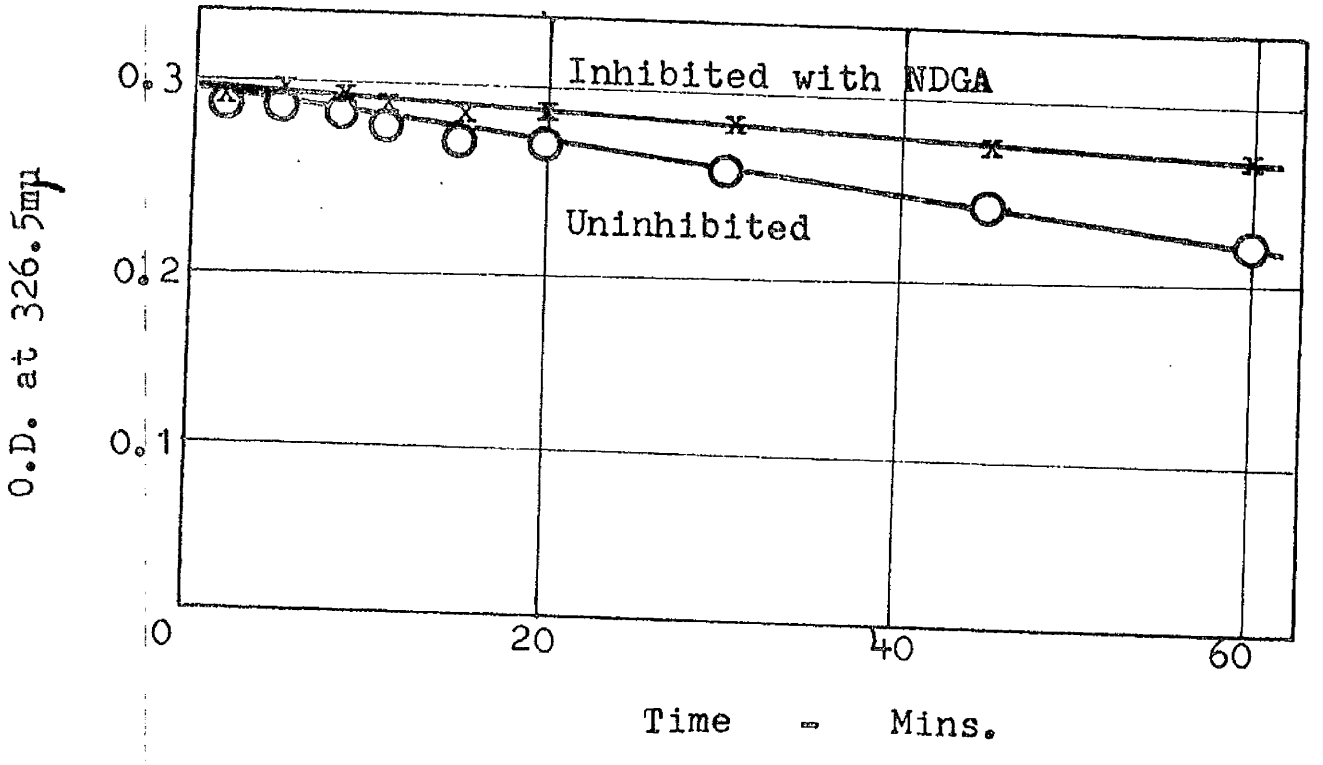
linoleate and carotene-oxidation have been observed concurrently for a lipoxidase catalysed reaction.

Similar studies with vitamin A substituted for carotene are even more limited if indeed there are any such. Therefore, it was decided to briefly investigate this lipoxidase catalysed coupled reaction, and also to study the effect of antioxidants on both the primary and secondary oxidation.

For these studies it was considered that an aqueous assay system would serve best and accordingly the systems described in Appendix IVa & b were devised. These systems are similar in type to that in Appendix I with the addition of vitamin A acetate which was added in ethanolic solution giving rise to a milky emulsion system.

The use of more than one assay system for these present studies, and other studies in the course of work for this thesis, is considered to serve a two-fold purpose. Firstly, a change in system often means an improvement in assay conditions made in the light of experience from the earlier assay. Secondly, such changes are considered to act as safeguards to the results obtained. These systems are often quite complex involving many variables. Results obtained with one system may apparently be due to a factor being examined. If similar results can be shown over two or more systems the results and conclusions drawn from them will be made more certain.

Fig.24 Stability of Vitamin A Acetate in Aqueous Buffer Solution in the Presence and Absence of Antioxidant.



Stability of Vitamin A Acetate in Aqueous Buffer Media in the Presence of Linoleic Acid.

An ethanolic solution of Vitamin A acetate was added to McIlvaine's phosphate-citrate buffer pH 6.5 containing an emulsion of linoleic acid (1.3×10^{-3}M). This gave an overall vitamin A acetate concentration of 2.3×10^{-4}M. This system was aerated by means of a magnetic stirrer and at suitable time intervals samples (1ml.) were taken into 60% ethanol (25mls.). The vitamin A acetate present was estimated by measuring the optical density of the ethanolic solution in light of wavelength 326.5mμ using an Optica spectrophotometer.

As may be seen from Figure 24, the vitamin A acetate is fairly stable there being only a slight loss in the course of an hour. Whether this loss was due to oxidation of the vitamin or due to precipitation of the vitamin out of the emulsion on to the glass walls of the reaction vessel was not clear. Some NDGA (2.4×10^{-5}M) was added to the reaction system and as may be seen (Figure 24) this considerably reduced the vitamin loss.

It would seem that the loss of vitamin was due to oxidative degradation which probably occurred *via* a chain mechanism since the antioxidant NDGA and others could considerably reduce this loss.

Effect of Soya Extract on Linoleic Acid-Vitamin-A Acetate System.

A sample (1ml.) of standard soya extract was added to the uninhibited system above and at time intervals samples (1ml.) of

Fig.25 Effect of Soya Extract on Linoleic Acid-Vitamin A Acetate Coupled Reaction.

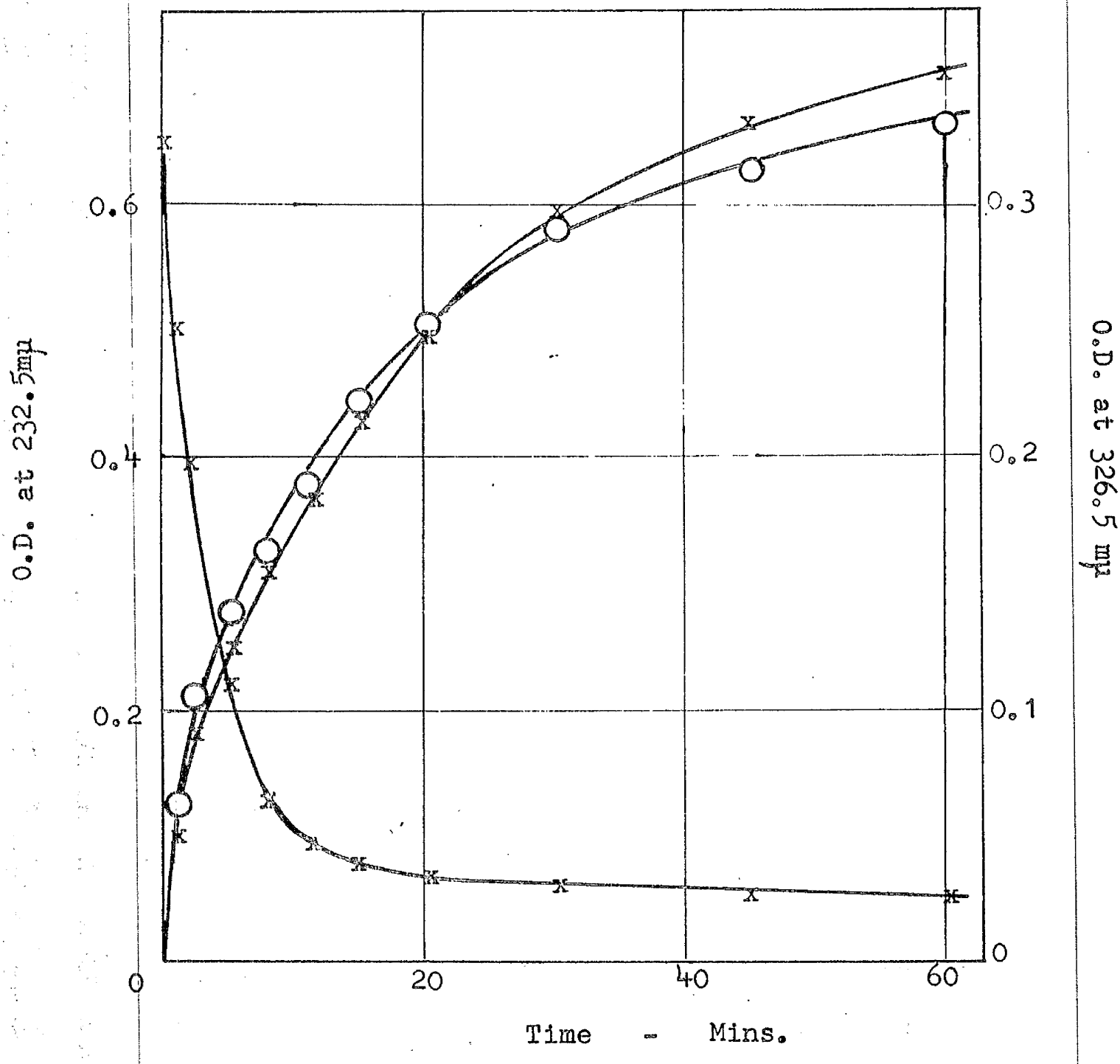
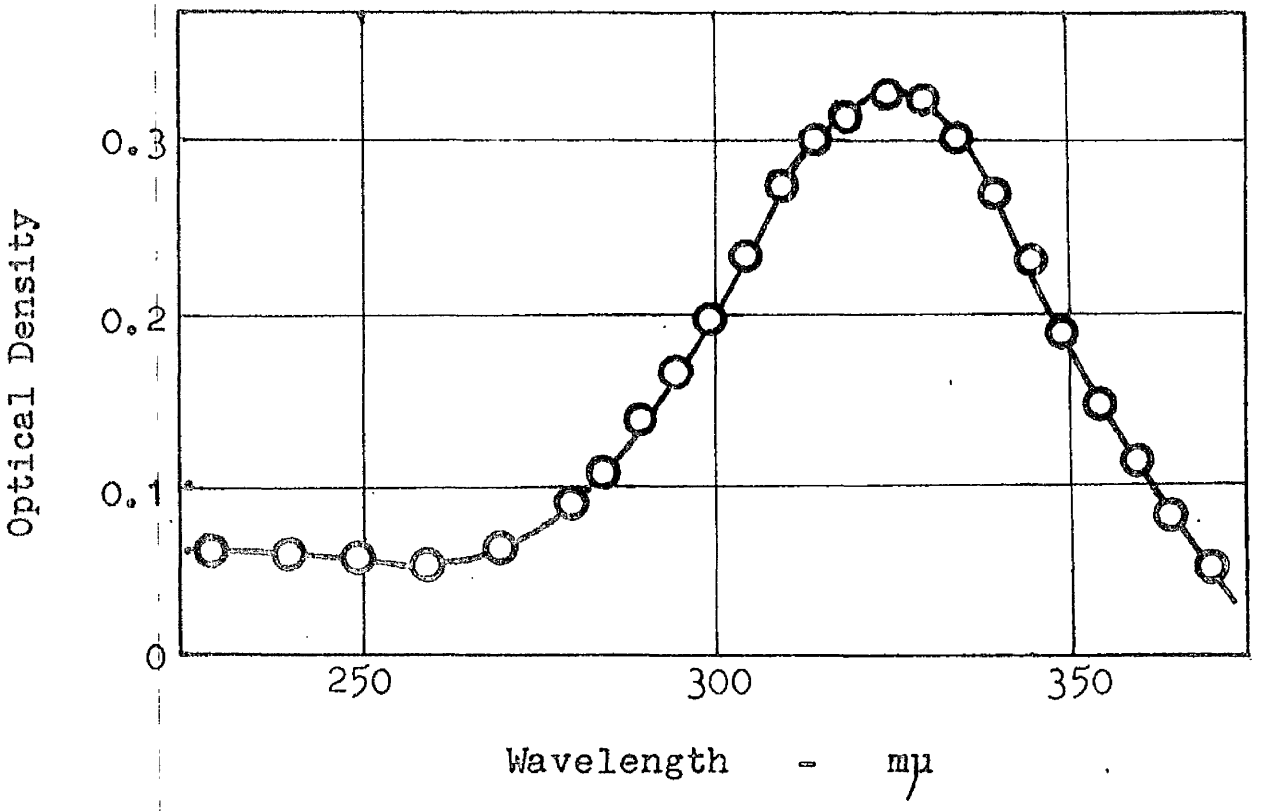


Fig.26 Spectrum of Vitamin A Acetate in Ethanol.



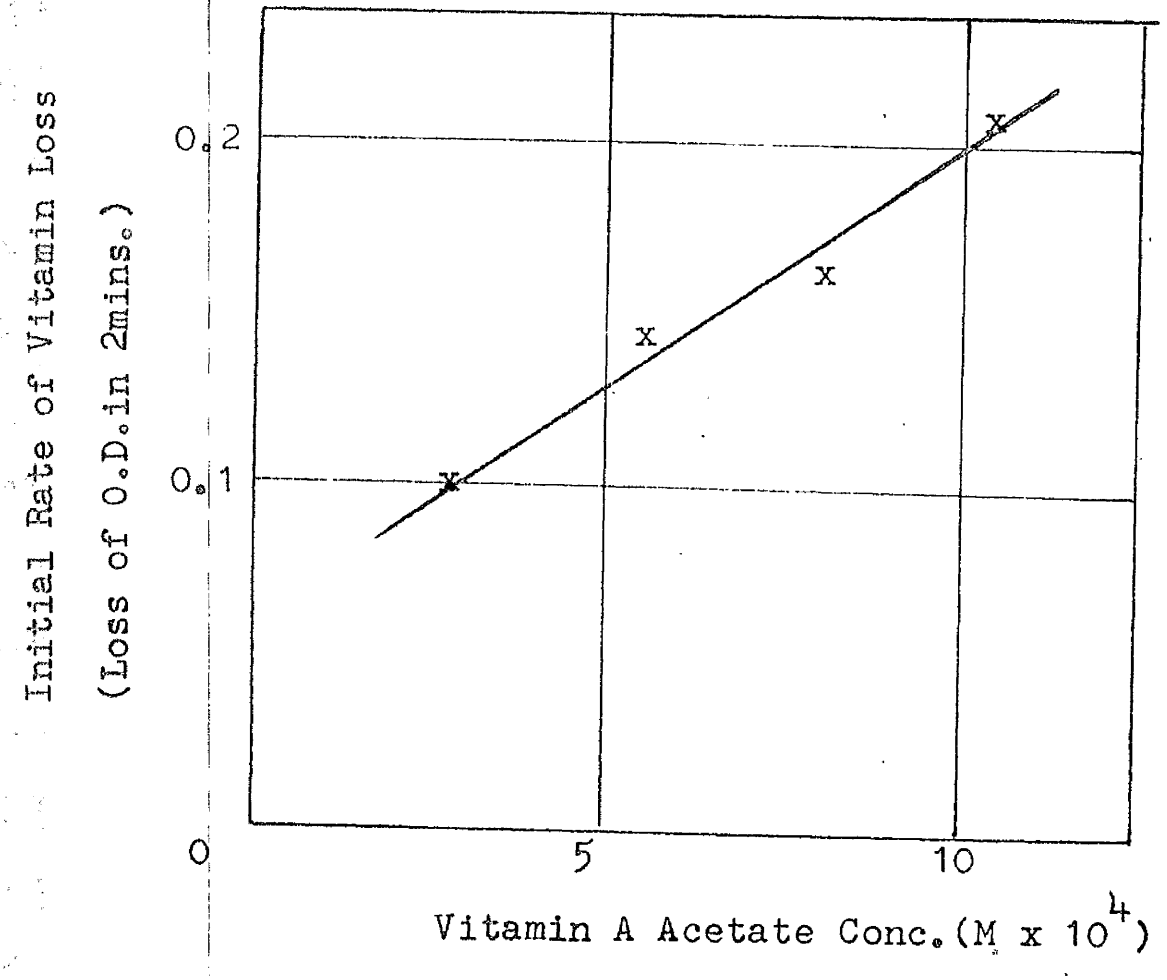
reaction mixture were pipetted into 60% ethanol (25mls.). The optical density of these samples at 232.5 and 326.5mμ was measured and the results of this are shown in Figure 25 where it is seen that there is a rapid loss of Vitamin-A acetate concurrent with the initial oxidation of linoleic acid. This is similar to findings of others who have worked with linoleate-carotene systems. They have shown rapid loss of carotene during the initial stages of both catalysed (82,106) and autoxidative (89,105) linoleate oxidation.

Also shown in Figure 25 is a curve of conjugated diene formation of linoleic acid in an identical lipoxidase catalysed reaction from which the vitamin-A acetate has been excluded.

The presence or absence of the vitamin-A has little notable effect on the rate of linoleic acid oxidation. This result can be related to that of Tookey et al. (82) who showed that addition of carotene had little influence on conjugated diene formation, while it contrasts with findings of Holman (106) who showed that the presence of carotene reduced diene formation.

It should be noted that vitamin-A acetate absorbs at 232.5mμ (Figure 26). It is impossible to know whether absorption at 232.5mμ decreases or increases as a result of vitamin degradation. In drawing the curves of diene formation in Figure 25 and in all subsequent diagrams it is assumed that this absorption does not materially alter as a result of such degradation. Support for this assumption is found in a study of vitamin-A acetate degradation in the presence and absence of

Fig.27 Effect of Vitamin A Acetate Concentration on the Rate of its Degradation.



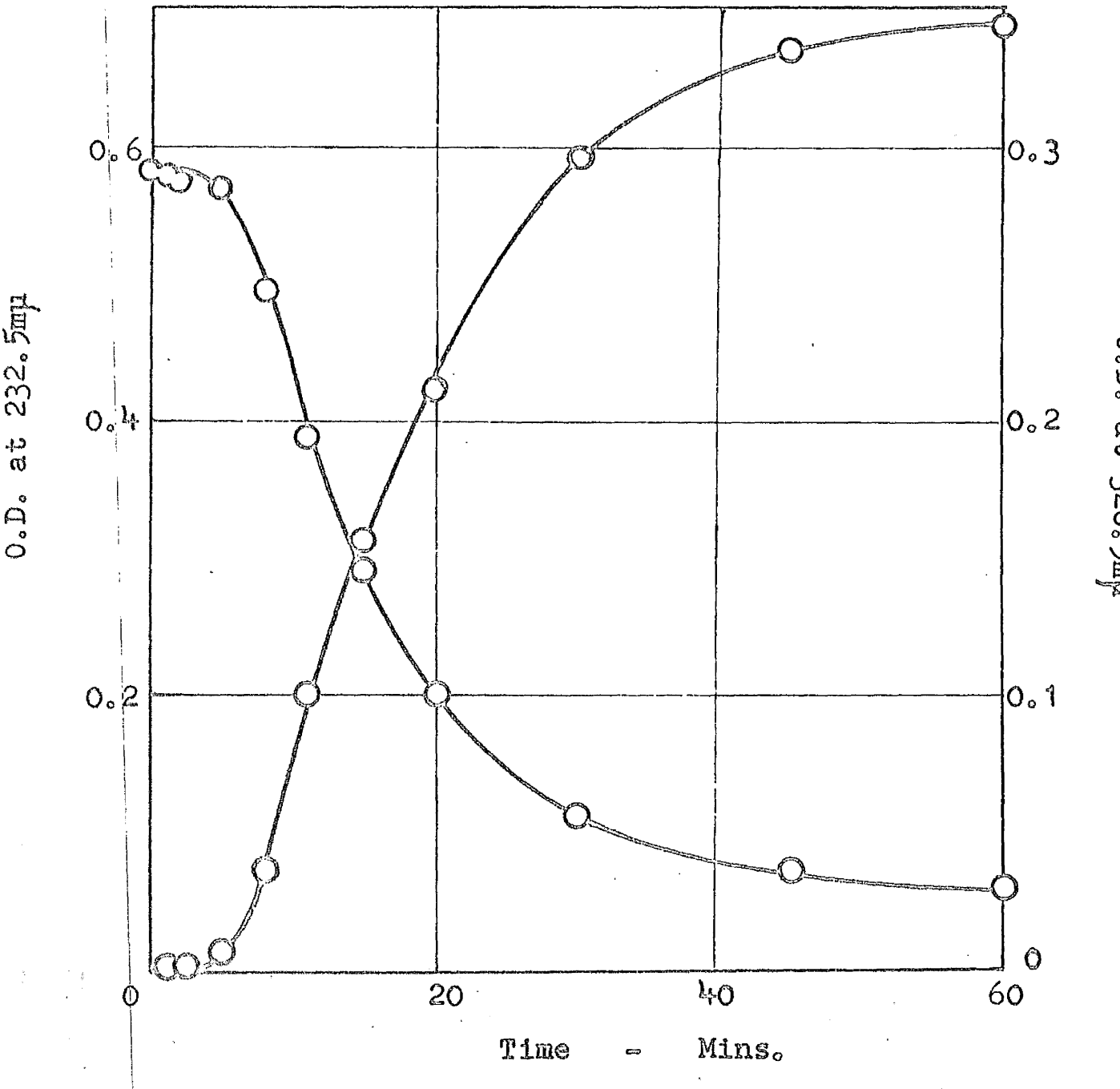
oxidising methyl linoleate by Holman (88) who concluded that these degradation products have little effect on absorption in the conjugated diene range.

Effect of Vitamin-A Acetate Concentration on the Rate of Vitamin-A Degradation.

Using the same system of emulsified linoleic acid in phosphate-citrate buffer, the effect of vitamin-A acetate concentration on the rate of its degradation was studied. The range of concentration examined was 2.8 - 11.2 x 10^{-4}M and the degradation of vitamin-A again was very rapid initially in all cases. This rate only declined as vitamin became exhausted or as linoleate oxidation decreased. A plot of the vitamin degradation after first two minutes against vitamin concentration shows an approximately linear increase in degradation rate with concentration within the range of concentrations studied (Figure 27). Both Holman (106) and Tookey et al. (82) obtained similar results in their studies of coupled linoleate-carotene reactions catalysed by lipoxidase.

The diene and peroxide contents of these reaction mixtures were determined concurrently and these both followed an approximately similar course to a reaction in which no vitamin-A was present. This points to the fact that, in producing vitamin degradation, there is little or no loss of conjugated linoleate hydroperoxide formation and also that the products of vitamin-A degradation are not peroxidic.

Fig.28 Effect of NDGA on Coupled Linoleic Acid-Vitamin A Acetate Reaction Catalysed by Soya Extract.



The effect of NDGA on the Coupled Linoleic Acid-Vitamin-A Acetate Reaction Catalysed by Linoxidase.

Knowing from earlier work that NDGA was capable of completely inhibiting lipoxidase activity at the level being used in the foregoing reactions, the effect of this antioxidant was examined in this system. It was added to a reaction mixture containing linoleic acid (1.3×10^{-3}M) and vitamin-A acetate (2.8×10^{-4}M) at a level of 2.2×10^{-5}M. To this reaction mixture, which was being stirred, was added standard soya extract (1ml.). Samples (1ml.) into 60% ethanol (25mls.) at time intervals gave rise to the curves shown in Figure 28. This clearly shows that NDGA is successful in preventing vitamin-A loss by virtue of its inhibition of the lipoxidase catalysed linoleic acid oxidation. Immediately the NDGA has been used up the primary oxidation reaction occurs along with the coupled secondary reaction.

The Effect of Antioxidants on the Coupled Linoleic Acid-Vitamin-A Acetate Reaction Catalysed by Linoxidase.

It had been previously shown that, of the antioxidants examined, only NDGA was a satisfactory inhibitor of primary lipoxidase action under the present conditions of low antioxidant concentration in a reaction with reasonably high enzymic activity. Nevertheless it was considered possible that some antioxidants might afford protection of the secondary reaction

without inhibiting the primary reaction.

For these studies a system was devised incorporating linoleic acid (1.3×10^{-3}M), vitamin A acetate (5.6×10^{-4}M) and the inhibitors (2.8×10^{-5}M) in phosphate-citrate buffer at pH 6.5. In order to observe such effects with the antioxidants it was considered necessary to lower enzymic activity. Accordingly the reaction system was not stirred and only 0.25 ml. standard soya extract was added. The details of this system are given in Appendix IVa.

The inhibitors used are shown in Table XII. They were used on an equimolar basis to facilitate comparisons and the concentration used represents approximately 2% of the linoleic acid and 5% of the vitamin A acetate concentrations. The reactions were carried out in groups of four, three being inhibited with different antioxidants and the fourth being uninhibited. This allowed comparisons to be made within each group without assuming reproducibility of enzyme activity in the soya extracts. The results, which were obtained in the usual manner by taking samples (1ml.) into 60% ethanol (25mls.) so that linoleate and vitamin oxidation could be followed concurrently, are recorded in a similar manner, i.e. the inhibited reactions with their corresponding control reaction.

The effects of the antioxidants in this system were similar to those obtained before with regard to the primary lipoxidase activity. All the antioxidants were ineffective as inhibitors of linoleic acid oxidation with the exception of NDGA which

Table XII. Effect of Antioxidants in Preventing Vitamin A Acetate Loss in the Coupled Linoleic Acid-Vitamin A Acetate Reaction Catalyzed by Lipoxidase.

Antioxidant	Opt.Dens. at 326.5mμ after					Inhibition.
	Zero	2 min.	8 min.	20 min.	45 min.	
None	0.565	0.545	0.450	0.410	0.325	
^{t}Butyl-phenol	0.575	0.535	0.445	0.415	0.320	None
2.6-di-^{t}butyl-phenol	0.570	0.540	0.475	0.450	0.375	Fair
BHA	0.570	0.560	0.520	0.485	0.420	Good
None	0.575	0.570	0.490	0.402	0.325	
BHT	0.565	0.590	0.534	0.465	0.388	Fair
2.6-di-^{t}butyl-methoxy-p-cresol	0.590	0.600	0.560	0.511	0.452	Good
2.6-di-^{t}butyl-dimethyl-amino-p-cresol	0.578	0.576	0.480	0.428	0.376	Fair

Table XII. (contd.)

Antioxidant	Opt.Dens. at 326.5mμ after					Inhibition.
	Zero	2 min.	6 min.	20 min.	45 min.	
None	0.590	0.556	0.456	0.422	0.320	
4.4'-bis-2.6-di-^{t}butyl-phenol	0.600	0.583	0.512	0.480	0.412	Good
4.4'-methylene-bis-di-^{t}butyl-phenol	0.600	0.570	0.514	0.498	0.450	Good
4.4'-methylene-bis-6-^{t}butyl-cresol	0.605	0.573	0.555	0.547	0.524	Very Good
None	0.580	0.544	0.450	0.400	0.330	
NDGA	0.610	0.596	0.606	0.591	0.570	Excellent
Santoquin	0.590	0.530	0.538	0.518	0.520	Very Good

produced 100% inhibition throughout the course of reaction and santoquin which showed a notable inhibition of the primary reaction. Indeed, in some cases, the antioxidants appeared to have a slight pro-oxidant effect with respect to the control reaction. This was somewhat surprising in view of the results for the screening of these compounds for the primary reaction alone, because, while most of the antioxidants were ineffective, some, e.g. BHA, showed slight inhibition of lipoxidase activity. The results of the effect of the antioxidants on the primary oxidation were not tabulated, it being considered unnecessary since they largely would have repeated earlier reported results.

The effects of all the antioxidants on the secondary degradation of vitamin-A are shown in Table XII, where the initial and subsequent levels of vitamin-A are recorded. The levels recorded are those at zero, 2, 8, 20 and 45 minutes from commencement of reaction. As may be readily seen, some of the antioxidants effectively inhibited to a considerable degree the vitamin-A loss while others were ineffective. The general classification used to describe the inhibition in this table is slight 0-15%, fair 15-30%, good 30-50%, very good 50-80% and excellent >80%.

It is noteworthy that the order of effectiveness of the antioxidants for this aqueous catalysed reaction is similar to that for the autoxidative reaction in the agar gel assay, using linoleic acid as substrate. Thus the bis-phenols and santoquin

are best under both circumstances. The excellence of NDGA in the aqueous catalysed system is due, of course, to its efficiency as an inhibitor of the primary lipoxidase activity.

Further Studies of the Efficiency of NDGA, BHA and Santoquin as Inhibitors of Primary and Secondary Reaction in the Coupled Linoleate-Vitamin-A Reaction Catalysed by Lipoxidase

Having shown that it was possible to inhibit the secondary vitamin-A destruction both with and without inhibiting the primary linoleic acid oxidation in the general assay, it was decided to investigate further this phenomenon on a limited scale. In this connection, studies were carried out with the antioxidants NDGA, BHA and santoquin. These antioxidants were selected for study because, as has been shown in the foregoing general study, they represent different classes of antioxidants. NDGA completely and santoquin partly inhibited both primary and secondary reaction, while BHA inhibited only the secondary reacti These antioxidants also are among the most commonly used in industry.

The assay used for these studies was considerably different from the general assay. Before use, the substrates, linoleic acid and methyl linoleate were heated at 200° C for 30 minutes in sealed evacuated ampoules. This procedure has been used by Lea (119) to destroy any trace amounts of peroxides present in unsaturated fat substrates. Ethanolic solutions of fatty

Fig.29 Effect of Antioxidants on Coupled Linoleic Acid-Vitamin A Acetate Reaction Catalysed by Soya Extract.

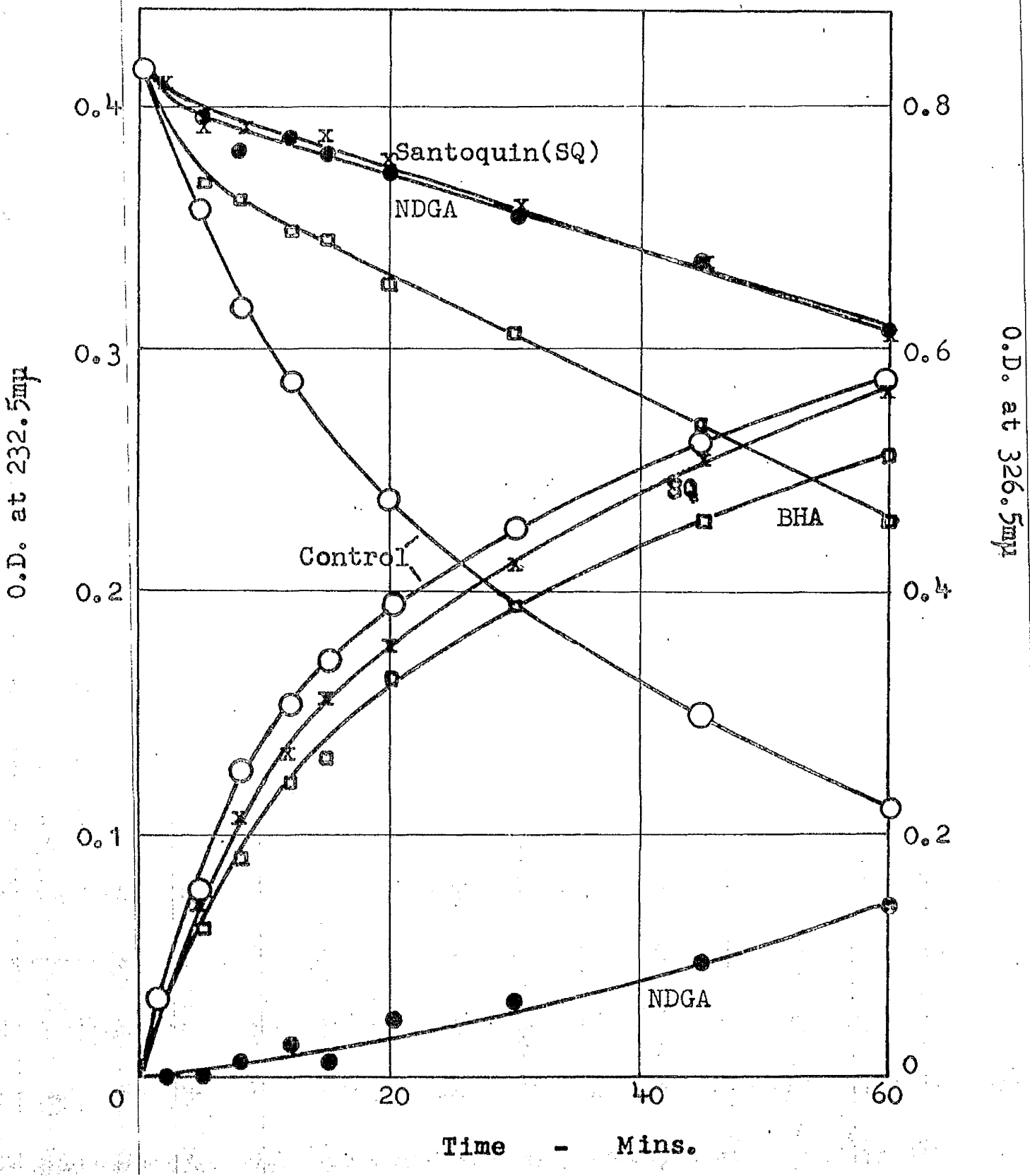
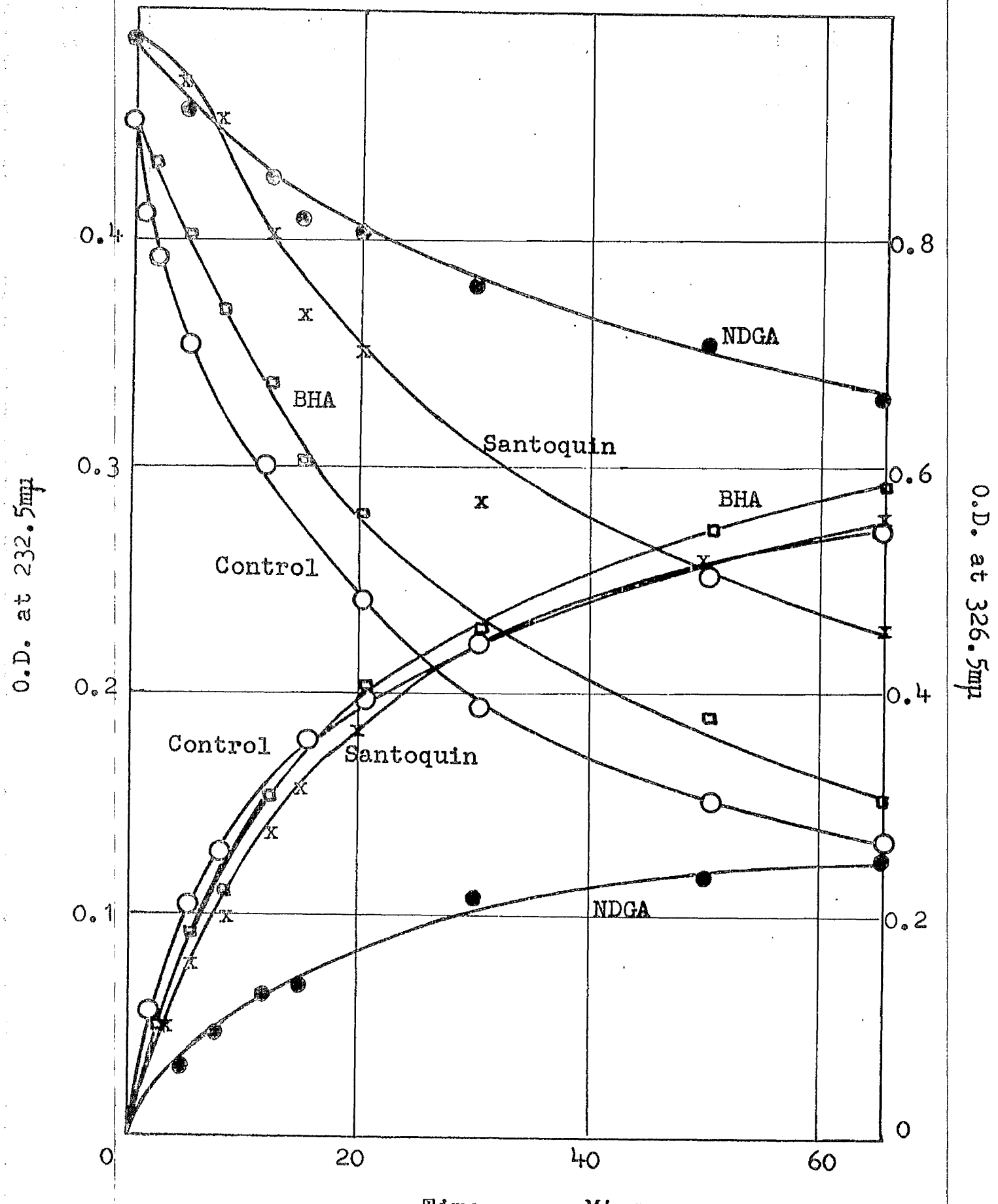


Fig.30 Effect of Antioxidants on Coupled Methyl Linoleate-Vitamin A Acetate Reaction Catalysed by Soya Extract.



substrate, vitamin-A acetate and inhibitors were then added to phosphate-citrate buffer pH 6.5 to give overall concentrations of 6.5×10^{-4}M linoleic acid or 6.2×10^{-4}M methyl linoleate, 2.2×10^{-4}M vitamin-A acetate and 6.1×10^{-6}M inhibitors. This reaction mixture was then homogenised in a top drive homogeniser to ensure a finely dispersed, stable emulsion system. Two portions (25mls.) were pipetted into separate reaction flasks to which standard soya extract (0.1ml.) was added. This permitted duplicate assays to be performed simultaneously. The course of reaction was followed by taking samples (1ml.) into 60% ethanol (10mls.) to allow spectrophotometric measurements to be made. The details of the assay are given in Appendix IVb.

In comparison to the general assay, this assay incorporated the inhibitors at about one fourth of the earlier overall concentration at a level approximately 1% of the fatty substrate and 3% of the vitamin-A acetate. The amount of catalyst used was lower to compensate for the lower inhibitor concentration. As can be seen (Figs. 29 & 30) the results obtained are similar to those of the general assay with some notable differences.

With the primary fat oxidation only NDGA is an effective inhibitor, while santoquin and BHA have little or no effect. This is true with both substrates. However, it is notable that at this level of NDGA it is not 100% effective as an inhibitor throughout the course of the reaction with either substrate.

Fig.31 Effect of NDGA Concentration on Coupled Methyl Linoleate-Vitamin A Acetate Reaction Catalysed by Soya Extract.

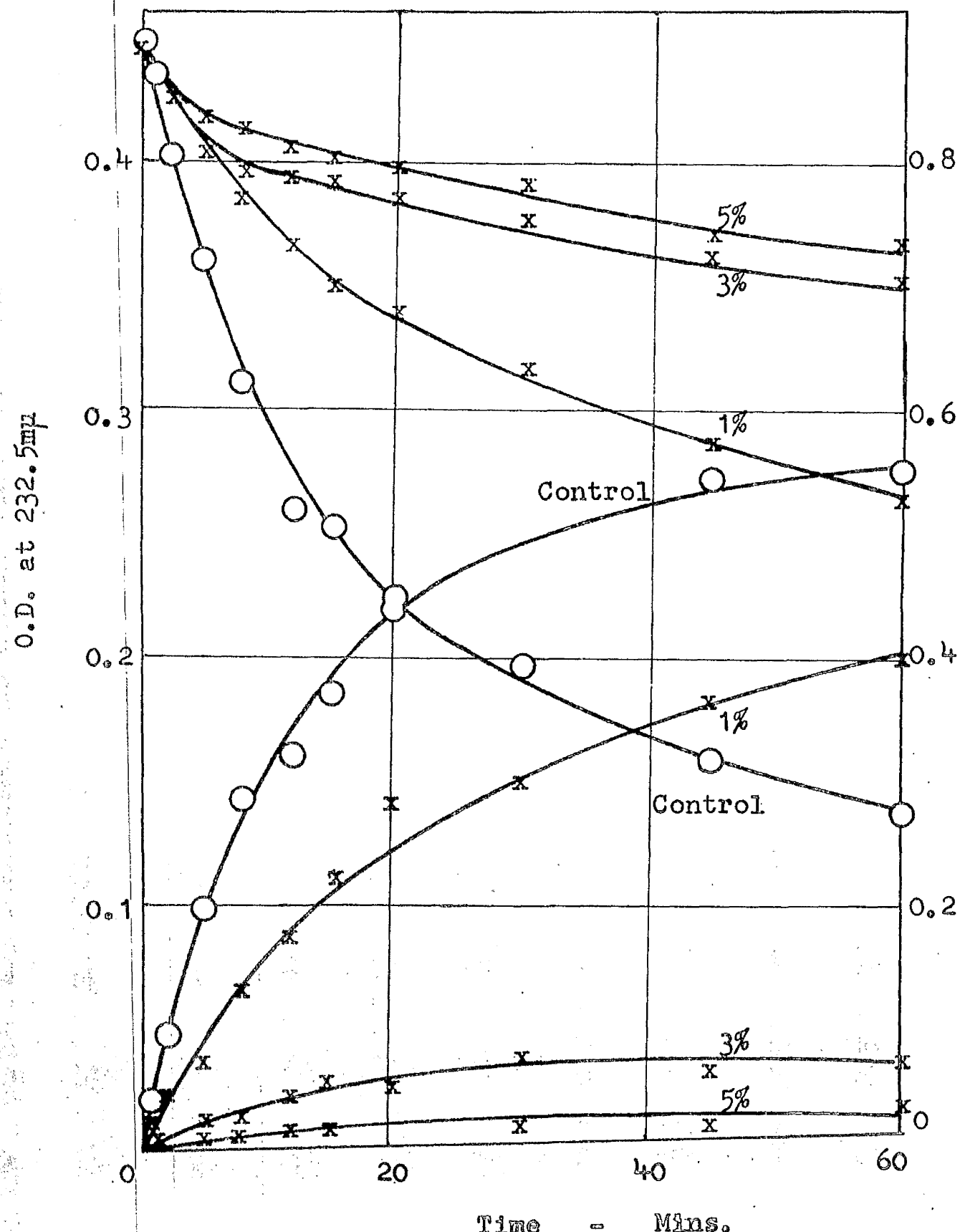


Fig.32 Effect of Santoquin Concentration on Coupled Methyl Linoleate-Vitamin A Acetate Reaction Catalysed by Soya Extract.

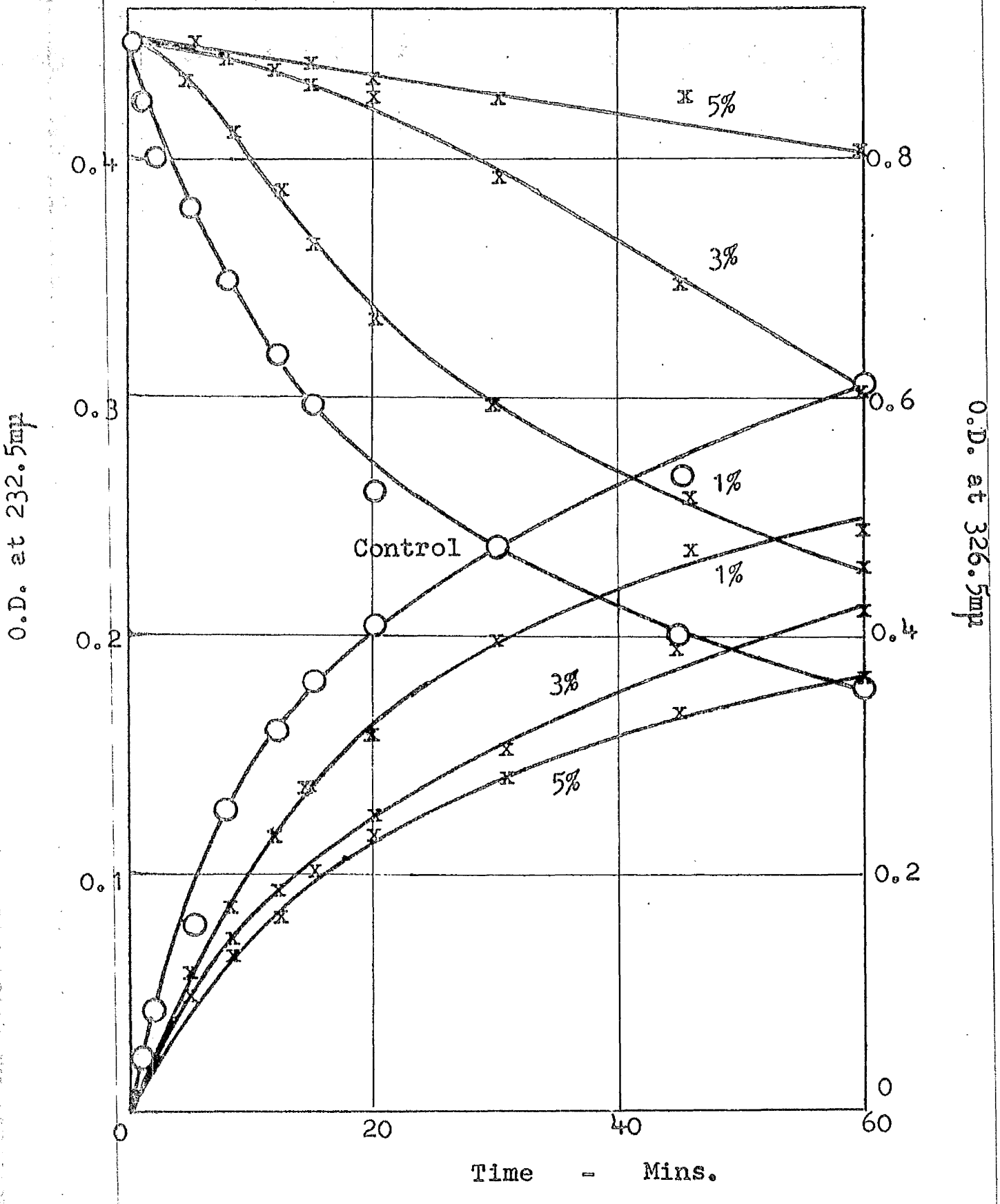
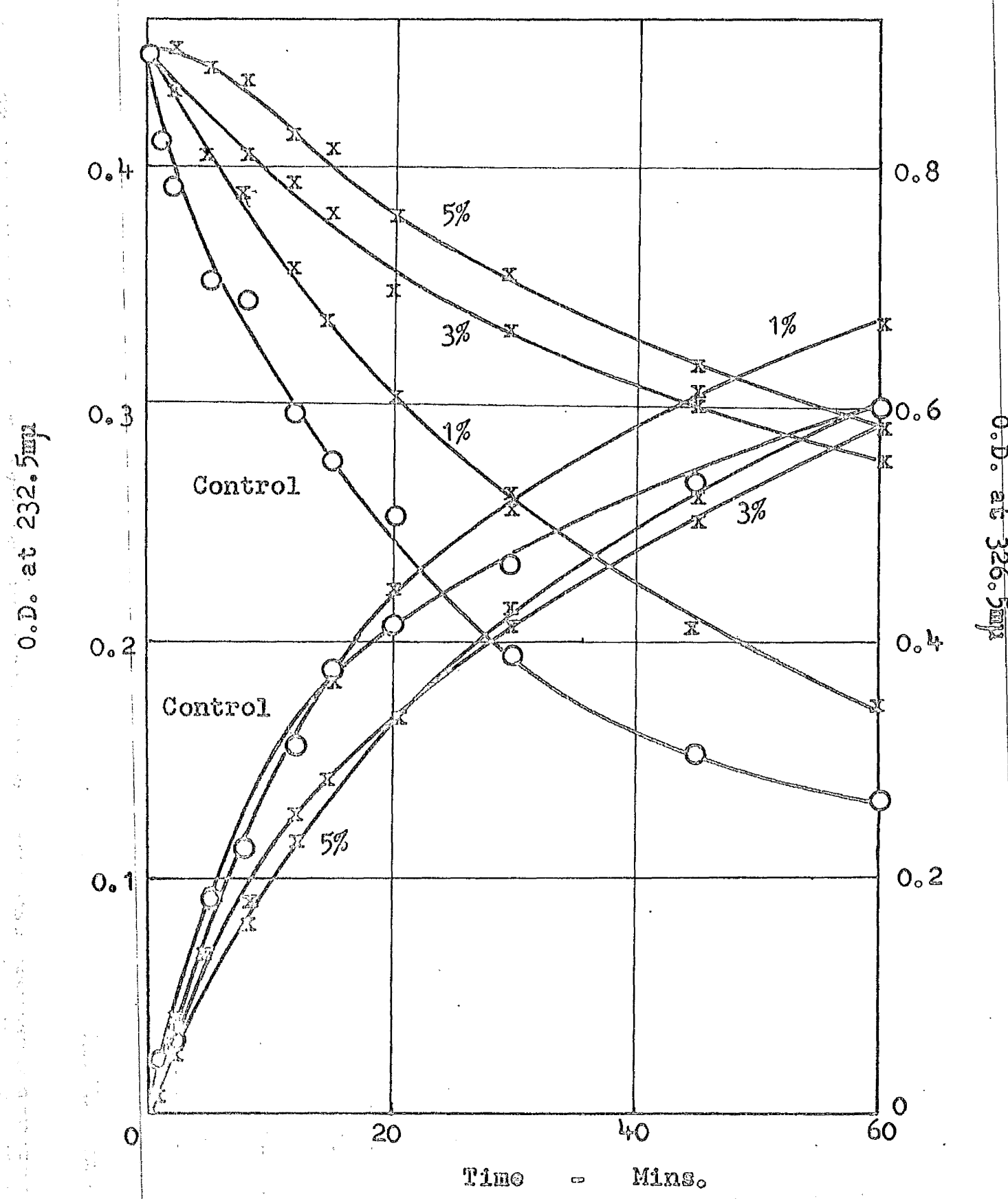


Fig.33 Effect of BHA Concentration on the Coupled Methyl Linoleate-Vitamin A Acetate Reaction Catalysed by Soya Extract.



With linoleic acid it is initially almost entirely effective but the rate of conjugated diene formation increases as reaction time increases. With methyl linoleate, NDGA is at no time totally effective but merely slows the reaction rate throughout the course of reaction. This lower efficiency of NDGA with the ester substrate was noted previously in Section II of this thesis.

All three antioxidants are effective in inhibiting vitamin-A acetate loss to some degree with both fatty substrates. With methyl linoleate the order from best to poorest is NDGA (~50%), santoquin (~20%) and BHA (~15%) while with linoleic acid all three are more efficient with NDGA (~70%) and santoquin (~65%) on a par and BHA (~30%) somewhat less effective.

In Figures 31,32 and 33 are shown the effect of various concentrations of these antioxidants on the coupled methyl linoleate-vitamin-A acetate reaction. The concentrations of inhibitor used are 0.61, 1.8 and 3.1 x 10^{-5}M which represent 1, 3 and 5% of the fatty substrate concentration respectively.

In all cases increasing concentration of antioxidant increases the inhibition of vitamin-A acetate destruction. The effect of inhibitor concentration on the primary reaction varies with the antioxidant involved. Thus BHA at all concentration has little inhibitory effect on the primary reaction and indeed at the 1% level appears to have a slight pro-oxidant effect, while Santoquin shows increasing inhibition parallel to concentration increase as does NDGA with which inhibition is all

but 100% at the higher 3 and 5% levels.

Conclusions and Discussion.

In this section the following major points have been made:-

1. Vitamin-A acetate is rapidly destroyed in the presence of oxidising linoleate catalysed by lipoxidase. The destruction of vitamin has little effect on the course of linoleate oxidation.
2. Increased vitamin-A acetate degradation occurs as the concentration of vitamin is increased.
3. NDGA, by inhibiting primary lipoxidase activity, also inhibits secondary vitamin destruction.
4. Other antioxidants, which are ineffective in preventing primary lipoxidase activity can inhibit secondary vitamin destruction. The magnitude of this inhibition varies from antioxidant to antioxidant but it is notable that the order of antioxidant efficiency is similar to that shown for autoxidation studies using the agar plate assay already described.
5. The efficiency of an antioxidant for protection of vitamin-A varies according to the fatty substrate present.
6. Antioxidant efficiency increases with antioxidant concentration.

The first two points are similar to observation made by Tookey et al. (82) for a lipoxidase catalysed linoleate-carotene

reaction. These results are somewhat surprising in view of the two mechanisms of lipoxidase action postulated by Bergstrom and Holman (42) or by Tappel et al.(67). The former mechanism is the chain mechanism which postulates that secondary oxidations occur by abstraction of hydrogen atoms from the secondary substrate by the intermediary fat free radicals. Thus the secondary substrate acts in a manner similar to antioxidants, i.e. as chain breakers. Thus the oxidation of one molecule of secondary substrate should cause the loss of formation of several molecules of linoleate hydroperoxide.

The mechanism of Tappel et al. (67) is a purely enzymic one but the secondary oxidation is viewed as occurring by donation of a hydrogen atom by the secondary substrate to the fat free radical on the enzyme surface. This causes the loss of one mole of linoleate hydroperoxide for every mole of secondary substrate oxidised.

The present investigations show that, even at advanced levels of vitamin-A oxidation, there is little, if any, loss of linoleate hydroperoxide formation, as compared with a reaction in which no vitamin is present. A possible explanation of this would be that the lipoxidase-linoleate reaction initiates an oxidative chain reaction in vitamin-A. If this were the case the loss of one molecule of linoleate hydroperoxide could cause the loss of considerable numbers of vitamin-A molecules depending on the length of chain.

An alternative explanation, tentatively put forward by Blain (125), is that vitamin destruction may not only be associated with the intermediate free radicals involved in linoleate hydroperoxide formation but also with the subsequent destruction of this hydroperoxide by haematins or other lipoperoxidase activity present in soya extracts.

A further but much less likely explanation would be that vitamin-A acted as a chain carrier for the linolenate. This would require that intermediate linoleate free radicals would abstract a hydrogen atom from vitamin-A, giving rise to a free radical which, in turn, could abstract a hydrogen atom from a linoleate molecule giving rise to a linoleate free radical, which would then carry on the chain mechanism in the linolenate. Thus there would not be a great total loss of hydroperoxide product, while at the same time, the vitamin would be degraded.

Of the first two explanations, the first - that involving a chain in vitamin-A degradation - has a stronger basis. Vitamin-A by itself can be prevented from degrading by the addition of antioxidants. This would suggest that this degradation occurred *via* a chain mechanism. Similarly, it would seem perfectly reasonable to expect that, in coupled oxidations of unsaturated fats and vitamin-A, the vitamin degradation will be *via* a chain mechanism. This should apply equally to reactions which occur in either lipid or aqueous media. It should also be true for both autoxidations and catalysed reactions. Here, where the effect of antioxidants

on the lipoxidase catalysed oxidation of linoleate-vitamin-A was studied, this postulate is supported through the demonstration of inhibition of vitamin-A degradation by antioxidants which have negligible effect on the primary linoleate oxidation both here in the coupled reaction and with linoleate oxidation alone. This inhibition by these antioxidants would be readily explained if they were acting as chain breakers of a vitamin-A radical chain. The fact that the order of effectiveness of these antioxidants is largely similar to that obtained in autoxidative studies with the linoleate-carotene-agar plate assay is suggestive of the fact that the process in both cases is similar. That similar results to those here were not obtained with the lipoxidase catalysed agar plate assay may be due to the fact that the initial measurements were made after one hour and not in the earlier stages as here or that higher enzyme activity or carotene bleaching rate occurred due to the initial elevated temperatures.

The second possible explanation of vitamin-A destruction in the coupled linoleate oxidation catalysed by lipoxidase also has a certain sound basis. Blain (125), in suggesting that vitamin destruction may be associated with a lipoperoxidase breakdown factor causing degradation of linoleate hydroperoxide, points out that Holman (111) using pure lipoxidase found that increasing carotene concentration caused a decrease in linoleate oxidation. He calculated that one molecule of β-carotene

prevented formation of 43 molecules of linoleate hydroperoxide. The work of Tookey et al. (76) and the present work was carried out with crude soya extracts in which lipoperoxidase activity has been demonstrated (52,53). If this hydroperoxide breakdown caused most of the vitamin destruction, the apparent linoleate formation would be little affected by the presence of the vitamin-A.

In the present work it was noted that in the presence of antioxidants the linoleate formation was slightly higher than in their absence. The antioxidants could be functioning as inhibitors of the breakdown factor and hence of the vitamin. However, the fact that the most successful inhibitors of vitamin-A degradation in the lipoxidase catalysed coupled linoleate oxidation were not always the inhibitors which produced the highest linoleate hydroperoxide values weakens this argument. Also the fact that the most successful inhibitors of the secondary oxidation were the same in both the autoxidative and catalysed reactions weakens this argument, since in the autoxidative process there will be no lipoperoxidase factor, apart from trace metals which should be inactivated by the citrate of the buffer.

It is considered that the present findings that antioxidents may inhibit secondary substrate degradations without comparably affecting the primary reaction may be applied more widely in the fat oxidising field. Thus it would seem possible

that similar findings could occur in coupled reactions in a single lipid phase as well as in biphasic systems. Certainly Lea (119,120), working with antioxidants in purified esters of seed oils, has noted that the bleaching of a trace amount of carotene present occurred at varying peroxide values dependent on the antioxidant and substrate being investigated.

It is considered that the present arguments should be restricted to carotene and vitamin-A coupled reactions and should not be indiscriminately applied to other secondary degradations known to occur in conjunction with unsaturated fat oxidation. It may well be that these other degradations fall within the sphere of the present speculations but further particular study would be required to prove or disprove this.

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APPENDICES

APPENDIX I.

SOYA LIPOXIDASE ASSAY.

Reagents and apparatus.

Substrates.

The substrates used were pure linoleic acid and methyl linoleate as supplied by the Hormel Institute, Austin, Minnesota. These substrates were stored in evacuated ampoules at -20° C, each ampoule containing 0.1gm. Prior to use in the assays an ethanolic solution of the substrates was prepared. The usual concentrations used were 10 or 5 mgs./ml.

Reaction buffers.

This was McIlvaine's phosphate/citrate buffer (126). This buffer allowed the use of a wide range of pH, if required, but in the main the pH used was 6.5. This latter buffer was made up by adding 14.2mls. 0.2M disodium hydrogen phosphate to 5.8mls. 0.1M citric acid and diluting this to 100mls. before checking the pH.

Soya extracts.

Soya beans were milled and passed through a 16 mesh sieve. The resultant coarse meal was defatted for 6-8 hours with diethyl ether in a Soxhlet apparatus and then dried off with gentle heating under a small I.R. lamp. This prevented moisture

condensing on the meal during ether evaporation. The defatted meal was more finely milled being passed through a 60 mesh sieve and the resultant flour was used for the preparation of soya extracts.

Soya extracts were prepared by extracting defatted soya flour (0.5gms.) with water (40mls.). The flour and water were stirred slowly for approximately 10 minutes to ensure thorough mixing. This aqueous extract was then spun at 4000rpm for 20 minutes in an MSE Major Centrifuge. The clear supernatant was filtered through a No. 1 Whatman filter paper and the filtrate was used for experiments.

This extract was termed the Standard Soya Extract. It was prepared freshly every day and during the course of the day was stored in a refrigerator to minimise enzyme inactivation.

Inhibitors and Antioxidants.

The unsaturated compounds, investigated in Section II as possible inhibitors of lipoxidase, were made up in ethanolic solution at a concentration of 0.5mgs/ml.

The polyphenolic antioxidants were dissolved in ethanol at a concentration of 3.4×10^{-3}M and when present in reaction mixture gave an overall concentration of 3.2×10^{-5}M.

Method.

The general reaction mix consisted of:-
25mls phos./citrate buffer pH 6.5 + 1ml. ethanolic substrate

solution + standard soya extract.

For inhibitor or antioxidant studies, the potential inhibitors were added in 0.25mls. ethanol.

In the above reaction mix, a substrate concentration of 10mgs/ml. gave an overall concentration of 1.32×10^{-3}M linoleic acid and 1.26×10^{-3}M methyl linolenate. Sometimes other substrate concentrations were used but there was always an addition of 1ml. ethanolic substrate solution. The molar concentrations of substrates are stated throughout the thesis.

Various volumes of standard soya extract were added and these are noted. In general, the volume added was not greater than 1ml. Occasionally, if constant volume was required the standard soya extract was diluted to allow the same volume of enzyme solution to be added.

For reactions with methyl linoleate where the substrate was homogenised, the procedure was somewhat different. The ethanolic substrate solution (2mls.) was added to buffer (50mls.) and this was homogenised in a top drive homogeniser for 1 minute. Two portions (25mls.) of this were pipetted into reaction flasks to which soya extracts were added.

On addition of enzyme solution to all reaction mixtures a stop clock was started and at various time intervals samples (1ml.) were taken into ethanol or 60% ethanol which stopped the reaction. The volume of ethanol used was 25mls. or 10mls. depending on the sensitivity necessary to allow peroxide and

diene determinations to be made.

Peroxide Determinations.

The assay used to make these determinations was the thiocyanate assay according to Koch, Stern and Ferrari (50). To carry out this assay which was performed in absolute ethanol it was found necessary to purify the ethanol as supplied in bulk, otherwise the assay was useless.

The ethanol purifications consisted of refluxing the bulk ethanol with a little sodium hydroxide and aluminium foil for half-an hour. The ethanol was then distilled and this purified ethanol was used for all purposes.

For the determination of the peroxide content of a solution of 25mls. ethanol containing a 1ml. sample of reaction mixture, the following procedure was used.

0.2mls. concentrated hydrochloric acid was added to clear the solution which was slightly milky. 0.025mls. of 5% ferrous ammonium sulphate solution in 3% hydrochloric acid was added and 30 seconds after this addition 1ml. of a 20% ammonium thiocyanate solution was added. After a further 3 minutes the optical density of this solution at 480mμ was read against a blank of ethanol in a Unicam SP 600 spectrophotometer. A blank determination of a reaction mixture to which heat inactivated soya extract had been added was made.

For determinations on solutions of smaller volume it was

found satisfactory to scale down the above assay.

Conjugated Diene Determination.

The optical density of the solution obtained by addition of 1ml. samples of reaction mixture to 25 or 10mls. 60% ethanol was measured at 232.5mμ in an Optica C4 spectrophotometer. The blank solution, against which the sample solutions were read, was identical but for the addition of heat inactivated soya to the reaction mixture from which the blank sample was taken.

APPENDIX II.

PREPARATION OF CRUDE LINOLEATE HYDROPEROXIDE.

Linoleic acid or methyl linoleate (500mgs.) in ethanol (12mls.) was added to McIlvaine's phosphate/citrate buffer pH 6.5 (1 litre) which had been previously oxygenated by bubbling oxygen through it for 15 minutes. Standard soya extract (30mls.) was added and the reaction mixture was swirled for 15 minutes. After this time N/10 hydrochloric acid (160mls.) was added to stop the reaction. The linoleate was extracted with three portions of petroleum ether 30-40°C (200mls.) and this extract was dried over anhydrous sodium sulphate. The desiccant was filtered off and the petroleum ether removed under vacuum. The linoleate remaining was taken up in ethanol (50mls.) and the solvent was evaporated off a sample (4mls.) to determine the quantity of linoleate present.

From this was calculated the total lipid recovered and further ethanol was added to obtain an overall concentration of 5mgs/ml. ethanol. This solution was stored at -20°C and remained fairly stable for several weeks.

The diene content of these solutions was determined and their hydroperoxide content estimated, assuming an extinction coefficient for conjugated linoleate hydroperoxide of 28,000. For linoleic acid a hydroperoxide content of between 35 and 55% was obtained and for methyl linoleate, the hydroperoxide content was between 25 and 35% for different preparations.

APPENDIX III

AGAR PLATE ASSAY.

Reagents and methods.

Reaction buffer.

pH 6.5 McIlvaine's phosphate/citrate buffer diluted to one fifth concentration.

pH 3.8-8.0 McIlvaine's phosphate/citrate buffer at one fifth dilution.

Agar.

Kobe agar (Hopkins and Williams) which was made up at 1.5% concentration i.e. 1.5gms. agar in 100mls. buffer solution.

Ionagar No.2 (Oxoid), which was made up at 0.75% concentrati i.e. 0.75 gms. agar in 100mls. buffer solution.

Substrates.

The substrates used were linoleic acid, methyl linoleate and trilinolenate (Hormel Institute) and pelargonic acid (BDH).

These substrates were dissolved in acetone A.R. at a concentration of 10mgs/ml.

β-Carotene.

β-Carotene (Roche Products) was stored in evacuated ampoules at -20°C, each ampoule containing 10mgs. Prior to use the carotene was dissolved in acetone A.R. (60mls.) with

gentle heating for 20-30 minutes.

Antioxidants.

The antioxidants used were dissolved in acetone A.R. at a concentration of 1.5×10^{-3}M.

Catalysts.

Lipoxidase was as contained in standard soya extract.

Haemoglobin used was bovine (Sigma) at a concentration of 2.8mgs/ml. water for the first assay and for the second assay at a concentration of 3.7mgs/ml. water. These concentrations gave an overall concentration of 2.5×10^{-7}M haemoglobin or 10^{-6}M haematin in both assays. These molar concentrations are based on a molecular weight of 68,000 for haemoglobin which contains four haematin units per molecule.

Method.

(a) Kobe agar was boiled in phosphate/citrate buffer to give a 1.5% solution which was alloved to cool to 65°C. This solution (15mls.) was poured into a calibrated test-tube and the acetone solutions of substrate (0.5mls.), β-carotene (1.2mls.) and antioxidants (0.1ml.) were added with stirring. For reaction mixes without substrate or antioxidants equivalent volumes of acetone were added.

The contents of the test-tube were then poured into transparent plastic boxes of cross-sectional area 23 sq.cms.

and allowed to cool. All operations were carried out with minimal exposure to light.

After one hour during which any major changes in gel opacity could occur, the residual carotene was measured by placing the boxes vertically in an absorptiometer (EEL Spectra) cell compartment and reading optical density at 450mμ against a blank identical but for the exclusion of carotene. The gels were then stored at 20° C in a blackened desiccator which was flushed with oxygen to accelerate bleaching. They were removed at suitable time intervals for further measurements of residual carotene.

For the catalysed reactions, which were carried out with methyl linoleate as substrate, the procedure was identical. The catalyst solutions (0.1ml.) were added along with the other reagents to the warm agar solution prior to pouring into storage boxes. It was found to be unnecessary to store under an oxygen enriched atmosphere, an air atmosphere giving a satisfactory rate of bleach.

All catalysed reactions were performed in the presence of an autoxidising control reaction which gave the initial value of carotene, present in these assays.

(b) For the improved agar plate assay the buffer, substrates, β-carotene, antioxidants and soya extracts were all prepared in the same way as before. The haemoglobin solution was

increased in concentration as mentioned above.

For this assay the agar used was Ionagar No.2. This gave a more rapid rate of bleaching as well as a clearer gel at a lower agar concentration. The concentration was 0.75%.

The procedure was as before except that 20mls. of agar were used. To this the same volume of substrate, antioxidant and catalyst solutions were added but the addition of β-carotene solution was increased to 2mls. to obtain a suitably high initial optical density.

The warm agar mixture was poured into small optically clear Petri dishes of cross-sectional area 31.5 sq.cms. which were stored as before.

APPENDIX IV.

ASSAY OF COUPLED LINOLEATE-VITAMIN A ACETATE REACTION CATALYSED BY LIPOXIDASE.

(a) Reagents and method

Reaction buffer.

McIlvaine's phosphate/citrate buffer pH 6.5 diluted to one fifth strength was used.

Substrates.

Hormel Institute linoleic acid at a concentration of 10mgs/ml. ethanol was used. This gave an overall concentration of 1.3×10^{-3}M in reaction mixture.

Vitamin A. Acetate.

This was a Roche Product and was made up at 5,10,15 and 20 mgs/ml. ethanol giving overall concentrations of 2.8, 5.6, 8.4, and 11.2×10^{-4}M respectively in the reaction mixture.

Antioxidants.

For the general screening reaction the antioxidants were dissolved in ethanol at a concentration of 3×10^{-3}M, which when added to the reaction mixture gave an overall concentration of 2.8×10^{-5}M.

Lipoxidase.

This was obtained from the standard soya extract.

Method.

The reaction mixture consisted of:-
2.5mls. phos./citr. buffer + 1ml. substrate + 0.5mls. vitamin A acetate + 0.25mls. antioxidant + standard soya extract.

The quantity of standard soya extract added, depended on the assay being carried out and the volume is stated throughout the text. For the general assay the volume added was 0.25mls.. For control reactions where no antioxidant was present an equivalent volume of ethanol was added.

On addition of soya extract a stop clock was started and samples (1ml.) of reaction mixture were pipetted into 60% ethanol (2.5mls.) at suitable time intervals. The optical density of these solutions at 232.5 and 326.5mμ was measured against a blank identical but for exclusion of vitamin A acetate and the addition of heat inactivated soya. The spectrophotometer used was the Optica C4.

(b) Reagents and method.

Reaction buffer. as before.

Substrates

Linoleic acid and methyl linoleate (Normel Institute) at 5mgs/ml. ethanol were used. These gave a concentration of 6.5 and 6.2 x 10^{-4}M respectively in the reaction mixture.

Antioxidants.

NDGA, BHA and santoquin were all made up in ethanol at concentrations of 2.8, 8.4 and 14 x 10^{-4}M. In the reaction mixture these gave an overall concentration of 0.61, 1.8 and 3.1 x 10^{-5}M respectively.

Method.

The reaction mixture consisted:-
50mls. buffer + 2mls. substrate + 2mls. antioxidant + 1ml. vitamin A acetate.

This was homogenised using a top drive homogeniser to produce a finer emulsion system. Two portions (25mls.) were pipetted into separate reaction flasks to which standard soya extract (0.1ml.) was added. Samples (1ml.) were pipetted into 60% ethanol (10mls.) at suitable time intervals thereafter and these were used to measure the optical density at 232.5 and 326.5 mμ so that the course of reaction could be followed.