THE UNSATURATED-FAT OXIDASES

with Reference to

Their Coupled Reactions in Cereal Flours

by

John Hawthorn, B.Sc., A.R.T.C., A.R.I.C.

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ProQuest LLC.
789 East Eisenhower Parkway
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"The bread I eat in London is a deleterious paste mixed up with chalk, alum, and bone-ashes, insipid to the taste, and destructive to the constitution. The good people are not ignorant of this adulteration; but they prefer it to wholesome bread, because it is whiter than the meal of corn. Thus they sacrifice their taste and their health, and the lives of the tender infants, to a most absurd gratification of a misjudging eye."

Dr. Tobias Smollett, 1771.

ACKNOWLEDGEMENTS

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Some of the pigment analyses reported in Section III were carried out by Mr. E.C. Styles, B.Sc., who also developed the analytical method used.

In a conversation at the commencement of this work Dr. A. Banks, B.Sc., F.R.I.C., of the Torry Research Station, Aberdeen, directed the writer's attention to haem catalysis of unsaturated-fat oxidation. This early emphasis modified his approach to the problem and led to a valuable saving of time.

His greatest debt of gratitude is to Professor James P. Todd, Ph.C., Ph.D., F.R.I.C. without whose constant encouragement and practical assistance this work could not have been brought to conclusion.

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PREFACE

The purpose of this investigation was a study of the destruction of carotenoid pigments by coupled reactions taking place when the nutritionally essential fatty acids of their esters undergo oxidation catalysed by the unsaturated-fat oxidases. The end in view was the application of the results to problems associated with hazards to health arising from the use of Agene and similar powerful oxidising agents in wheat flour.

In 1946, Sir Edward Mellanby reported in the British Medical Journal that dogs fed on diets of flour or bread which had been treated with the widely used Agene (nitrogen trichloride) as a bleaching and "improving" agent developed hysterical running fits, and eventually died. This work has since been amply confirmed, and cases of Agene poisoning in man have been reported.

Unfortunately, bread of generally acceptable quality cannot be economically baked without the use

of bleaching agents to remove the carotenoid pigmentation of raw flour, and "improvers" to impart these mechanical properties to flour proteins which are necessary if loaves of good volume and satisfactory texture are to be obtained. Without the use of such agents loaves baked from the flours available in this country have a yellow tint, a dense heavy texture and an unattractive appearance.

Of a number of possible alternatives to Agene, the least objectionable so far proposed is incorporated in a British Patent of 1951 in the names of J. Rank and J. G. Hay. In this process, which involves high speed mixing of flour and water batters to which a small addition of soya-flour is normally made, no chemical oxidising agents are added, and the syndrome associated with Agene poisoning in man disappears when affected persons are fed on the resulting bread. Moreover, the bread produced is of acceptable quality, being in most respects equal to or better than that made from Agene-treated flour.

The Rank and Hay process is now used in some parts of England and on an extensive scale in Scotland. Its main disadvantage is the costly machinery involved and the degree of technical skill required in its operation. The process was developed on an ad hoc basis, but in private communications, Hay has suggested that the changes observed in flour characteristics are due, at least in part, to the action of wheat lipoxidase, and that the addition of the small quantity of soya has the effect of supplementing the inherent lipoxidase of the wheat.

A study of lipoxidase with a view to throwing further light on this important process was therefore put in hand. The complex nature of the problem was soon apparent, and to assist in achieving a rapid solution a group of six workers has been concentrating on these studies. Arising from their efforts, a broad understanding of the reactions involved in the Rank and Hay process has been achieved, and a simpler and more

direct process has been proposed. Assurances have been given by the appropriate Government Departments that legislation will be introduced to exclude the use of Agene in flour as soon as a satisfactory alternative process has been established.

The experimental approach to the problem was visualised as following the lines indicated below.

1. A method for studying the coupled bleaching of carotenoid pigments by unsaturated-fat oxidases would be sought so that the behaviour of these enzymes under a variety of conditions could be described in terms of carotene bleaching. This part of the work is dealt with in Section I of this thesis for soya-bean lipoxidase, and in Section II for catalase, catalase being selected as an example of a haem compound behaving as an unsaturated-fat oxidase.

2. A survey of the anatomical distribution of the

unsaturated-fat oxidases in the wheat berry would be made, and the range of activity encountered in wheat flour would be determined. This work has been carried out by Blain and will be referred to from time to time in this thesis.

3. A method for applying the information so gained to the Rank and Hay process under controlled conditions would be required. This work forms a major part of Section III of this thesis.

As the first member of the research group referred to above, it fell to the writer to open up the field of investigation rather than to make a detailed study of a particular part of that field. In the following sections a rather wide variety of experimental work is described, some of which is now being studied in more detail by other members of the group.

SECTION I

THE BLEACHING OF CAROTENE

BY

SOYA-BEAN LIPOXIDASE

SECTION I

THE BLEACHING OF CAROTENE BY SOYA-BEAN LIPOXIDASE

The Discovery and Early Literature of Lipoxidase

Two recent reviews by Bergstrom and Holman (1,2) have surveyed the extensive literature on lipoxidase and related enzyme systems. No attempt at a comprehensive account of the previous work on lipoxidase will therefore be made, but rather it is desired to draw attention to these aspects of the subject of special interest in the present work.

The earliest observations of reactions attributable to lipoxidase seems to be that of Haas and Bohn (3) who reported in 1928 that when small quantities of raw soya-meal were added to bread doughs, the bread produced was whiter than that prepared without such additions. They later took out patents (4) covering a breadmaking process making use of this bleaching property of unprocessed soya-meal. In 1931, Hauge reported that losses of vitamin A took place during the weathering

of lucerne (5), and in 1935 he published a further account of these losses (6) in which he demonstrated that an enzyme system, or systems, were involved.

Meantime, in 1932, Andre´ and Hou (7) had demonstrated the presence of a fat oxidising system in soya-beans to which they gave the name of "lipoxydase", while in 1935, Wilbur and his co-workers (8) reported that the addition of raw soya-beans to the ration of dairy cows reduced the vitamin A content of the milk produced. In 1936, Frey, Schultz and Light (9) showed that losses of vitamin A potency in animal test diets were associated with carotene destruction brought about by soya-benas.

By 1939 it was known (a) that soya-beans, white beans, radish and potatoes contained systems capable of destroying carotene and (b) that soya-beans contained a system capable of oxidising fats. The first system was known as "carotene oxidase" and the second had been called "lipoxydase".

In 1939 and 1940 three important papers were published. In the first of these Sumner and Dounce (10) showed that "carotene oxidase" was capable of catalysing the peroxidation of fats. In the second, Sumner and Sumner (11) showed that the destruction of carotene by "carotene oxidase" was very much more rapid if fat was simultaneously present in the system. The third paper by Tauber (12) was published independently and almost simultaneously with the second, and it demonstrated conclusively that the soya "carotene oxidase" of previous workers was, in fact, an unsaturated-fat oxidase, the carotene being destroyed by the oxidation products.

This clarification awakened wide interest and was followed by a large number of publications in the next few years. The term "carotene oxidase" was replaced by "lipoxidase" from the original "lipoxydase" of André and Hou, and lipoxidase activity was reported in a wide range of plants, notably in the Leguminosae,

the Solanaceae and the Labiatae. Soya-beans remain today the most potent known source of lipoxidase.

The Isolation and Properties of Soya-Bean Lipoxidase

The isolation of lipoxidase from soya-beans was necessarily dependent on the development of a method of assay. In 1943, Balls, Axelrod and Kies developed an assay (21) based on the rate of bleaching of carotene in a water/ethyl linoleate suspension, and achieved a 115-fold concentration of the enzyme on a protein nitrogen basis. In 1945, Cosby and Sumner, also using an assay system based on carotene bleaching (22) achieved a 60-fold concentration of the enzyme. In 1946, Theorell, Bergstrom and Akeson (23) developed an improved system of assay based on the fact that, in the course of enzymic oxidation, linoleic acid develops an ultraviolet absorption band in the region of 234 mμ due to the formation of conjugated double bonds. Using this improved assay, Theorell, Holman and Akeson (24,25) obtained a crystalline lipoxidase

preparation which was found to be homogeneous by several criteria.

Their method consisted essentially of fractional precipitation of acetate buffer extracts of ground defatted soya-beans, followed by electrophoresis on a preparative Tiselius apparatus. Crystallisation was achieved by dialysis of fractions from the Tiselius apparatus. The crystalline preparation represented a 150-fold concentration of a water extract on a dry matter basis.

From studies of electrophoretic mobility, Theorell Holman and Akeson (24) calculated the isoelectric point of lipoxidase to be approximately pH 5.4, and from consideration of sedimentation and diffusion constants, the molecular weight was calculated to be 102,400. While analyses for heavy metals have not been carried out on the highly purified crystalline material, a specimen known to be 94% pure was found to contain only iron as a possible metallic component (24). The concentration of iron found required a molecular

weight of 370,000 to permit the presence of one atom of iron per molecule, The absorption spectrum of the crystalline material is that of a common protein (24) with a relatively high peak at 280 mμ, and is free from the bands which characterize other iron containing enzymes. Inhibition studies with metal complexing agents (26, 27, 28) such as cyanide and diethyl-dithiocarbamate also make it unlikely that heavy metals are active centres in the enzyme. The active centre of lipoxidase is thus unknown, although Holman, Panzer, Schweigert, and Ames, (29) who determined the amino-acid composition of the enzyme detected an unknown constituent in their chromatograms.

Substrate Specificity and Mode of Action of Lipoxidase

A large number of fatty acids have been examined as possible substrates for lipoxidase, (30, 31, 32, 21, 33, 34) and it now seems clear that only the naturally occurring isomers of linoleic, linolenic and arachidonic acids in which all double bonds are cis, are attacked

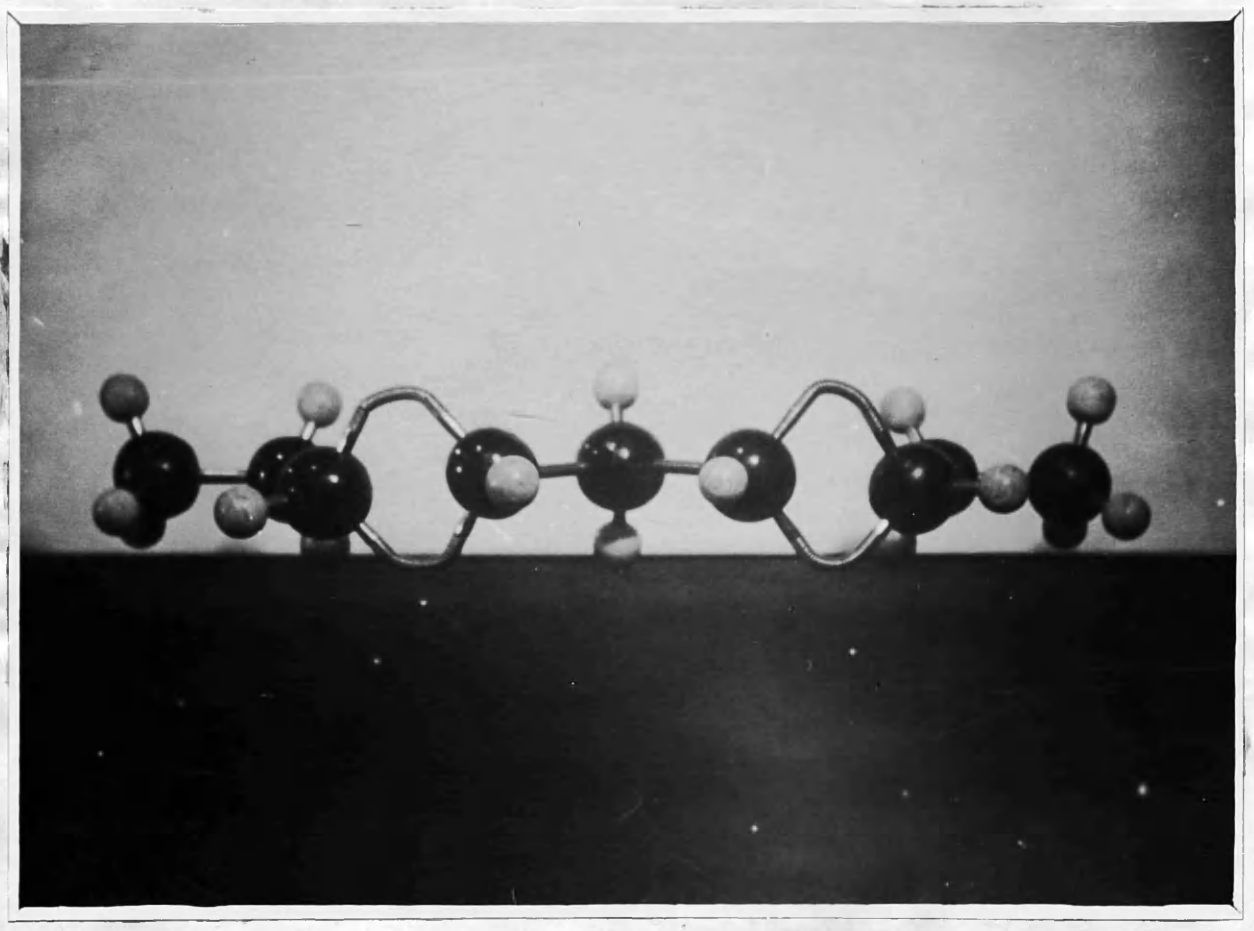


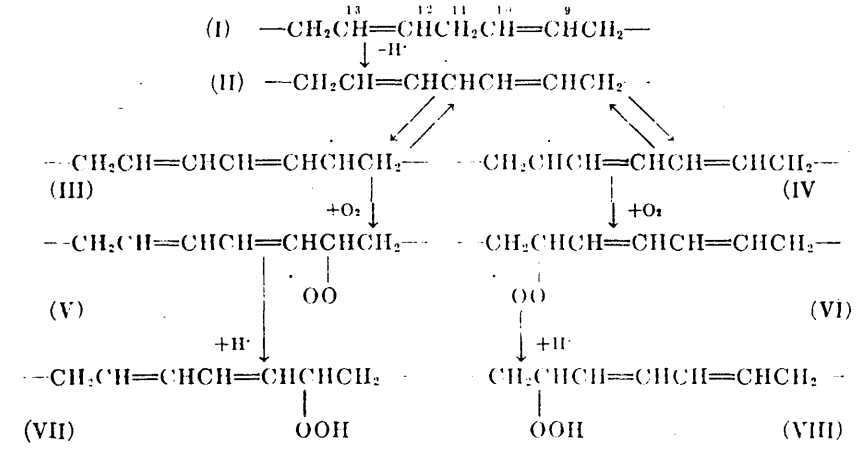
Fig. I

by the enzyme. Elaidinized forms are not attacked, nor, for example, is the conjugated isomer of linoleic acid. It was thought at one time that the presence of a double bond in the 9, 10 position in the fatty substrate was necessary for the reaction to proceed, but it is now believed that the position of the first double bond in relation to the carboxyl group is not critical, since this bond in arachidonic acid is probably in the 8, 9 position. The system attacked is the methylene interrupted, multiply unsaturated structure in which the double bonds are cis. Fig I illustrates a model showing the simplest form of this structure.

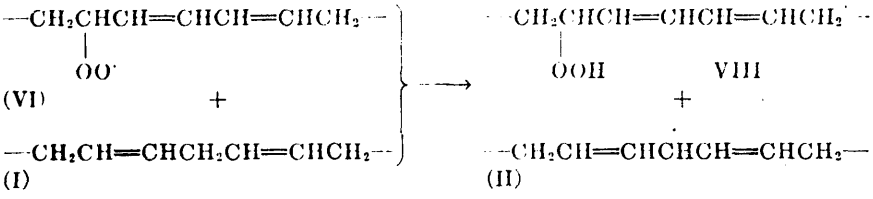
It is worth emphasising that the three fatty acids attacked are these which are regarded as being nutritionally essential.

The reaction mechanism proposed by Farmer(35) in 1943 for the autoxidation of unsaturated compounds has been suggested by Bergstrom & Holman (2) as a possible mechanism for the enzyme reaction (Fig. 2a).

The free radical mechanism proposed for the autoxidation of unsaturated compounds by Farmer *et al.*[70] has been suggested as a possible explanation for the enzymatic reaction.[11]



The oxidation of linoleic acid, once started by the enzyme, could continue by means of a chain reaction. For example, the radicals V or VI could accept an H atom from linoleic acid:



This would give rise to another radical, II, which would go through the same cycle.

Fig. 2a

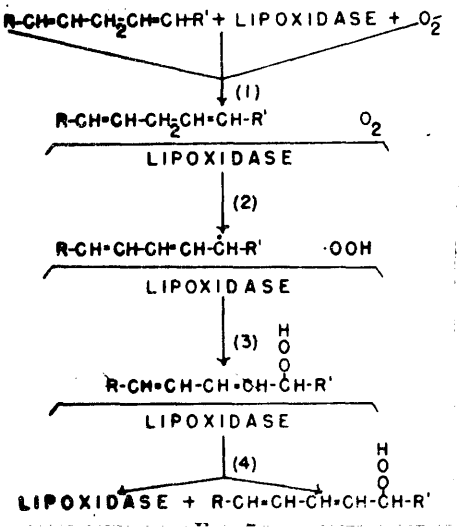


Fig. 2b

Such an explanation would involve the catalysis of a chain reaction by an enzyme, and the possibility of *in vivo* chain reactions of this type is still a matter for debate.

In a recent kinetic study of the lipoxidase reaction by Tappell, Boyer and Lundberg (36) a more convincing explanation of the reaction mechanism, which does not involve the postulation of a chain reaction, is put forward. In the same paper a study of antioxidant inhibition of the reaction is described and explained on the basis of their proposed mechanism. Nevertheless, it is the opinion of the present writer, in view of evidence to be brought forward later in this thesis, that the heterogeneous nature of lipoxidase catalysis demands that caution should be exerted before coming to conclusions on reaction mechanism based on formal kinetic data. With this reservation in mind the mechanism proposed by Tappel *et al* is shown in Fig. 2b.

The Assay of Lipoxidase.

The assay of lipoxidase has always presented certain practical difficulties which arise from the fact that the enzyme reaction is heterogeneous, the water-soluble enzyme reacting with an essentially water-insoluble substrate. Clearly, observed reaction velocities may be expected to reflect the interfacial area of the system employed.

The methods of assay which have been used depend on one or another of the following:

1. The measurement of peroxide values by iodimetry or by the ferric thiocyanate colour reaction (13, 31, 37, 38).

2. The measurement of oxygen uptake of a suitable system in a Warburg apparatus (12, 14, 27, 28, 36, 40, 41, 47).

3. The measurement of the ultraviolet absorption band

at about 234 mμ which develops with the diene conjugation produced in the fatty acid substrate as oxidation proceeds (23, 33, 36, 48, 50).

4. The measurement of the bleaching of a carotenoid added to the reaction system (11, 21, 22, 43, 45, 46, 47, 49).

The direct measurement of peroxide values as a measure of lipoxidase activity had largely fallen out of use by 1945. In the Renner method (38) it had been found that enzyme activity fell off rapidly during the course of the reaction, while the thiocyanate method proposed by Sumner (37) in 1943 did not attract the attention of other investigators. Indeed, in 1945 Sumner himself, in proposing yet a further new method based on carotene destruction, opened his paper (22) with a brief survey of existing methods and concluded that "there appears to be no satisfactory method for determining lipoxidase activity". Of the methods subsequently proposed,

the most reliable and the one which has been most widely used in one modification or another is that proposed by Theorell, Bergstrom and Akeson (23) in 1946. The disadvantages of the previous methods were brought about by variations in the degree of dispersion of the fatty substrate or carotenoid pigment. Moreover, impure substrates of crude soaps were often used which doubtless introduced competitive inhibition. Theorell et al used pure sodium linoleate as the substrate in their system, and obtained more reproducible and homogeneous conditions by working at pH 9. In addition, they measured the products of the primary reaction by means of their ultraviolet absorption at 234 mu. With this method, they found that diene conjugation in the substrate was proportional to time and enzyme concentration over a wide range of conditions.

Tappel (50) and his colleagues have recently introduced an elegant modification of this method.

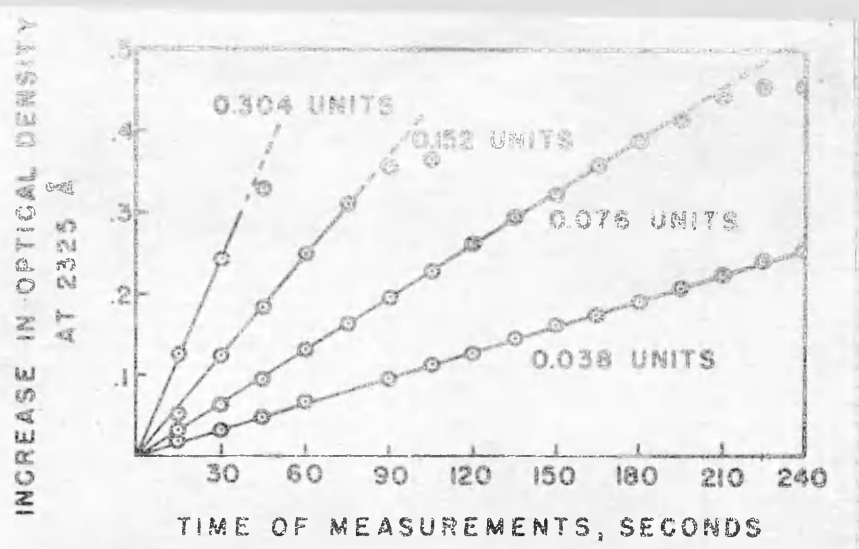


FIG. 1. Effect of lipoxidase concentration upon the reaction rate of linoleate oxidation as measured by the direct spectrophotometric method.

Units of lipoxidase concentration according to method of Bergström and Holman (7). Substrate was 0.0071 *M* sodium linoleate buffered at pH 9.0; temperature, 30°C.

Fig. 3a

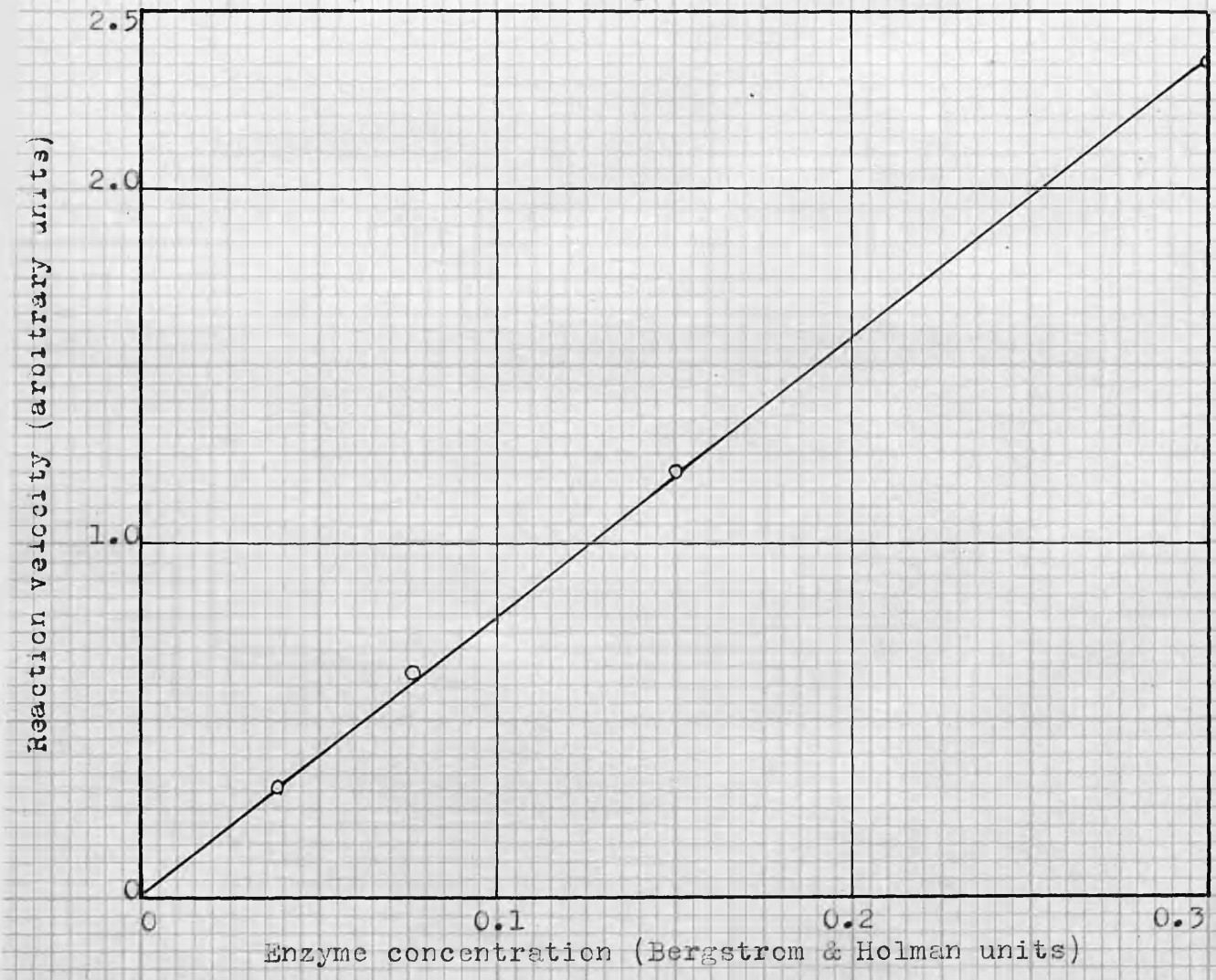


Fig. 3b

They use a controlled temperature cell compartment in their spectrophotometer and arrange to pass dry oxygen through the compartment. They are thus able to work at reaction temperatures of from 0° to 30° with a control of 0.1°. The reaction is carried out in a silica cell, they dry oxygen preventing fogging of the cell at low temperatures. To the cell are added 0.8 ml of 7×10^{-3} M linoleate in oxygen-saturated 0.1N ammonium hydroxide/ammonium chloride buffer at pH 9.0, and 0.2 ml. of a properly diluted enzyme solution. The cell contents are rapidly mixed and measurements of the optical density at 232.5 mμ are made every 15 seconds by simultaneously balancing the photoelectric circuit and observing a stop-watch.

Fig. 3a is taken from Tappel's paper and shows the optical density/time relationship observed by this method. Fig. 3b which has been obtained by calculation from Tappel's data, shows the linear relationship

between the slope of his initial zero order reaction curves and the concentration of enzyme used.

As a result of the development of such spectrophotometric assay methods in recent years, methods based on carotenoid bleaching have tended to fall into disuse. For many purposes it is clearly advantageous to work with the simplest possible system, and the introduction of carotene may be a complication which becomes a particularly burdensome when antioxidant studies introduce yet a further reactant.

Notwithstanding this, since in the present study the focus of attention was the possible role of lipoxidase in bleaching the carotenoids in wheat flour, it was necessary to re-examine early work on the bleaching of carotenoids and to devise a suitable reaction system for the study of these bleaching reactions under as wide a range of conditions as possible.

Although the use of other methods of lipoxidase assay will be referred to from time to time, most of the remainder of this work will be devoted to the consideration of reaction systems for the study of carotene bleaching.

The Bleaching of Carotenoids and other Pigments

As already mentioned, the essentially coupled nature of the lipoxidase bleaching reactions was established in 1940, and it is relevant at this point to sum up what was known about the general conditions of this reaction in 1950 when the work described here was undertaken.

1. Carotenoids are not bleached by lipoxidase the absence of unsaturated fats.

2. For coupled bleaching to take place one or a mixture of the following fatty acids or their esters must be present:- linoleic, linolenic or

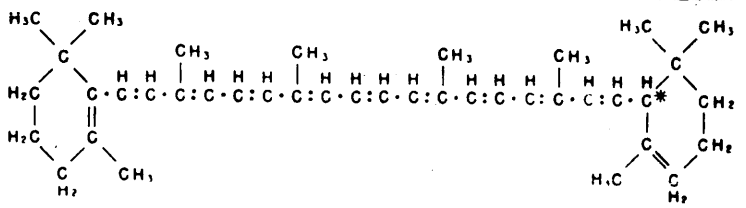
arachidonic acid.

3. For coupled bleaching to take place the carotenoid must be present while the catalysed oxidations of the fat is actually proceeding. A carotenoid added to the resulting mixture of fatty peroxides after completion of the catalysed reaction is not bleached.

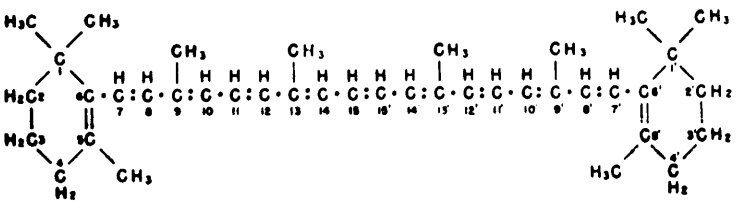
4. The system must have free access to atmospheric oxygen for the reaction to proceed.

5. Nothing is known of the nature of the oxidation products of the carotenoid except that all vitamin A activity is lost.

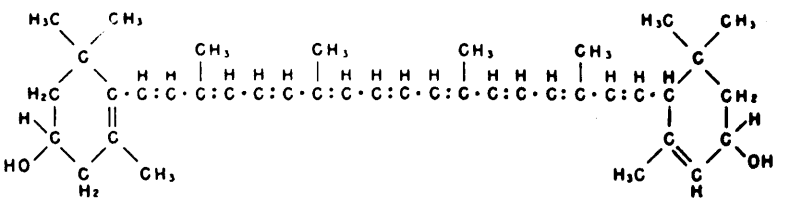
The bleaching reaction had been studied up to this date with the main object of its use as an assay method. The following substances were known to be bleached:- α and β - carotene, bixin (45), egg yolk xanthophyll (12), zeaxanthin (30), eschscholtziaxanthin (30), chlorophylls a and b (30) , and haemin (51). It was



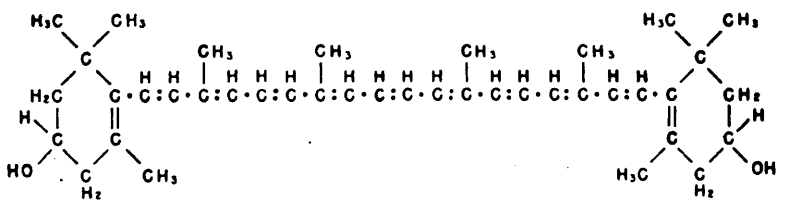
α-Carotene



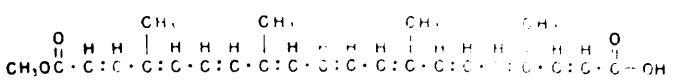
β-Carotene (Karrer numbering)



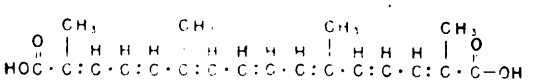
Lutein



Zeaxanthin



Labile or stable bixin



Crocetin

Fig. 4

also known that wheat flour pigments were bleached and that these consisted mainly of taraxanthin and xanthophyll, β-carotene being virtually absent (52). While work was in progress, Fukuba (47) showed that crocetin, the carotenoid of the saffron crocus, was bleached by the same reaction. Fig. 4 illustrates the chemical structures of some of these pigments.

In addition to these decolourisation reactions, Strain (30) noted that p-phenylenediamine was oxidised to a blue pigment, while dihydroxyphenylalanine was oxidised to a black melanin pigment in presence of lipoxidase and unsaturated fat. Ascorbic acid was slowly oxidised under similar conditions. It has also been pointed out (21) that certain leuco dyes are readily oxidised in a coupled fashion by lipoxidase and it has been suggested that the use of o-chlorophenol indophenol might form the basis of a colorimetric method of lipoxidase assay. In the presence of enzyme and absence of unsaturated fat none of these reactions take place.

Of the papers previously referenced on the assay of lipoxidase by carotenoid bleaching, four merit particular attention. In the earliest of these (21), Balls, Axelrod and Kies used a solution of ethyl linoleate (100γ) and carotene (43γ) in 1 ml. of 90%/ 10% acetone /ethanol. 5 ml. of this solution were run into 52.5 ml. phosphate buffer at pH 6.5, and an aliquot taken and added to a suitable volume of enzyme solution. Readings with time were observed on an Evelyn colorimeter. Such a system would be turbid and colour readings could not be made with a reasonable degree of accuracy.

In 1945, Cosby and Sumner (44) improved on this method by using gum arabic as a stabilising agent, thus allowing colour readings to be made on less turbid solutions. As substrate they used neutralised soya-bean fatty acids, and their carotene solution was prepared and stored separately from their substrate. Their procedure was as follows: 5 ml. stock carotene solution (7.5 mg.% in acetone and ethanol) are added

to a 250 ml. conical flask, and followed by 1 ml. of a 0.3% solution of neutralized fatty acids of soya-bean oil. 100 ml. of 0.1.M phosphate buffer containing gum arabic are then added and mixed. An initial colour value for the solution is obtained on a photoelectric colorimeter. To the suspension is added 1 ml. of enzyme solution and the mixture returned to the colorimeter cell. The time required for the colour reading to drop to half its original value is noted. The unit of lipoxidase is defined as contained in that volume of enzyme solution which gives half bleach in 300 seconds.

In 1947, Sumner and Smith (45) pointed out that carotene solutions are unstable and that carotene soon separates from aqueous digests. They proposed the use of bixin (the carotenoid of annatto) as being a relatively stable substance of greater colouring power than an equal weight of carotene, and less liable to separate from aqueous solutions. Their procedure with bixin solutions was very similar to the procedure used with carotene, a gum arabic buffer and soya-bean

fatty acids still being employed. They note that the reproducibility of the method depends on obtaining a clear homogeneous solution and mention precautions as to the order of mixing reagents to obtain a solution of minimum turbidity. In a second paper (46)published the following year they replaced their crude soya bean substrate with methyl linoleate and gave spectrophotometric data showing the relationship between the decrease of absorption due to bixin and the increase due to conjugation of diene in the linoleate substrate. They found a linear relationship between the ratio diene conjugation/bixin decolorization and reaction time. In this paper the use of gum arabic as a stabilizer was discarded. Instead the reaction was stopped with methanol and the protein precipitated by means of lead acetate. The spectrophotometer readings were taken on the centrifuged product. Warning is given that unless the described procedure is strictly adhered to bixin may be absorbed by the precipitated protein and removed from the solution.

While these methods due to Sumner and his colleagues

represented a distinct advance in carotenoid bleaching systems they were open to criticism on a number of grounds. The experimental section which follows draws attention to technical difficulties in applying these methods, but perhaps the most serious objection is that the systems were ill-characterised, could only be used at a fixed pH and that adequate data relating enzyme concentration, substrate concentration, reaction time, reaction temperature and substrate dispersion were lacking. This section describes work leading up to the development of a better characterised reaction system.

Experimental:-

1. A Provisional Carotene Assay System

A study of the bleaching of carotene by lipoxidase could not be effect by any of the spectrophotometric methods based on conjugation of diene, however satisfactory these might be for assay purposes. It was evident at the outset that attention must be given again to methods based on the direct observation of carotene

bleaching. Since the pigments in the technical systems of ultimate interest were more closely related to carotene than bixin, the method of Cosby and Sumner (22) was preferred as a starting-point for the investigation.

Cosby & Sumner's reagents and conditions as described on pages 22-23 were duplicated except that cotton seed fatty acids at the same concentration were used instead of soya-bean acids, and a number of assays were carried out on soya-bean extracts. The extracts were prepared by suspending 5 to 20 g. (depending on the activity) of ground defatted soya meal in 100 mls. of 0.1N. acetate buffer pH 4.5 shaking, allowing a stand two hours, shaking again and centrifuging. Colour readings were taken using a Spekker absorptiometer with Ilford spectrum blue filters. (No. 602) It would be profitless to detail these assays since it soon became clear that the system as described was inadequate for the purpose. The following are the main points of criticism.

1. In spite of the presence of gum arabic the solutions

were always slightly turbid.

2. The turbidity was not constant but increased during the reaction time.

3. Reagent blanks also increased in turbidity after preparation. The order of mixing the reagents appeared to affect the rate of development of turbidity.

4. The substrate of mixed fatty acids was not reproducible, three different batches of substrate giving different assay figures for the same soya extract.

5. The sensitivity of the method was low. For example it failed to detect activity in wheat flour which had been previously reported (37) to contain the enzyme in small quantities.

In spite of these objections it was found that fairly consistent results could be obtained for the same batch of substrate providing detailed attention was given

to manipulative techniques. Various samples of defatted soya meal examined by this method gave activities ranging from 50 to 75 Cosby & Sumner units per gram.

As a temporary solution to difficulties arising from non-reproducibility of substrate, about 500 g. of defatted soya was prepared and assayed with the original set of reagents. It was then sealed as a reference sample and stored at 2^{o}. By assaying the same sample with subsequent sets of reagents a factor was obtained which allowed a common basis for comparison of results.

The problems associated with turbidity led to a search for a more effective stabilising agent than gum arabic. The development of turbidity with time on the addition of substrate to the gum arabic buffer, could be followed on the Spekker as a steadily increasing galvanometer deflection. A range of possible stabilizing materials were tried, and the results are shown in Table 1.

Buffer.	pH.	Stabiliser.	Turbidity at 12 mins.	Bleaching Rate.
Phosphate	6.6	None	53	xxxx
Phosphate	6.6	C. & S. Gum Arabic	44	-
Phosphate	6.6	0.2% Purified Starch	64	-
Phosphate	6.6	0.2% Edifas I	44	xxx
Phosphate	6.6	0.4% Edifas I	77	xxxx
Phosphate	6.6	0.8% Edifas I	79	xx
Phosphate	6.6	0.05% Stergene	42	x
Phosphate	6.6	0.1% Stergene	26	xx
Phosphate	6.6	0.2% Stergene	4	x
Phosphate	6.6	0.05% Pur. Stergene	27	x
Phosphate	6.6	0.1% Pur. Stergene	5	xx
Phosphate	6.6	0.2% Pur. Stergene	1	x
Phosphate	6.6	0.2% Manucol I	54	xxx
Phosphate	6.6	0.2% Manucol II	79	xxx
Phosphate	6.6	0.2% Gly. Mono Stearate	77	xxxx
Phosphate	6.6	0.2% British Agar	86	xxxx
Phosphate	6.6	0.4% Japanese Agar	100	xxxx
Phosphate	6.6	0.5% Pectin	77	xxxx
Phosphate	6.6	0.05% Gum Tragacanth	96	xxxx
Phosphate	6.6	0.4% Edifas II	63	xxxx
Phosphate	6.6	0.4% Cellofas A	73	xxxx
Phosphate	6.6	0.3% Cellofas B	92	xxxx
Phthalate	4.0	0.1% Purified Stergene	15	720 secs.
Acetate	4.4	"	33	720 secs.
Phosphate	5.4	"	35	481 secs.
Phosphate	6.6	"	10	161 secs.
Phosphate	7.9	"	-2	125 secs.
Borate	8.7	"	6	137 secs.
Borate	9.9	"	1	720 secs.

TABLE I

The "Edifas" and "Cellofas A" are methyl ethyl cellulose derivatives while "Cellafos B" is sodium carboxy-methyl cellulose. "Stergene" is a proprietry wetting agent of the non-ionic type, In some tests the active material was salted out from the commercial article giving the "purified Stergene" of the table. In the first set of experiments relative bleaching rates are indicated as varying between -- (no bleach) to xxxx (very rapid bleach). The turbidities are recorded as arbitrary scale readings, the higher figures indicating greater turbidities. A consideration of the table shows that while Stergene as a stabilizer was very successful, increasing concentrations slowed down the reaction and indeed, in results not included in the table, no reaction was obtained at levels of Stergene higher than about 0.2% of the purified material. It is worth mention at this point that some recent investigations (36, 52) on the kinetics of the lipoxidase reaction have been carried out in systems emulsified with surface-active agents, the authors being apparently not fully aware of the inhibiting effects of these substances. The

possibility of steric congestion at the surface of aggregates of fatty acid molecules makes inhibition with increasing concentration of surface-active materials not unexpected.

Attention is particularly drawn to the second half of the table where comparative bleaching rates are shown as the time to 50% bleach for the same volume of enzyme solution under different conditions of pH. The apparent optimum at about pH 8 in phosphate buffer under conditions of zero turbidity offered a promising improvement on the gum arabic system. Evidence will be brought forward later to show that in the absence of a surface-active agent the pH relationships are of a much more complex nature.

The modifications of added Stergene (subsequently on a more highly purified sample of this material a concentration of 0.01% was found to be suitable) and phosphate buffer at pH 8.0 were adopted. Using this system it was found that after quite a high initial

bleaching rate immediately after the addition of the enzyme solution the reaction velocity decreased before the half colour figure was reached. This effect was particularly apparent at low enzyme concentrations. Accordingly the concentration of the carotene stock solution was reduced from 7.5 mg% to 1.25 mg% and the original 10 mm. absorption cell replaced by a 40 mm. cell. Under these conditions carotene destruction was found to be almost linear with time between the range of 20% and 70% carotene destroyed. When these modifications were referred to the behaviour of the standard enzyme preparation it was found that the reaction velocity for the same enzyme solution was about 20% lower than that observed for the original gum arabic buffer. This slight loss of sensitivity was more than compensated for by the greatly enhanced stability of the system.

With these improvements it was now possible to make a study of the substrate concentration relationships of the system. The lower curve of Fig. 5 shows a

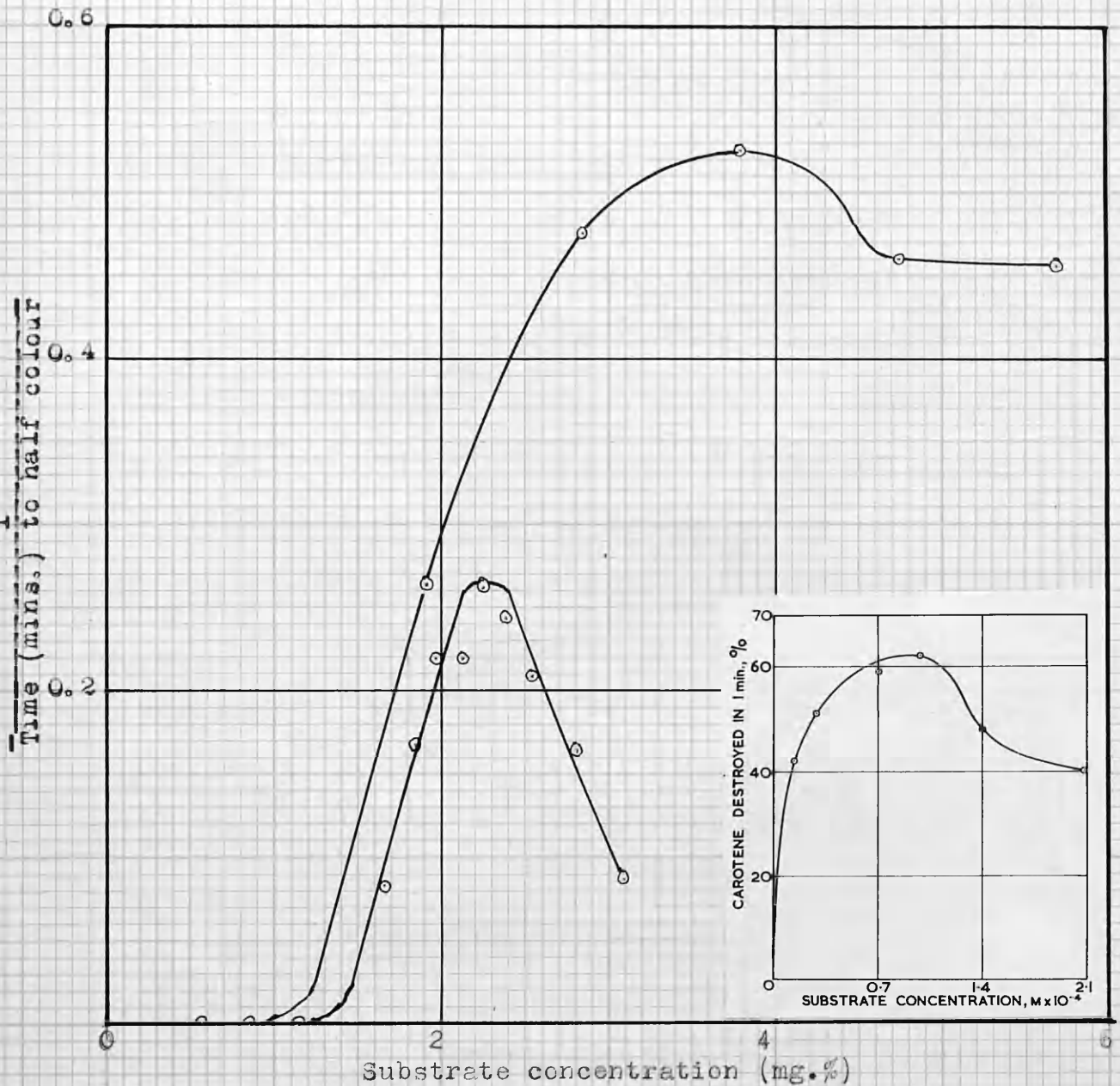


Fig. 5

Substrate concentration/reaction velocity curves for the Stergene system.

Upper curve: Sodium linoleate substrate

Lower curve: Cotton-seed fatty acid substrate

typical set of results. The fact that a curve of this degree of regularity could be obtained was reassuring from the point of view of stability and method of measurement of carotene destruction, but the sharply defined optimum and subsequent rapid decline in reaction velocities suggested some kind of competitive inhibition. Cotton-seed oil has been reported by Hilditch & Maddeson (54) as containing 23% oleic and 48% linoleic acids calculated as a percentage of the total fatty acids present, the remaining fatty acids being saturated. Holman (26) has reported competitive inhibition between oleic and linoleic acids in lipoxidase systems. It is clear from this that crude fatty acid substrates, however convenient, are unsuitable for work over a range of conditions.

Pure linoleic acid was prepared by bromination of cotton-seed oil (55), and a sodium linoleate substrate was used in observing the upper curve of Fig. 5. The obvious advantages of using a pure instead of a crude substrate lead to the immediate adoption of a standard

sodium linoleate solution in the assay. While a maximum is still obtained, it is spread over a wider range, and the rapid fall towards the base-line does not occur. The explanation for the maximum is not known but it is found in systems free from surface-active agents (56). For convenience, a curve from Appendix II, observed with a linoleic acid substrate in absence of added surface-active agent, is shown alongside. In comparing the curves it might be noted that 2.8 mg.% linoleic acid is 1×10^{-4}M.

Arising from these observations an improved method of assay was devised, which permitted the use of solutions free from turbidity, and whose substrate was reproducible. Moreover the sensitivity of the method was greatly enhanced. For example, using the same definition of units, samples of soya meal which with Cosby and Sumner's system assayed at about 60 units per gram, gave figures of 600 units per gram with the improved system. It might be noted however, that both of these systems containing a surface-active agent failed

to react until the substrate level was about 1 mg.%, while the comparison curve taken with a system free from added surface - active agent does not show this effect.

In Appendix I a detailed description of the lipoxidase-linoleate system containing added surface-active agent is given. This system was quite satisfactory and convenient for assay purposes, and was used in studies of lipoxidase stability in solution and on activating and inhibiting effects of wheat flour extracts. It was later superceded by a system free from surface-active agents (Page 44).

Experimental:-

2. The Stability of Crude Lipoxidase Solutions

The stability of lipoxidase extracts was a matter of some importance in the development of assay methods since the preparation of stable solutions could facilitate

comparative work from week to week. Acetate buffer at pH 4.5 was selected for extractions because the enzyme was known to be soluble under these conditions (24, 25), and because at this pH bacterial growth is largely inhibited although yeasts and moulds are not suppresseed.

The table below shows the results of a storage test on such an extract. The original solution was divided into two portions. Portion A was placed in a refrigator at 2^{o}, while portion B was stored at room temperature. The results are expressed as Cosby &Sumner units per hundred ml. of extract.

Storage Time (Days)	Portion A.	Portion B.
0	1380	1380
10	1380	- -
22	- -	1200
32	1400	- -

Thus extracts prepared as described do not lose measurable activity over a period of one month when stored at refrigator temperature. Although this information was adequate for manipulative purposes, it is of interest to note that a sample of a similar extract examined after sixteen month's storage at 2° still showed considerable activity.

Experimental:-

3. Inhibition of Lipoxidase by Wheat Flour Extracts

For reasons mentioned in the preface, it was of interest to examine the wheat berry with reference to its supposed lipoxidase content, and if possible to determine the distribution of the enzyme in the various parts of the grain. In these studies Blain (57) oberved that some wheat and flour extracts in acetate buffer at pH 4.5 appeared to inhibit the activity of soya-bean lipoxidase when examined in the Appendix I system. The acetate buffer extracts used were tedious to handle since they dissolved large quantities of

wheat protein giving viscous solutions which were difficult to clarify. As will be reported elsewhere, he later improved his extraction methods. Meanwhile it had occurred to the writer that the time-honoured "gluten washing" technique might well offer possibilities as a method for separating the bulk of the flour protein from this "inhibitor". Gluten washing is a traditional method used in the baking and milling industries for separating the gluten proteins from cereals and making a qualitative examination of their properties. It is carried out by preparing a dough from about an ounce of flour and appropriate amount of water (14.5 to 16 cc depending on the flour properties). If this ball of dough is gently kneaded in the palm of the hand for a few minutes under a very slow stream of tap water, the starch and some water soluble protein is washed out leaving a rubbery mass of protein known as "gluten". The test is commercially useful in that the properties of the bread dough made from that flour are to some extent reflected in the

properties of this gluten mass.

This procedure was adopted on a sample of 40 g. of unbleached flour which had had no chemical treatment. The washings were collected and amounted to a volume of 250 cc. After centrifuging the starch a somewhat cloudy supernatant fluid was obtained. The effect of this extract on lipoxidase solutions was tested using the system of Appendix I. Under the conditions of the experiment, the time required for 50% destruction of carotene was 208 seconds. Under the same conditions the presence of 0.2 ml. of the flour extract reduced the "half bleach" reaction time to 161 seconds. Thus, instead of obtaining an inhibiting solution as described by Blain an activating solution had been prepared.

There had been previous reports in the literature of lipoxidase inhibitors and activators. For example, the early work of Balls (21) and Theorell (40) had indicated that a heat-stable activator present in soya-beans was necessary for the proper functioning of the enzyme. Balls also found that purothionin, a crystalline

protein obtained from wheat, inhibited the reaction. Kies (58) isolated a crystalline polypeptide activator from soya- beans. On the other hand, Cosby and Sunner (22) failed to demonstrate an activator effect using their gum arabic substrate. Balls et al. found that gum arabic itself contained an activator. In his recent review, Holman (1) has pointed out that activation phenomena are only observed with certain types of substrates and that with these substrates, similar affects can be noted if surface-active agents are added. For example, with his homogeneous substrate of sodium linoleate at pH 9 no activator effect could be observed. He concludes that the activator effect is related to the physical state of the system used, and that emulsion systems require some surface-active agent to give optimum lipoxidase action.

Some, but not all of this information was available at the time this work was carried out and while it

seemed probable that the activation effects observed were similar to those quoted by Holman our system already contained a surface-active agent. It was therefore decided that a further investigation was justified.

Three samples of unbleached untreated flour were obtained and the gluten removed as described above. After centrifuging the starch from the gluten washings the solutions were submitted to fractional precipitation with ammonium sulphate, and the fractions examined on the assay system for activator or inhibitor effects.

Table II shows the results obtained on the first of these tests. The percentage activation is given by $\frac{(t_1 - t_2) \times 100}{t_1}$ where t_1 is time required for a given concentration of soya lipoxidase to bleach 50% of the carotene in the system, and t_2 is the time required for the same concentration of soya lipoxidase

to produce 50% bleach in the presence of an appropriate quantity of activator solution. Inhibition observations are treated by the same definitions and appear on the table as numerically negative activations.

Fraction.	Residue Volume	Activation %
1. Original Solution	-	22.6
2. 0.25% Saturation (S)	-	10.6
3. " (R)	50 ml.	-6.3
4. Fraction 2 (Boiled)	-	11.3
5. 0.5% Saturation (S)	-	35.7
6. " (R)	50 ml.	23.4
7. 0.64% Saturation (S)	-	-
8. " (R)	20 ml.	17.2
9. Saturated (S)	-	-29.0
10. " (R)	5 ml.	12.4

In all cases 2 ml. of the activator solution was added to the reaction system. The column "residue volume refers to the volume of water or very weak ammonium sulphate solution in which the precipatates from each fraction were dissolved. The letters "S" and "R" apply to the supernatant and residue of each fraction respectively.

TABLE II

The other two samples of flour examined showed a similar pattern of results and are therefore not detailed here. There is no real evidence in any of the samples of concentration of the activator or inhibitor in the various fractions, and at the time the work was carried out it was concluded that the effects observed were due to surface activity of the fractions when added to the assay system. The fact that activity remained after boiling (fraction 5 of the table) supported this view and it appeared that the observations were of the same nature as these of the early investigators already mentioned. One fact was difficult to explain. Some fractions were able to bleach the carotene slowly without the addition of enzyme solution.

This behaviour was not understood until the work on catalase described in Section II had been carried out. The inhibiting, activating and slow bleaching effects can all be explained on the asumption that the

extracts contained catalase. These phenomena are probably of some significance in the enzymic bleaching of bread doughs, and will be discussed in more detail in Section III.

Experimental:-

4. An Improved Carotene Assay System

The reaction system described in Appendix I, while a marked improvement on the gum arabic system of Cosby and Sumner, had certain disadvantages. The solutions used were free from turbidity at pH 8 but below pH 6 they became slightly cloudy. If the concentration of the surface-active agent was raised sufficiently to depress this effect, the reaction was inhibited for that level of substrate. The method thus lacked flexibility. Also the presence of a surface-active agent in the system was an undesirable complication especially on studies of the behaviour of the enzyme in the presence of inhibitors and antioxidants.

In modifications proposed by Blain, Hawthorn and Todd, (56, 57) and reported in the form of published work in Appendix II, these difficulties were largely overcome. The principal changes adopted were:-

(1) The reaction was carried out in simple buffer solutions without the addition of a surface-active agent.

2. The reaction was stopped after a selected reaction time (normally one minute) by the addition of strong caustic soda solution. The alkali cleared turbidity from the solution and enabled observations to be made at leisure.

3. The reagents used were more carefully characterised than hitherto.

4. The conditions under which the reaction was carried out, especially with regard to the addition of reagents, were more closely prescribed.

The general development of this improved system and the evaluation of its characteristics were due to Blain (57), but two aspects to which the writer gave special attention will be reported here.

Experimental:-

5 Standardisation of Carotene Solutions

Difficulties were experienced from time to time in the standardisation of carotene solutions due to (a) the marked tendency of crystalline carotene to auto-oxidise (b) the awkwardness of weighing the quantities used without having to resort to a microbalance and (c) confusion over blank readings in the enzyme reaction system due to variations in the degree of dispersion of the carotene (this is dealt with in detail in the next section on the effect of pH on the reaction system). To overcome these difficulties a spectrophotometric method of standardisation was adopted as follows.

0.0600 g. carotene (98% β - carotene from the British

Chlorophyll Co. Ltd.) is dissolved in 1500 ml. of redistilled acetone and the solution is made up to 2000 ml. with ethanol. (This quantity should completely dissolve but we have observed that the solubility of carotene samples in acetone/ethanol appears to vary somewhat, some samples being soluble to the extent of about 75 mg./litre .(44). These differences may be due to varying amounts of α - carotene and other impurities in the samples which have to be used. While a stock of pure β - carotene is held as an International Standard by the Medical Research Council this is not made available in useful quantities for investigations of this nature).

20 ml. of the resulting solution is diluted to 200 mls. with 75%/25% acetone/ethanol and the absorption curve obtained spectrophotometrically. Fig. 6 shows the curve obtained on the purest sample available to us as observed on the Hilger Uvispek spectrophotometer. The curve was also checked on a Unicam instrument. The characteristics of the solution were found to be

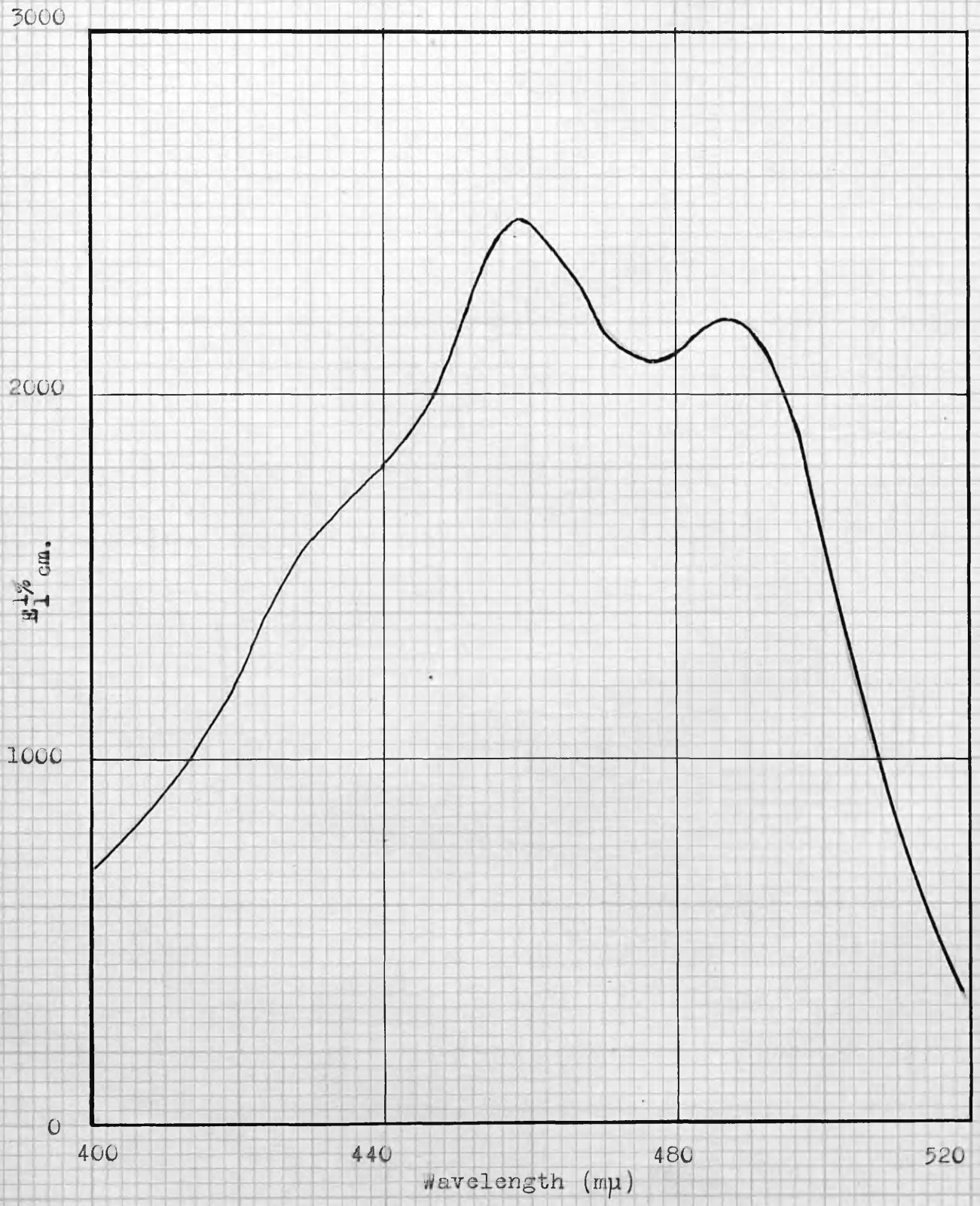


Fig. 6 Absorption curve of β-carotene in acetone/ethanol

$E^{1\%}_{1\ cm.}$ 458 mμ (max) = 2480

$E^{1\%}_{1\ cm.}$ 477 mμ (min) = 2100

$E^{1\%}_{1\ cm.}$ 487 mμ (max) = 2200

The writer is unaware of a previous spectrophotometric characterisation of β-carotene in an acetone/ethanol solvent, but the above figures are in close agreement with those recorded by Deuel (59) for β-carotene in 20% ether, 80% ethanol as follows.

$E^{1\%}_{1\ cm.}$ 453 mμ (max) = 2500

$E^{1\%}_{1\ cm.}$ 472 mμ (min) = 2100

$E^{1\%}_{1\ cm.}$ 480 mμ (max) = 2200

Using the figure of 2480 for the specific extinction coefficient at 458 mμ, the concentration of the stock solution weighed at 3 mg.% is calculated accurately and suitable quantities are diluted with the same solvent mixture to 1.5 mg.% for use as required in the

method described in Appendix II. The solutions thus prepared have been found to be stable over long periods of time and may be rapidly and conveniently checked as required in the spectrophotometer. When added at the rate of 2 ml. to 50 ml. of buffer, 1 ml. of substrate and 2 ml. of caustic soda solution as described in Appendix II, they will give an optical density reading of 0.37 to 0.39 under the conditions described. The instrument used may be either the Hilger Spekker absorptiometer with spectrum violet filters (No. 602) or the Unicam SP 600 spectrophotometer with the wavelength control set at 440 mμ.

Experimental:-

6. The pH Optimum of Lipoxidase

The changes in lipoxidase activity at various pH values has been the subject of controversy. As early as 1939 Sumner and Dounce, examining the lipoxidase destruction of carotene dissolved in olive oil, found maximum activity at pH 6.5 (10).

Using bixin with a substrate of soya-bean fatty acids, Sumner and Smith (45) later confirmed this observation. On the other hand, Holman (26) using the sodium linoleate substrate of Theorell et al. found high activity at pH 9 and over, but there was a marked decrease below this value. Holman's observations were independently observed by Franke et al. (60). Smith (61) suggested that this optimum at pH 9 was due to increased availability of the substrate at high pH values and gave experimental evidence of a correlation between the solubility of linoleic acid at various pH values and the enzyme activities oberrved by Holman. Using a substrate of methyl linoleate he again obtained an optimum at pH 6.5. Discussing these results, Holman and Bergstrom (1) point out that observed activities are made up of the inherent activity of the enzyme and the availability of the substrate, both of which vary with pH in this instance. In emulsion systems, the substrate concentration is the limiting factor, and since the internal surface of an emulsion varies with pH, the

optimum activity as measured will rather reflect the state of the emulsion than the behaviour of the enzyme. They suggest that the effect of pH on the inherent enzyme activity cannot be observed unless a substrate which is soluble over the whole pH range can be found. Fukuba (62) has recently found that the colloidal properties of polyoxyethylene linoleate are independent of pH as measured by solution turbidities and interfacial tension against xylene. Using this material as substrate he obtained an optimum between pH 6.5 and 7.0.

Anomalies in pH behaviour are by no means unknown in other enzyme systems. For example, the observed optimum pH of urease has been found to vary both with the buffer system used and the substrate concentration (63). Sherman and his co-workers (64) found variations in the pH optimum of malt amylase depending on the buffer concentration used. Furthermore, the observed optimum may alter during the purification of the enzyme.

Willstatter and his colleagues (65) found that several purifications of canine gastric lipase caused the optimum to move from about pH 5.9 to about pH 7.5. Considering these facts it seems not improbable that the optimum pH of at least some enzymes observed in in vitro systems may bear little relationship to the enzyme's behaviour in vivo. In considering the behaviour of lipoxidase, which may be considered to catalyse a reaction across a fat/water interface, it is unlikely that the pH of the medium is the same as the pH at the interface, if indeed at such an interface the conception of pH has any real meaning.

In the pH activity curves which are illustrated in Figs. 7 to 13, the behaviour of the lipoxidase linoleate system described in Appendix II is shown under a variety of conditions. Since it was considered undesirable to change the anions in the system, McIlvaine's buffer solutions (66) were used throughout in obtaining these curves. These buffers can be used between pH 3

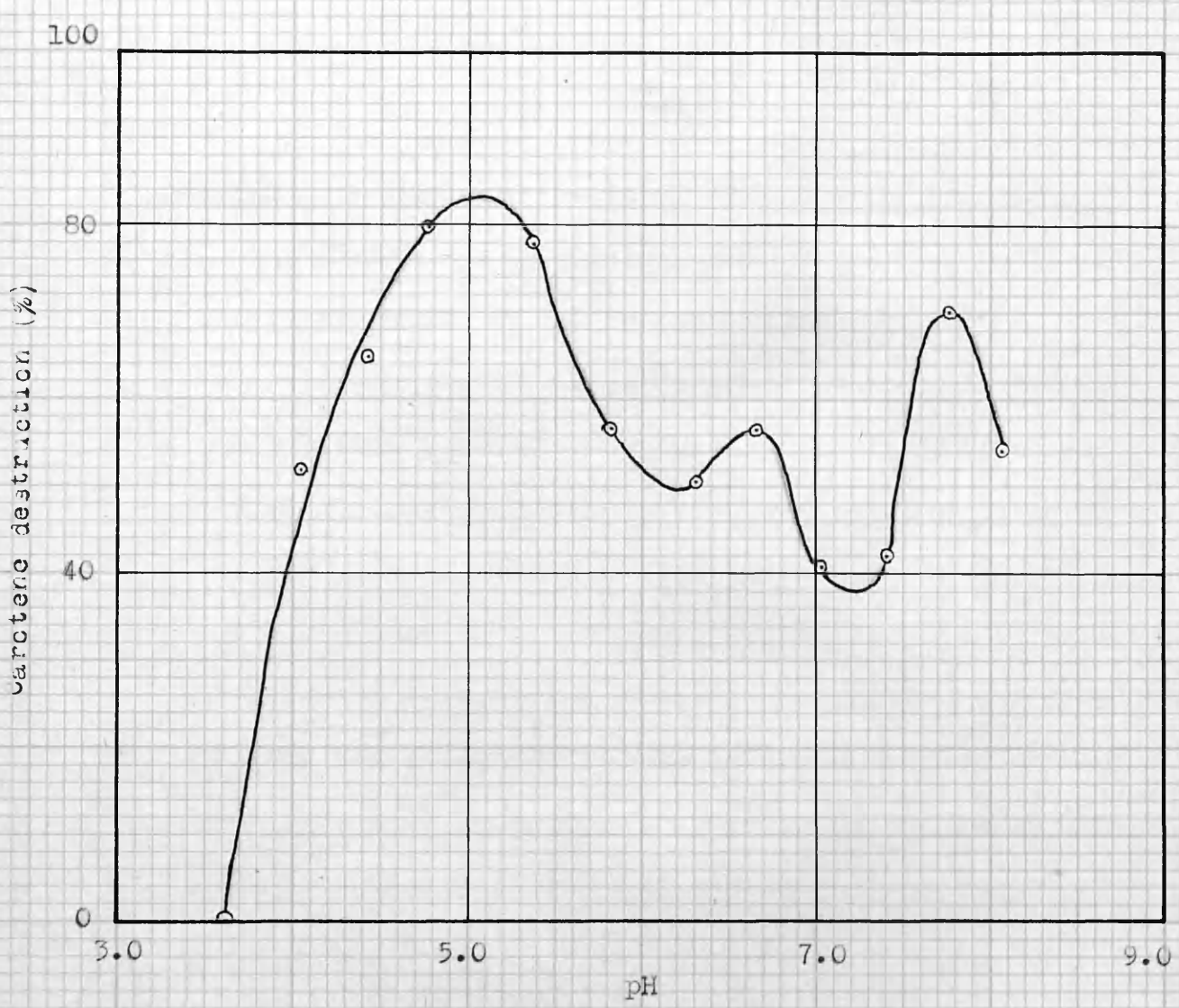


Fig. 7

The pH activity curve of soya-bean lipoxidase under the standard conditions of Appendix II

and pH 8 , and the observations are therefore restricted to this range. For convenience in making comparisons some of the illustrations in Appendix II are reproduced here.

Fig. 7 illustrates the effect of change in pH on the bleaching of carotene using the system described in Appendix II. This unusual pH activity curve has been confirmed many times and was felt to require some explanation. For example, using the Appendix I system a single optimum, was observed in the region of pH 8 (Table I). Again, it is not common to find more than one peak on a pH activity curve. In Appendix II evidence is given that the three peaks in Fig. 7 are directly related to the dispersion of the system. Briefly, the evidence cited there is as follows:

1. In the absence of enzyme and substrate, standard additions of carotene were made to the appropriate volume of buffer solution and no significent change

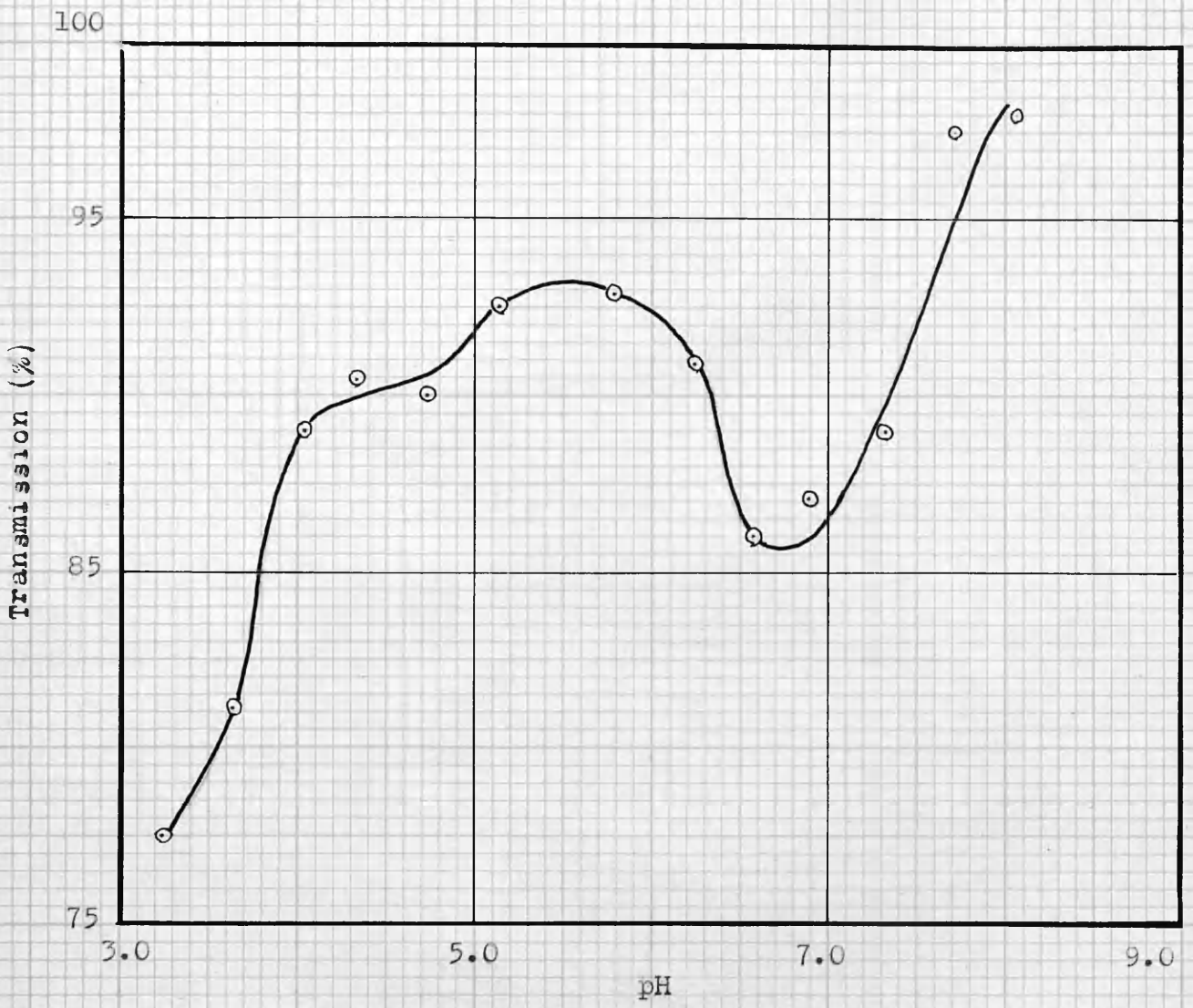


Fig. 8

The optical transmission of linoleic acid suspensions (2 mg.%) in 50 ml. of phosphate-citrate buffer solutions.

in optical density with change in pH was found over the range examined. Since a change in carotene dispersion with pH would have resulted in a change in optical density (spectroscopic evidence for this is given), it may be concluded that the dispersion of carotene in buffer does not change with pH.

2. In the absence of carotene and enzyme, dispersions of sodium linoleate in buffer do show such a change with pH. This change is presumed to be due to varying rates of precipitation of linoleic acid manifested as different turbidities produced in a given time interval at different pH values. The shape of the substrate turbidity pH curve reproduced in Fig. 8 (transmission plotted against pH) shows no obvious relationship with the activity curve of the enzyme.

3. When both substrate and carotene were added to

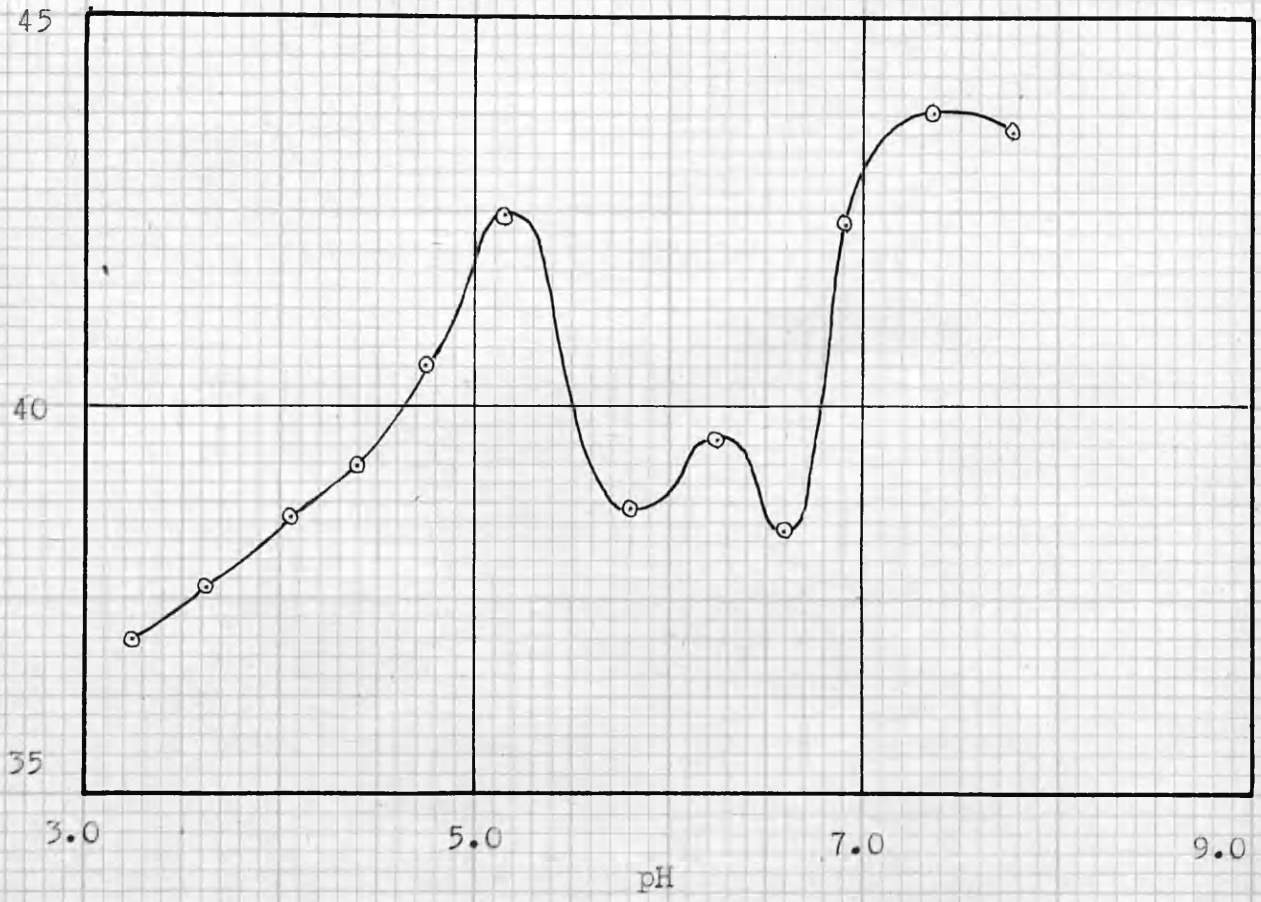


Fig. 9

The optical transmission of linoleic acid suspensions in presence of carotene under the conditions of Appendix II.

the appropriate volume of buffer solution in the absence of enzyme, and the transmission of the mixture read with change of pH, the curve shown in Fig. 9 was obtained. Since Fig. 9 is very different in form from Fig. 8, and since buffer containing carotene alone does not change in dispersion with pH, the presence of carotene must influence the dispersion of substrate. Arising from this, a comparison of Figs. 7 and 9 is significent. Both curves show three peaks at approximately the same pH values. From this coincidence of the peaks in the dispersion curve with those in the activity curve, it was concluded in Appendix II that the form of the pH activity curve reflected the dispersion of the system.

Further evidence confirming and extending this work has been obtained since the publication of Appendix II. In carrying out the enzyme reaction the normal procedure is to make the additions of substrate, carotene

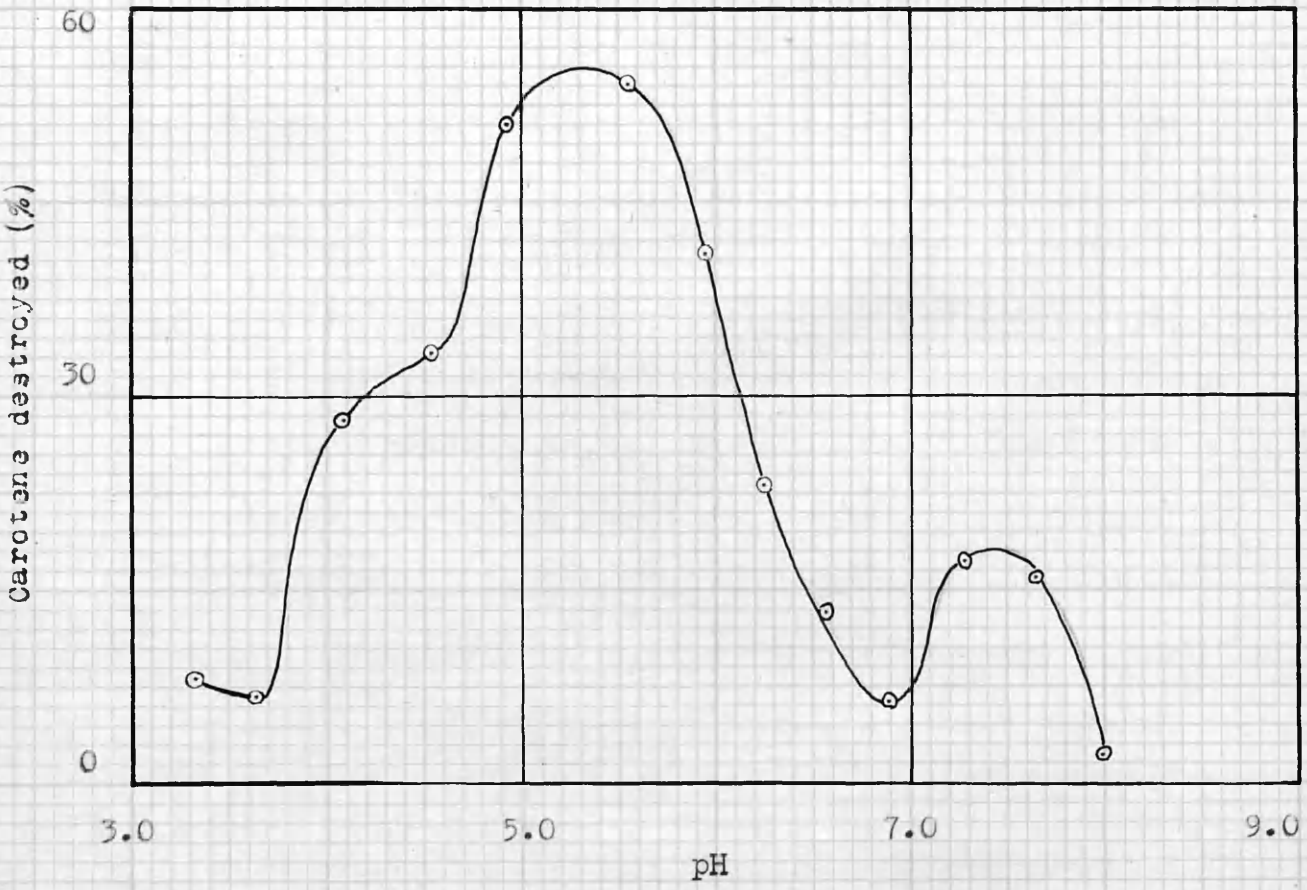


Fig. 10

pH activity curve as observed with a delay of three minutes between the addition of substrate and carotene.

and enzyme to the buffer in that order and as rapidly following one another as possible. As already shown, under these conditions the presence of carotene influences the dispersion of the substrate. However, in carrying out these reactions it had been observed that, at acid pH values precipitation of linoleic acid in the concentrations used was not immediate, but took place gradually and visibly over the first few minutes after the addition of the substrate solution. It was therefore felt that if a delay of, say three minutes, were introduced between the addition of substrate and carotene a curve reflecting the dispersion of substrate might be obtained. The experiment was tried and Fig. 10 shows the result. As anticipated, this pH activity curve much more closely resembles Fig. 8 than Fig. 9, particularly between pH 3 and 7.

These data show more clearly than any previously published figures the extent to which the observed activities of lipoxidase in <u>in vitro</u> systems are

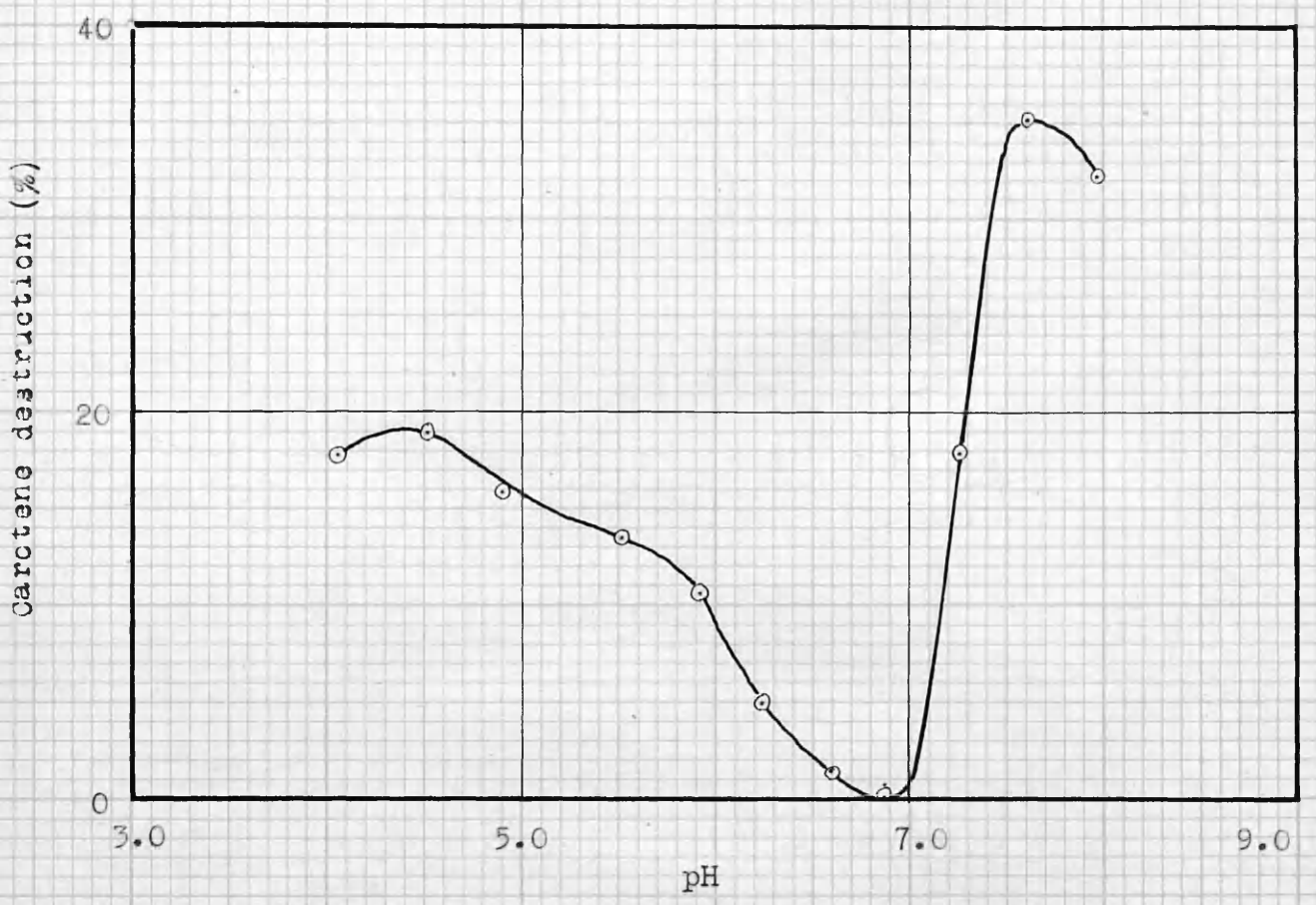


Fig. 11

pH activity curve at low substrate concentration (0.5 mg.% in the reaction system).

influenced by the colloidal state of the substrate. It is equally clear that the controversy as to the optimum pH of lipoxidase has arisen from this factor.

From the information presented up to this point, it is not possible to come to further conclusions on the inherent pH optimum of the enzyme. In considering this problem it was felt that pH activity curves determined under conditions of very high and very low substrate concentrations might give useful information. The results of these determinations are shown in Figs. 11 and 12.

The substrate concentration used in observing Fig. 11 was one quarter of that required to saturate the enzyme. (Fig. 7 in Appendix II shows the substrate concentration curve for the system.) Under these conditions, the absence of activity at pH 6.8, followed to the rapid rise to a maximum at pH 7.6 are the striking features of the curve. Reference

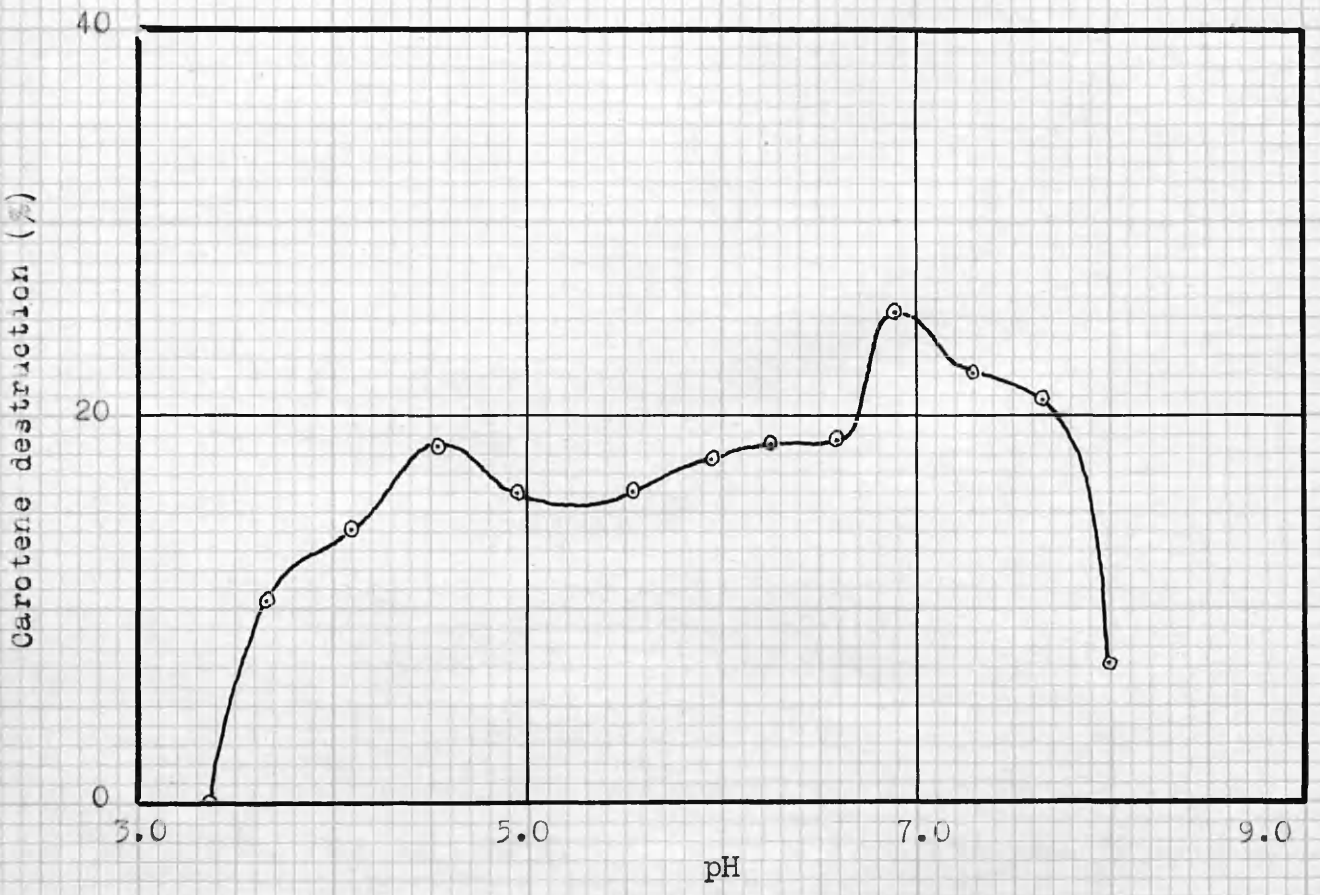


Fig. 12

pH activity curve at high substrate concentration (20 mg.% in the reaction system).

to Fig. 8 shows that rapid precipitation of substrate takes place at pH 6.8, and it might therefore be expected that, at this pH, little or no substrate would be available to the enzyme at low levels of substrate addition. The rapid increase in activity above pH 7.0 doubtless reflects the increased solubility of substrate in this region as shown in Fig. 8.

The substrate concentrations used in observing Fig. 12 were ten times that required to saturate the enzyme. In direct contrast to conditions of low substrate concentrations, the highest activity was found at pH 6.8. The previous curves have amply demonstrated that substrate availability is low at this pH. Hence, the presence of an optimum at pH 6.8 under conditions of high substrate concentration but low substrate availability indicates that the inherent activity of the enzyme must be high at this pH. Further, taking account of the other regions of Fig. 12 where substrate availability is

higher, it seems reasonable to interpret the curve as meaning that the inherent optimum of the enzyme lies between pH 6.6 and 7.3.

In considering these pH activity curves, it has been assumed that the concept of a pH optimum has a real meaning for a heterogeneous catalysis of the type under consideration. This may not be so, and it is certainly clear from these data that, under most experimental conditions, observed reaction velocities mainly reflect the colloidal state and mode of presentation of the substrate to the enzyme. By suitable adjustment of experimental conditions a pH "optimum" can be observed almost anywhere in the pH range 4 to 9, and while there are indications of an inherent optimum at pH 6.8, the nature of the lipoxidase reaction justifies caution in attaching fundamental significence to this value.

To conclude this Section, Table III summarizes the available data on the pH optima of lipoxidase.

Author.	Optimum pH.	Substrate.	Mode of Assay	Reference
Sumner et al.	6.5	Olive Oil	Carotene Bleaching	10
"	6.5	Soya-Bean Fatty Acids	Bixin Bleaching	45
Smith	6.5	Methyl Linoleate	Spectro-photometric	61
Holman	9.3	Sodium Linoleate	"	26
Franke et al.	9.3	"	"	60
Fakuba	8.9 - 9.2	"	Oxygen absorption	62
"	6.6 - 7.3	Methyl Linoleate	Carotene destruction	62
"	6.5 - 7.0	Polyoxyeth-ylene Linoleate	Oxygen absorption	62
Hawthorn	Over 8.0	Sodium Linoleate	Spectro-photometric	Fig.8
"	5.0	"	Carotene Bleaching	Fig.7
"	6.7	"	"	Fig.7
"	7.8	"	"	Fig.7
"	5.0	"	"	Fig.11
"	7.5	"	"	Fig.11
"	7.6	"	"	Fig.12
"	6.8	"	"	Fig.13

TABLE III

SECTION II

THE UNSATURATED-FAT OXIDASE ACTION OF CATALASE.

SECTION II

THE UNSATURATED-FAT OXIDASE ACTION OF CATALASE.

Preliminary Considerations

It has long been known that haem compounds are capable of catalysing the oxidation of unsaturated fats. For example, in 1924, Miss M.E. Robinson in Cambridge studied the effect of haem compounds on linseed oil emulsions (67). She used Barcroft manometers to measure the oxygen uptake of the oil and concluded, (a) that the blood pigments haemoglobin, methaemoglobin and haemin are efficient catalysts of the auto-oxidation of linseed oil and (b) that the concentration of iron present as blood pigment necessary to produce the same catalytic effect is very much smaller than that required if the iron is present in the form of an inorganic salt.

The reactions catalysed in this fashion are so similar to the lipoxidase reactions that confusion as to the existence of animal lipoxidases has arisen.

In the section which follows some aspects of the behaviour of catalase as an unsaturated-fat oxidase are described, and means of distinguishing between lipoxidase and the unsaturated-fat oxidase action of haem compounds are suggested.

The choice of catalase for study in this connection requires some explanation. The presence of cytochrome c, peroxidase and catalase in flour raised the possibility that one or all of these substances might be concerned in the bleaching reactions involved in the Rank and Hay process in addition to lipoxidase. A general survey of the unsaturated-fat oxidase activity of these substances was therefore put in hand, and substantial progress had been made with catalase and cytochrome c. The writer's interest in catalase stems from two papers by Holman (21, 68) and from some observations of his own on the effect of hydorgen peroxide on bread doughs which will be discussed in Section III.

In the papers mentioned, Holman made a study of the

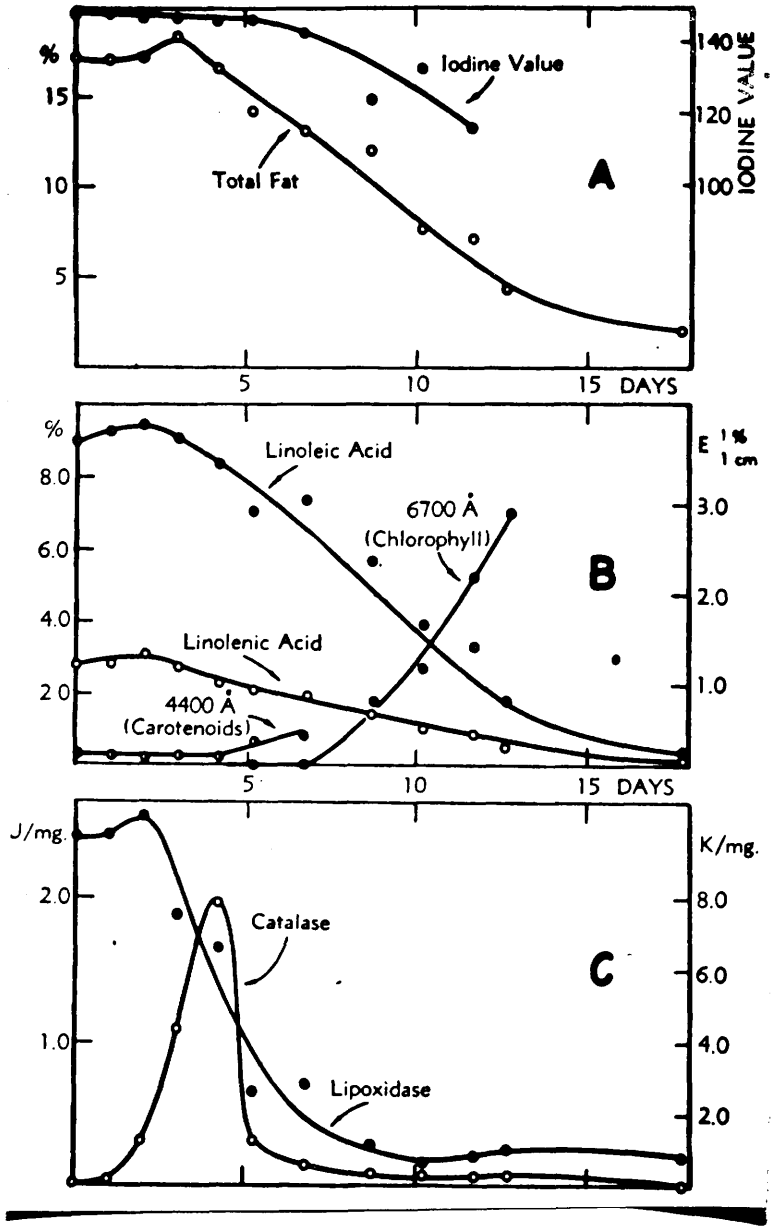

%
15
10
5
Iodine Value
Total Fat
A
140
120
100
IODINE VALUE
5
10
15 DAYS
%
8.0
6.0
4.0
2.0
Linoleic Acid
6700 Å
(Chlorophyll)
B
Linolenic Acid
4400 Å
(Carotenoids)
E 1% 1 cm
3.0
2.0
1.0
5
10
15 DAYS
J/mg.
2.0
1.0
Catalase
C
Lipoxidase
K/mg.
8.0
6.0
4.0
2.0

Fig. 13.

relationship between lipoxidase activity and fat composition in germinating soya beans. As is usual in the germination of oil bearing seeds the total fat decreased throughout the experiment. Contrary to expectation, the lipoxidase activity also decreased sharply two days after planting and there was a similar and preferential decrease in the linoleic and linolenic acid content of the fat. At the time of greatest decrease in lipoxidase activity there was a marked rise in catalase activity followed by a subsequent sharp decline as the lipoxidase disappeared. Holman's data are reproduced opposite in Fig. 13. Discussing these results he says"Some connection between catalase activity and fat metabolism has been suspected by workers in this field. It has been found, for example, that catalase is easily prepared in good yields from adipose tissue (69). Unpublished experiments have demonstrated that there is probably a complex formation between linoleate peroxide and catalase as measured in the recording spectrophotometer by Dr. Chance. A shift in the absorption spectrum upon the mixture of linoleate, lipoxidase and catalase takes place indicating some binding of catalase. This shift does not take place with other

combinations of these reactants. However, the complex is not dissociable in presence of oxidizable substrates as is the case with catalase, hydrogen peroxide and ethyl alcohol."

Holman's implication of a relationship between lipoxidase and catalase in germinating soya-beans is of possible interest in connection with the physiological function of catalase. In spite of the ubiquity of catalase (70) its function in living tissue is obscure. It has long been regarded as a scavenger of the hydrogen peroxide known to be formed during the reduction of oxygen to water in metabolic processes. Haemoglobin, for example, is readily destroyed by hydrogen peroxide and the function of catalase might be concerned in the protection of blood pigment from the effects of peroxide accumulations. Lemberg and Legge (71) after detailed discussion are inclined to support this point of view, but Theorell (72) and Keilin and Hartree (73) tend rather to emphasise the importance of the peroxidatic function of the catalases. The peroxidatic theory is based on Keilin and Hartree's observations that, in the presence

of a hydrogen-peroxide-producing biological system, alcohols may be oxidised by catalase in a complex reaction (73). Chance (74) suggests that the distinction between peroxidases and catalases is artificial, and that their behaviour may be expressed in the following equations,

$$E + S \rightleftharpoons ES$$

$$ES + AH_2 \rightleftharpoons E + A + SH_2$$

where the enzyme-substrate complex ES reacts with a donor molecule AH_2 to produce reduced substrate and oxidised donor. This reaction mechanism applies equally well to catalase, AH_2 and S being identical when catalase is decomposing hydrogen peroxide to oxygen and water.

Recently, further evidence has been put forward by Chance (95) who has identified the spectrum of catalase hydrogen peroxide complex I in respiring suspensions of Micrococcus lysodeikticus. A refined spectrophotometric method was used and it was found possible to demonstrate in vivo the effect of nitrite in reducing the steady rate

concentration of complex I. The same effect had been previously demonstrated *in vitro*, the role of nitrite being that of the donor molecule AH_2. This paper provides the first direct evidence for the peroxidatic function of catalase *in vivo*.

The reasons for selecting catalase as an example of a haem protein acting as an unsaturated-fat oxidase, were associated with the possibility that the presence of catalase might influence the lipoxidase reaction in the Rank and Hay process.

Haem Proteins as Unsaturated-Fat Oxidases

Banks (13) found that rancidity in the oil of frozen herring is stimulated by a heat labile system in the lateral band of red muscle, and he subsequently showed that haem proteins are involved in this form of spoilage. The properties of this haem system were quite unlike those of soya-bean lipoxidase. The optimum activity was found at low pH values and no measurable oxidation took place when the system was homogeneous, though oxidation was rapid when the unsaturated fatty substrate was in suspension in buffer at pH 6.6. In

experiments with haematin as a pro-oxidant, spectroscopic examination showed that the catalyst was rapidly destroyed during the course of the reaction.(14) In a more recent paper (75), Banks has drawn attention to the importance of cytochrome c oxidase as well as haemoglobin as pro-oxidants for herring oil.

Lea (15) also observed an oxidase system in pig muscle which accelerated fat rancidity and showed optimum activity about pH 4.5. Watts and Peng (76) attributed the fat oxidase activity of pork muscle to the myoglobin and haemoglobin present. Reiser (16) found that the peroxidising and bleaching effects of bacon tissue extracts involved more than one catalyst, and that extracts containing no demonstrable haemoglobin still exhibited activity. Sullivan (1) showed that rabbit muscles showed strong unsaturated-fat oxidase activity, while Hove (17) found similar activity in the gastric mucosa of rats. With the exception of Reiser's experiments, all the cases cited could be attributed to the presence of haem compounds in the systems. There is therefore little evidence for the belief that animal

lipoxidases of a similar nature to soya-bean lipoxidase exist.

Kies (51) made a comparative study of haemin and lipoxidase, but did not publish her results in detail. She found that purified haemin was completely inactive in reaction systems containing 10% acetone, while lipoxidase retained its activity under these conditions. She found that haemin and cytochrome c inhibit lipoxidase activity while catalase and haemoglobin do not, and confirmed the observations of Haurowitz (87), Banks (14) and others, that the haem compound is itself destroyed when catalysing the oxidation of unsaturated fat.

Three recent papers by Tappel (19, 20, 77) have re-examined this problem and form the most complete study to date.

Tappel used oxygen uptake as measured by the Warburg apparatus to follow the progress of the reactions, and pure linoleic acid or sodium linoleate at pH 7 and pH 9 as substrates. He considered the pH 7 solution to be heterogeneous while the system at pH 9

was homogeneous. He first applied his system to the study of pork muscle extracts and found that the pro-oxidant activity of the extracts was largely inhibited by heating to 80°. This behaviour was similar to that of soya-bean lipoxidase and different from that of crystalline haemin which is not inactivated by heat. A previous study by Lea (15) with similar results had suggested to him the possibility of an animal lipoxidase. On the other hand, Watts (76, 79) had pointed out that heat coagulation of haemoglobin and myoglobin could lead to effective removal of haem from the system, and Tappel was able to verify this suggestion experimentally. He then attempted to repeat Reiser's work in which activity had been found in tissues free from demonstrable haemoglobin (16). Tappel was unable to verify Reiser's observations, and, concluding that pork tissues are free from lipoxidase, suggested that Reiser's method for detecting traces of haemoglobin was not sufficiently sensitive. As a criterion of distinction between lipoxidase and haem catalysis, Tappel suggested the differentiating effect of homogeneous and colloidal linoleate. (In 1944, Banks (14) had pointed out that

haem will not oxidise homogeneous linoleate nearly so readily as colloidal linoleate but Tappel was apparently unaware of this paper.)

In a second paper (20) Tappel studied a wide variety of animal tissues, and found that most of the linoleate oxidation catalysed by extracts from the tissues of fish, rats, cattle, chicken and turkey, could be ascribed to their content of haem compounds.

In a third paper (77) Tappel dealt with more fundamental aspects. Using activity measurements based on iron-porphyrin concentration, he found the catalytic activity of cytochrome c, haemin, haemoglobin and catalase to decrease in the order named. He found low activity and long induction periods before oxidation by catalase was observed, and suggested that breakdown of catalase may have ensued during this time interval. Induction periods were found for all haem compounds tested, the more reactive the compound the shorter being the induction period. He concluded that the reaction kinetics were of the type -

$$\frac{dO2}{dt} = K(\text{haematin catalyst})^{\frac{1}{2}}$$

The suggestion of a chain reaction implicit in this relationship was confirmed by the action of chain-breaking antioxidants.

From these three papers it seems clear that the so-called animal lipoxidases are in fact haem proteins in which the haem group is the active catalyst although its activity is modified by the protein moiety. Although the end products are similar, the reactions of haem and lipoxidase are kinetically dissimilar and lipoxidase is known to be free from any haem grouping. It therefore seems advisable to use the phrase "unsaturated-fat oxidase activity" to describe the reactions in a general sense and to restrict the term "lipoxidase" to the soya enzyme or to other unsaturated fat oxidases of plant origin where the possibilities of haem oxidation have been eliminated.

<u>The Coupled Bleaching of Carotene by Haem Systems</u>.

No systematic study of the coupled bleaching of

carotenoids by such systems has been published, and indeed the literature is singularly free from mention of this phenomonen. In 1941 Brocklesby and Rogers (81) found that vitamin A was rapidly destroyed at room temperature by extracts of salmon liver and it is probable that this was a coupled oxidation initiated by an unsaturated-fat oxidase. Reiser (16) demonstrated carotene bleaching with bacon extracts, while Hove (17) found that water extracts of minced rat stomachs destroyed carotene in presence of methyl linoleate. In 1947, Watts and Peng (76) studied haemoglobin-catalysed peroxidation of lard in which carotene had been dissolved. They found a correlation between the percentage of carotene destroyed and the peroxide number developed by the fat.

Tappel (88) has recently demonstrated that α-tocopherol, in presence of oleic acid undergoing haematin catalysed oxidation, exerts a protective function on vitamin A or carotene if these are simulteneously present. He suggests that α-tocopherol may function *in vivo* by inhibiting haematin catalysis of unsaturated fat and simultaneously "sparing" vitamin A. This suggestion

is in conformity with previous findings, particularly in connection with the occurence of fat peroxides and polymerised fats in vitamin E deficient animals. (For example, see references 71, 89, 90, 91, 92, 93, 94). The carotene bleaching system he used was no better characterised than those of previous workers.

In view of this rather scanty literature, a further examination of coupled bleaching of carotenoids by haem pigments seemed justified. Exploratory experiments with haemoglobin, haematin, and cytochrome c indicated that the carotene bleaching system of Appendix II was well suited for the study of coupled carotenoid bleaching by haem catalysis. The work on catalase described here is being paralleled by Blain (57) on cytochrome c.

Previous work on catalase as an unsaturated-fat oxidase had indicated that it was comparatively inactive. Simon et al. had found that at concentrations of 3×10^{-7}M it was completely inactive as measured by oxygen uptake with linoleic acid at pH 7.3. Tappel (77) found it the least active of the haem proteins which he examined for

unsaturated-fat oxidase activity. Nevertheless, as will be shown in the following pages, under favourable conditions catalase is a very active fat pro-oxidant, its activity being most marked at low concentrations.

Experimental:-

1. The Purification of Catalase.

Catalase has not yet been isolated in a pure state from a plant source, but a variety of methods are available for the purification of animal catalases (83, 84, 85, 86). The use of crude plant catalases was undesirable because of the possible presence of lipoxidase in the extracts, and it was decided to purify an animal catalase for use in this work. It is true that the main centre of interest in the work is a plant product, but it was felt that a reasonably pure sample of animal catalase would at least provide a model for the behaviour of catalases in general.

Tauber and Petit (82) have recently described a simple procedure for the isolation of liver catalase which was adopted in this work. Since it was not considered strictly necessary to obtain crystalline catalase for the

end in view, a further simplification of Tauber's method was adopted.

Fresh cow liver was coarsely minced and then disintegrated in a high-speed macerator for two minutes. To 650 g. of the resulting material was added 700 ml. of distilled water and the mixture stirred with an electric stirrer for six minutes. The volume of the mixture was about 1150 ml. The mixture was transferred to a 3-litre beaker, and 0.4 volumes of redistilled acetone added in successive small portions with stirring. Stirring was continued for one minute after the last portion of acetone had been added, and the mixture filtered in two portions through large Whatman No. 12 papers. About 850 ml. of filtrate was obtained. The filtrate was kept overnight at 4° and a precipitate formed. Without removing this precipitate, a further 196 ml. (0.23 volumes) of acetone were added with the same precautions as before to precipitate the remainder of the catalase. The precipitate was removed by filtration and dissolved in 50 ml. of distilled water. Undissolved material was removed by centrifuging, and the solution was then dialysed against

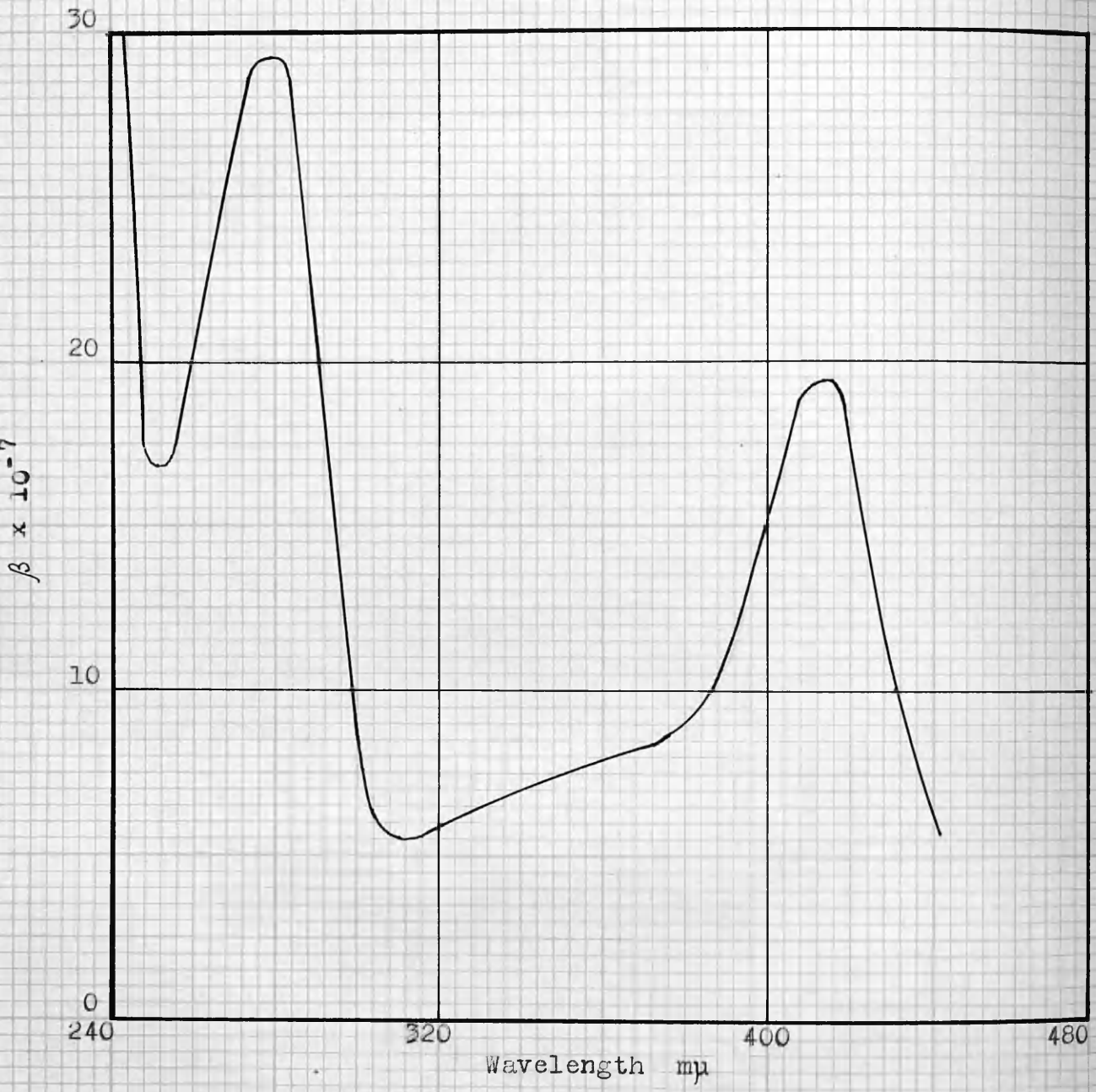


Fig. 14

The absorption spectrum of the catalase preparation used.

distilled water for two hours and the inactive precipitate which formed removed by centrifuging. If dialysis is too prolonged catalase may crystallise and be lost with the inactive precipitate.

By this procedure about 50 ml. of a black solution, ruby red when observed in thin layers by transmitted light, was obtained.

The absorption spectrum of the preparation is shown in Fig. 14. In general form it is almost identical with that published by Tauber and Petit. β is calculated on the assumption that the molecular weight is 225,000.

A comparison of the absorption characteristics of Tauber's purest crystalline preparation and those of the present preparation are given below.

	Tauber	Present Prep.	Purity
β (276 mμ) =	82.2×10^7	29.2×10^7	35.5%
β (404 mμ) =	63.6×10^7	19.4×10^7	30.5%

The apparent purity of the present preparation in terms of Tauber's material is shown in the last column calculated for both peaks. On these data our preparation would appear to contain at least 30% catalase on the same dry solids basis used by Tauber.

Activity measurements using Jolles' modification of von Euler and Josephson's method (96) gave a Kat. f. value of 12,000 as against the corresponding value of 32,000 for Tauber's preparation. Based on activity measurements, the present preparation was therefore 37.5% catalase on a dry solids basis, which, all factors considered was in good agreement with the absorption data. Dilutions of this preparation were used in the subsequent work. The preparation was found to store well at about 4° and a check on its characteristics on completion of the work showed no significant change.

Experimental:-

2. The Coupled Bleaching of Carotene by Catalase.

Using the lipoxidase assay system described in Appendix II, a study was made of the behaviour of catalase

as a bleaching agent for carotene in the presence of linoleic acid. Any departures from the procedures and concentrations used in the lipoxidase system were slight and are mentioned at the appropriate places in the text.

It was soon found that, where precautions were taken to prevent any trace of auto-oxidation in the linoleic acid substrate during preparation and storage, bleaching did not take place during the short reaction times preferred in this system. Banks (14) noted that pre-formed peroxide must be present for catalysis by haem proteins to take place. Tappel (77) observed induction periods in haem catalysis. Lovern (97) suggested that the haem proteins are not themselves catalysts, but that a linoleate-peroxide-haem complex is the active catalyst. As already mentioned, spectrophotometric evidence for the existance of this complex has been obtained (68). It was found that where a freshly prepared, nitrogen-protected substrate was left overnight in a petri dish exposed to the air, or where a substrate had been allowed to lie in a bottle for a week, catalase readily brought about carotene bleaching. Bleaching rates did not increase to

a maximum and then remain constant as the substrate aged, but after a time began to decrease. This was almost certainly due to the polymerisation of the peroxides formed as the following simple observations suggest. A freshly prepared substrate was found to be inactive in the system. A few drops of a very old, highly oxidised substrate were added and the system remained inactive. A few drops of new substrate which had been oxidised by lipoxidase just previously were now added and the substrate became active. (Pre-formed peroxides are not necessary for lipoxidase catalysis.) In order to observe the behaviour of catalase it was therefore necessary to use "aged" substrates in which a certain amount of auto-oxidation had taken place. The results must therefore be considered as giving the general pattern of the behaviour of the system, but owing to the complexity of the auto-oxidation of linoleic acid, it was considered virtually impossible to conduct all the experiments with substrate containing equal amounts of pre-formed peroxides. Strictly quantitative reproducibility is not attainable until substrates containing comparable amounts of preformed peroxide can be prepared without undue polymerisation or

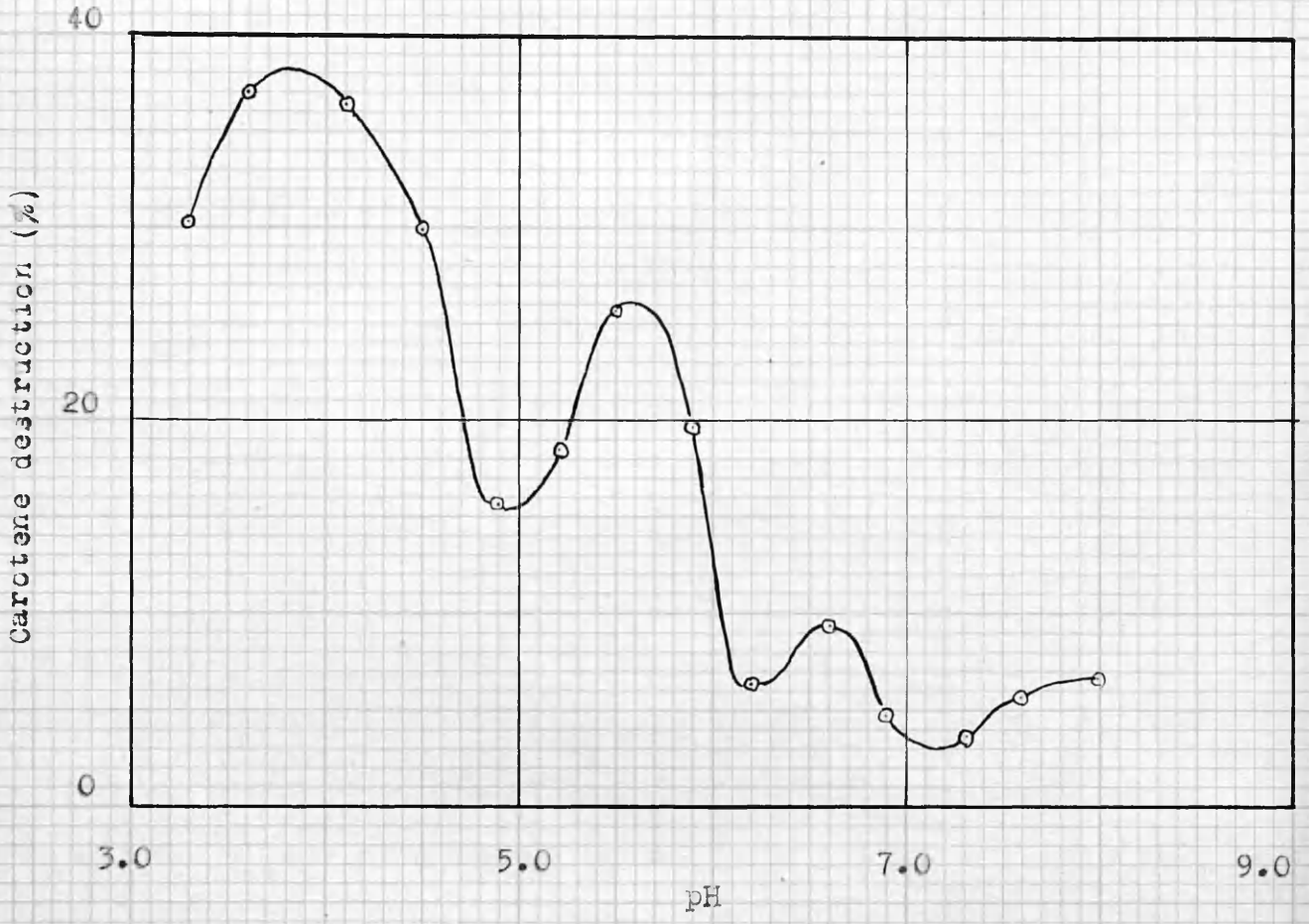


Fig. 15

The pH activity curve of catalase as an unsaturated-fat oxidase.

decomposition of the hydro-peroxides. Blain (57) has observed that pre-formed peroxides must be present before cytochrome c will act as an unsaturated-fat oxidase.

Experimental:-

3. The pH Optimum of Catalase.

Fig. 15 shows the pH activity curve of catalase as an unsaturated-fat oxidase. A comparison of this curve with that for lipoxidase in the same system (Fig. 7) and at the same substrate concentration shows certain similarities. Both show three peaks and in both cases activity is low in the region of pH 7. Fig. 8 indicates that, at this pH, substrate availability is at a low level. The appearance of the catalase maximum activity at pH 3.7 as against 5.1 for lipoxidase affords a possible means of distinguishing between the two forms of catalysis. Blain (57) using cytochrome c as an unsaturated-fat oxidase has obtained a curve of almost identical form to the catalase curve shown here.

Reference to Fig. 8 will show that at pH 3.7 linoleic acid is precipitating rapidly, and the finding of an

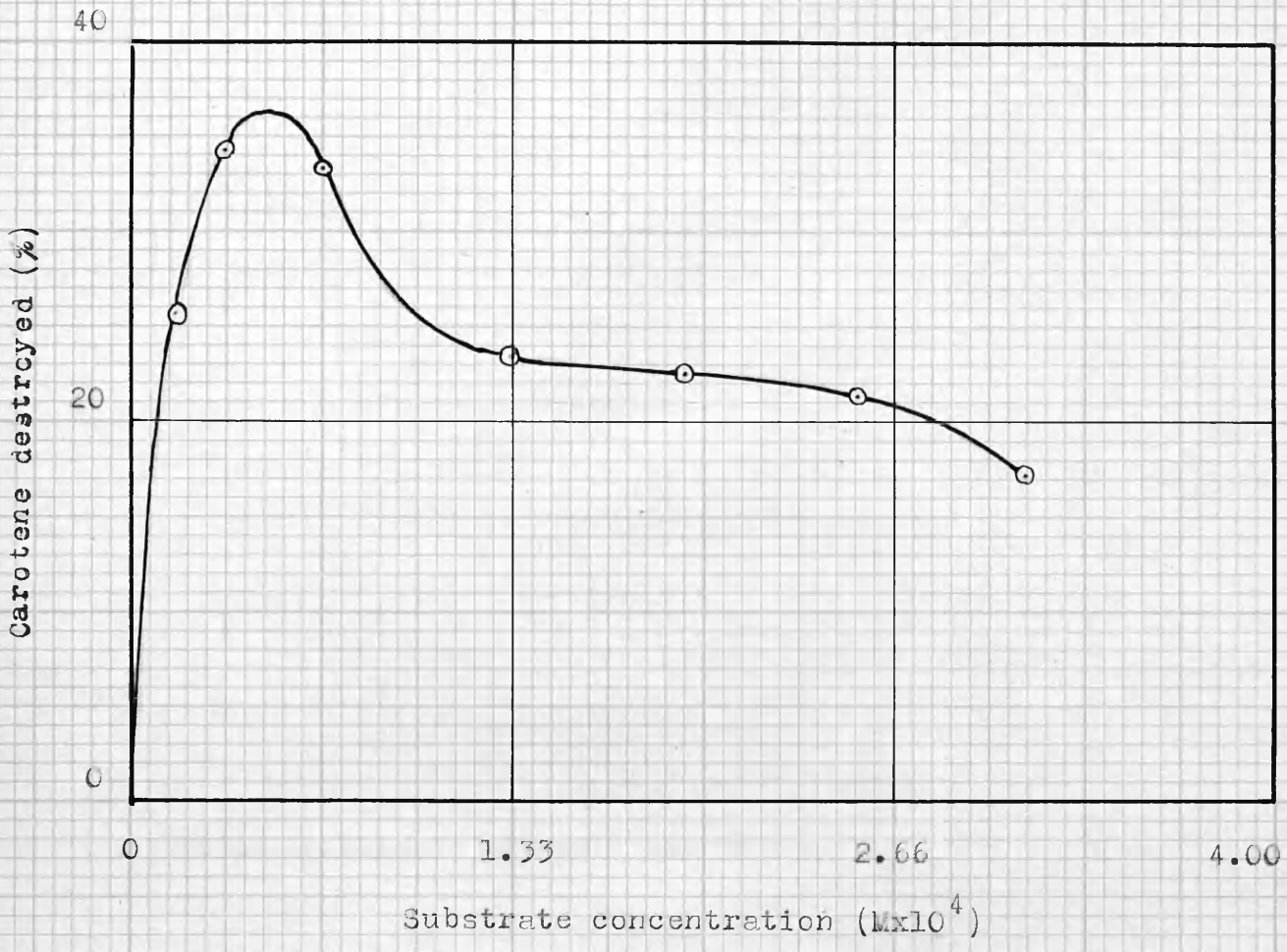


Fig. 16

Substrate concentration curve for the destruction of carotene by catalase at pH 3.8.

optimum in this region is in good agreement with the findings of Banks (14) that haem catalysis is most effective in a heterogeneous system and at low pH values. Reiser (16), working over the range pH 8.0 to 5.5 concluded that haem catalysis of carotene destruction was more effective at lower pH values.

Experimental:-

4. The Substrate Relationships of Catalase.

Fig. 16 shows the substrate concentration curve for catalase, which again presents similar features to that for lipoxidase as shown in Fig. 5. The data were obtained at pH 3.6 and a catalase concentration of 0.2 p.p.m. The shape of this curve appears to be affected by the initial degree of peroxidation of the substrate. In results not reproduced here, it has been observed that when the substrate is new and contains just enough preformed peroxide to permit the reaction to proceed very slowly a rather irregular curve is obtained with the reaction falling away to zero at a substrate concentration of about 2.5×10^{-4} M. The explanation for this falling off in activity at high levels of substrate is not known.

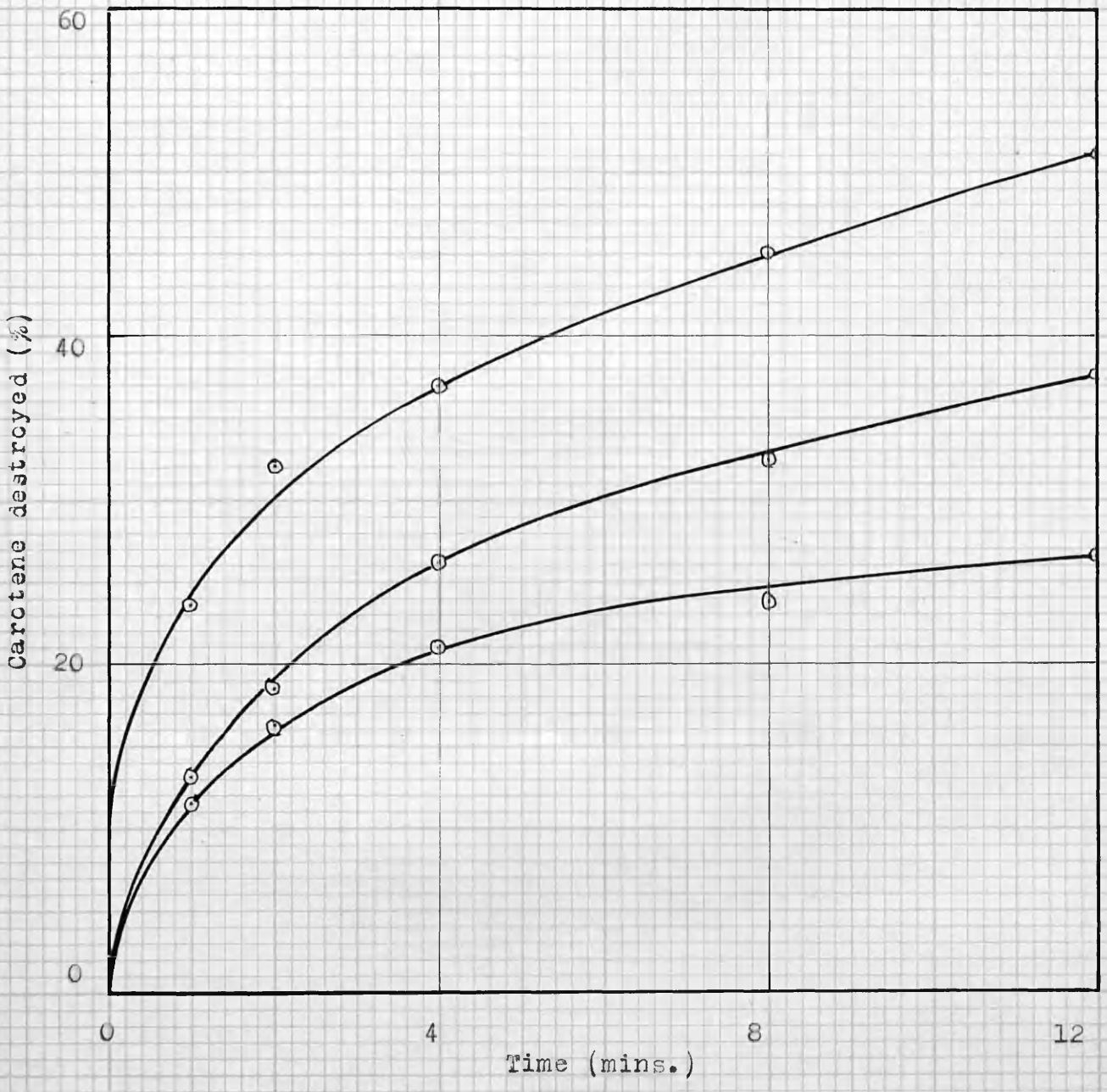


Fig. 17

Carotene destruction/time curves at pH 3.8 with substrate containing variable quantities of preformed peroxide.

Catalase concentration: 6.2 p.p.m.

Experimental:-

5. The Effect of Time on Catalase-Induced Carotene Destruction

The catalase carotene-destruction/time curve is shown in Fig. 17. The three curves, which were obtained with substrates of different ages and stored under different conditions, clearly indicate the influence of auto-oxidation of substrate on the reaction. The upper curve was obtained with a substrate solution which had been stored for three months at 4^{o} in a stoppered bottle. The middle curve was derived from a substrate prepared under nitrogen and then exposed to air for five days before use. The lower curve was observed on a substrate ten weeks old, after storage at a higher concentration than the previous substrates in a bottle with a very large air-space. Since these latter conditions would tend to encourage polymerisation of peroxides, the low activity of this solution would be expected.

These results are quite different from the corresponding lipoxidase curve shown in Fig. 9, Appendix II. As against the rapid initial reaction and

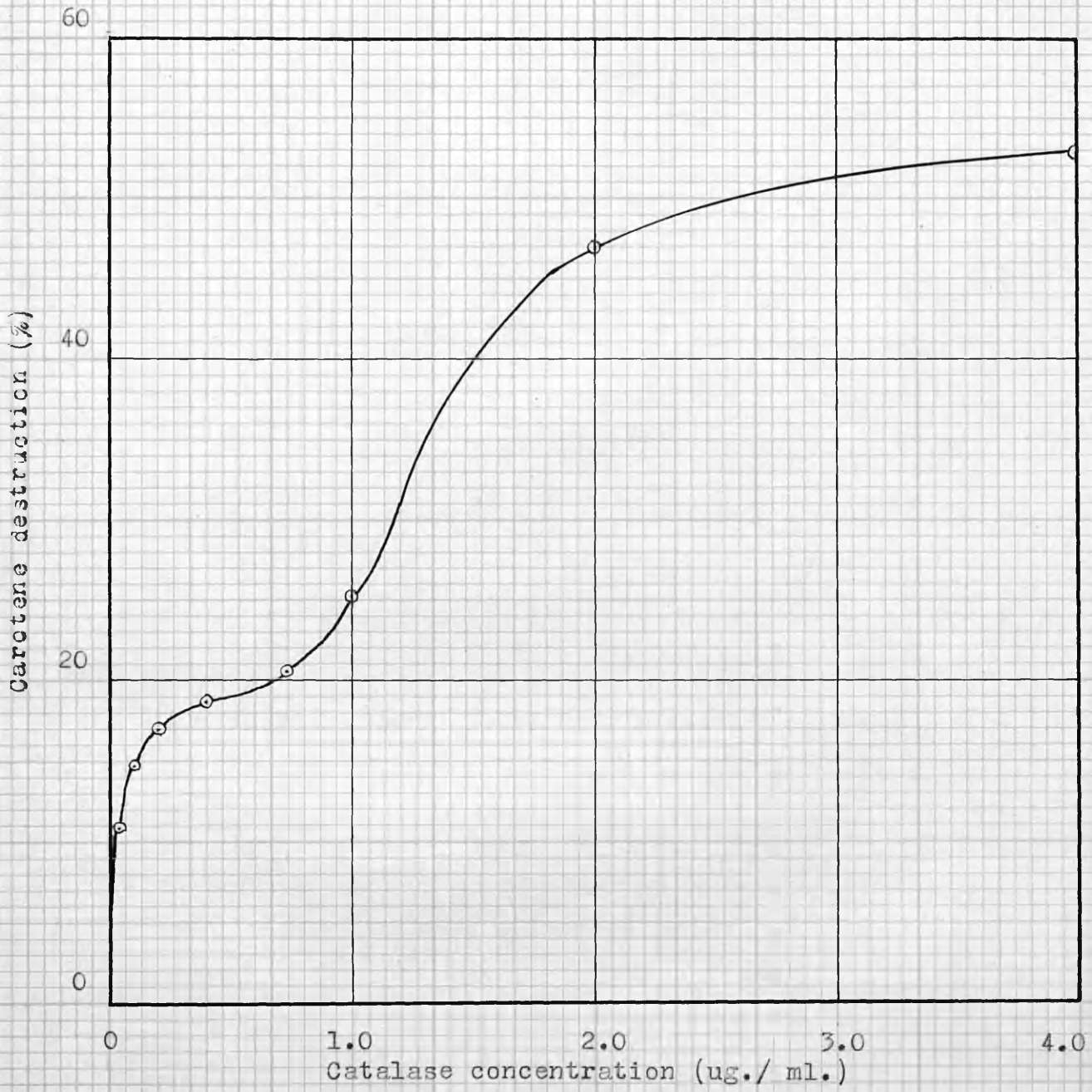


Fig. 18

The relationship between carotene destruction and catalase concentration in a system containing preformed fatty peroxides

subsequent decline in reaction rate shown by the catalase system, the lipoxidase reaction rate only falls off slightly during the course of the reactions.

Experimental:-

Destruction

6. The Effect of Catalase Concentration on Carotene

Fig. 18 illustrates the complexity of the relationship between catalase concentration and carotene destruction. The data were obtained at pH 3.8 and a linoleic acid concentration of 3.7 mg.% (approximately 1.3×10^{-4}M). The reaction time was one minute. The effectiveness of catalase in destroying carotene, considered in terms of carotene destroyed per unit concentration of catalase is very high at low catalase concentrations. Table IV, which is calculated from the data on which Fig. 18 is based, illustrates this point.

Column I indicates the catalase concentration in the reaction system in γ per ml., while column II shows the carotene destroyed (γ) in the first minute of the reaction for each γ of catalase present in each ml.

of the solution.

I Catalase Conc.	II Carotene Destroyed
0.05	64.8
0.10	43.5
0.50	11.4
1.00	7.5

TABLE IV

The significance of these figures is almost certainly of importance in relation to the development of rancidity in stored foodstuffs. It will be discussed again in Section III.

Experimental:-

7. Cyanide Inhibition of Carotene Destruction by Catalase.

Using oxygen uptake to follow the progress of the reaction, Robinson (67) found that cyanide did not inhibit haem catalysis of unsaturated-fat oxidation except at very high concentrations. Barron and Lyman (98)

came to a similar conclusion although they found that haemochromogens were inhibited by cyanide. Watts and Peng (76) found that the catalytic effects of pork muscle extracts in fat peroxidation were not affected by 0.01 M cyanide at pH 5.3. Reiser (16) found that 0.01M cyanide had no effect on the carotene bleaching activity of haemoglobin solutions.

As will be recalled, in the lipoxidase carotene bleaching system of Appendix II, the reaction was stopped at the required time by the addition of 2 ml. of a 20% caustic soda solution. During the exploratory work on catalase and other haem compounds it was found that this addition did not effectively stop the reaction although it proceeded very slowly thereafter. No such drift was observed with soya lipoxidase solutions, but a drift was observed with lipoxidase assays on wheat germ extracts (57) which are rich in cytochrome and catalase. When 20% caustic soda was replaced by 10% caustic containing 5% potassium cyanide no further trouble with drifting was encountered. Lipoxidase is not inhibited by cyanide (26).

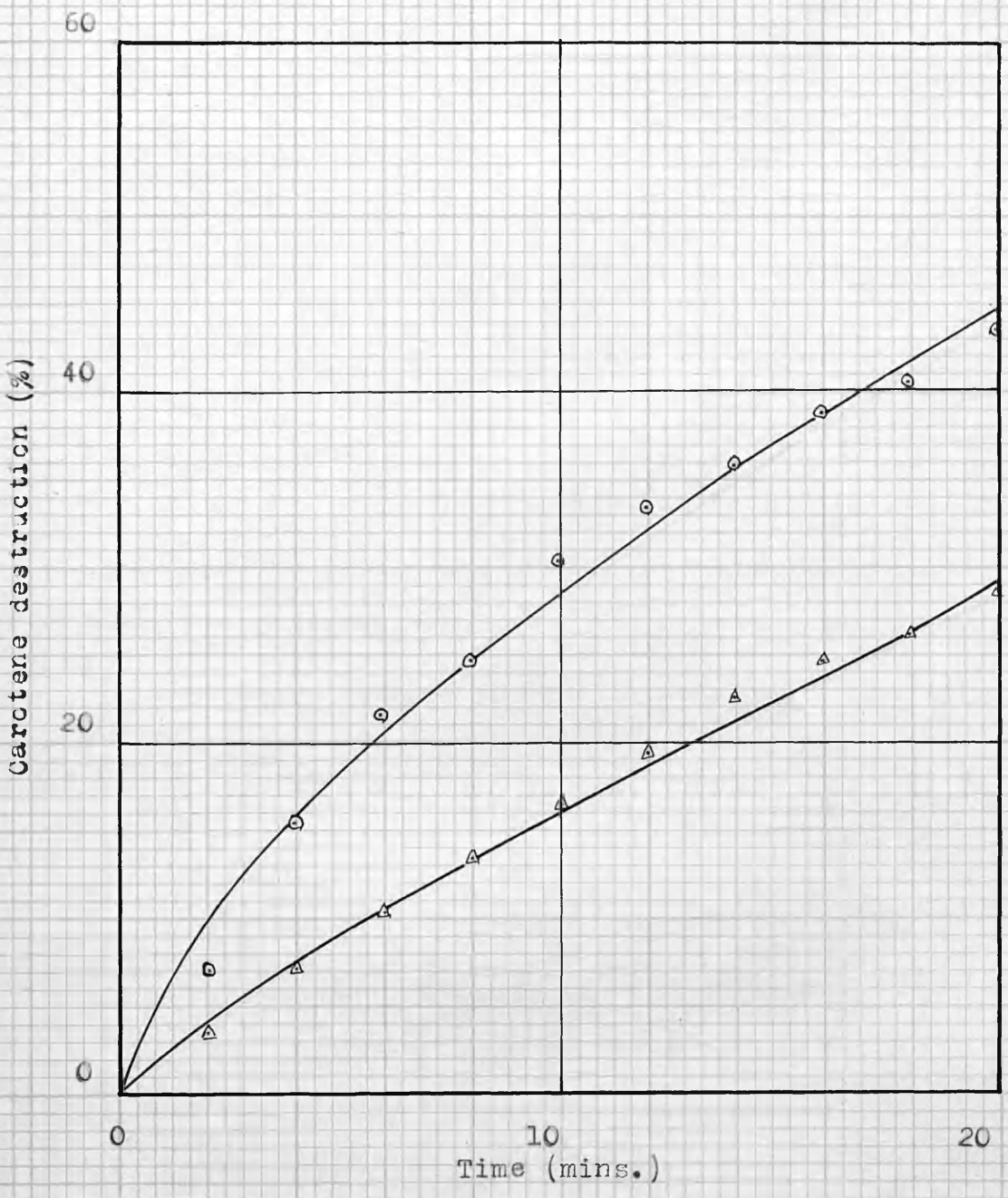


Fig. 19

Cyanide inhibition of catalase-induced carotene bleaching.

In Fig. 19 the effect of 2 x 10^{-4}M cyanide on the catalase system at pH 7.8 is shown. The catalase concentration was 10^{-8}M and the upper curve shows the progress of this reaction in the absence of cyanide, the marked reduction in the lower curve being due to the presence of 2 x 10^{-4}M cyanide.

Data were also obtained at pH 3.6, where a 21% inhibition was recorded at the same cyanide level as against the 35% of Fig. 19. Owing to the low buffer strengths employed in the system the cyanide concentration could not be increased beyond 5 x 10^{-4}M without danger of altering the pH, but this level at pH 3.6 gave a 26% inhibition. Compared to the concentration of catalase these cyanide concentrations are enormous, and it is unlikely that this cyanide effect is that of a specific enzyme poison. It is interesting to note that the cyanide effect is smaller in acid solution where the catalase bleaching effect is greatest.

Experimental:-

8. The Effect of Catalase on Lipoxidase Systems

Apart from Holman's work on germinating soya-beans, the only previous observations on the behaviour of fatty systems containing both lipoxidase and haem were made by Kies (51) and Tappel (19), and both investigators used oxygen uptake as a measure of the course of the reaction. Kies found that crystalline bovine haemin and cytochrome inhibit lipoxidase completely whereas catalase, peroxidase and haemoglobin do not. The reaction conditions and relative concentrations are not stated. Tappel's data were obtained with a view to distinguishing between haem and lipoxidase catalysis, and the few figures which he records for mixtures of the two catalysts suggest that, with a homogeneous linoleate substrate, lipoxidase is a more effective catalyst in presence of haemin or bacon adipose tissue extracts.

Table V summarizes the results obtained from two series of observations made on the behaviour of mixtures of catalase and lipoxidase in carotene bleaching.

Catalase Concentration (p.p.m.)	Inhibition (%)	
	I	II
0.005	18	-
0.015	5	14
0.050	16	-12
0.150	-40	-22

TABLE V

The concentration of lipoxidase in the reaction system was constant throughout and the reaction was carried out at the lipoxidase optimum at pH 5. In this region, as reference to Fig. 15 shows, catalase, although not at its optimum pH, is still capable of effectively bleaching carotene.

The inhibition of the reaction by catalase is expressed as a percentage of the lipoxidase bleach obtained in the absence of catalase, and a negative inhibition means that a greater bleach was obtained with the mixture than with lipoxidase alone. In no case did the bleach obtained with a mixture equal the sum of the

separate bleaching effects of the two enzymes when acting on the system alone. For example, in one experiment lipoxidase alone gave a 32% bleach while 0.15 p.p.m. catalase gave an 18% bleach. A mixture of the two gave 41% bleach. Under the various conditions tried there was always some inhibition of the overall reaction in this fashion.

Although not entirely consistent, (a 1% error of observation appears as a 4% error in the inhibition columns) the table shows clear evidence of interaction of the two systems even under conditions of very low levels of catalase. The data recorded here are too limited to do more than suggest a field of investigation, but taken in conjunction with the work of Holman on germinating soya-beans and Chance's observation of a non-dissociable catalase linoleate-peroxide complex in the presence of lipoxidase, the phenomena described may well be of fundamental importance.

Of practical importance in connection with the present problem is the inhibition of lipoxidase by small

concentrations of catalase. Some, at least of the effects reported in Table II on inhibition of lipoxidase with flour extracts must have been due to the presence of catalase in these extracts since their pattern of behaviour was very similar to that of the lipoxidase catalase mixtures described here.

Experimental:-

9. Methods of Distinguishing Haem Proteins and Lipoxidase in Unsaturated-Fat Oxidase Systems.

In early reports of lipoxidase activity in various plant tissues, no attempts were made to distinguish between lipoxidase and haem catalysed oxidations. Since these tissues would certainly contain both catalase and peroxidase, it is difficult to say with certainty whether lipoxidase occurs in plant tissues other than those of the soya-bean. Much of the work on the occurence of lipoxidase in plant tissues might well be re-examined in the light of present knowledge of haem oxidations. It therefore seems appropriate to summarize the means of distinguishing between the two types of catalysts.

When one or the other system is present no real difficulty exists, but mixtures of the two in evenly balanced proportions such as might be found in many plant tissues could give rise to difficulty. For example, Blain (57) finds marked lipoxidase activity in wheat germ, but this material is a relatively rich source of catalase and is also known to contain cytochrome and peroxidase. The behaviour of wheat germ in the carotene bleaching system of Appendix II is intermediate with that of haem and lipoxidase and Blain concluded that both systems are active in his preparations.

Using oxygen absorption measurements, Kies (51) found that haemin activity was completely inactivated by 10% acetone while lipoxidase activity was not. Banks (14) found that haem proteins were ineffective catalysts in homogeneous systems and this observation was developed by Tappel (19, 20) as a basis of distinction between lipoxidase and haem catalysis in animal tissues. In these papers Banks and Tappel also reported that pre-formed peroxide is necessary for haem catalysis. The carotene bleaching system described here is

particularly sensitive to this effect and can readily be used to distinguish between the two systems, providing they are not simultaneously present.

No single test is sufficient for mixtures of the two enzymes. Comparison of carotene bleaching curves recorded in Fig. 15 to 19 for catalase and Fig. 2, 7, 9 and 10 of Appendix II for lipoxidase suggest that pH/activity curves and carotene-destruction/time curves offer other means of distinction. High activity at pH 3.7 for haem catalysis where lipoxidase is almost inactive, as well as the shape of the carotene-destruction/time curve all offer possibilities of distinction. In addition, haem catalysis will proceed slowly after the addition of 1 ml. 10% caustic soda solution to the carotene assay system while lipoxidase activity is arrested under these conditions. Taking account of all these differences the carotene bleaching system described offers excellent means for distinguishing between the two catalysts, and for detecting their presence in mixtures. The differences are summarized below.

Test	Lipoxidase	Catalase
Reaction system pH 3.8	slight activity	very active
Peroxide-free substrate pH 5.0	very active	inactive
System + 1 ml. 10%NaOH	inactive	detectable activity
Carotene-destruction/ time curve	steady rate	rapid initial rate.

Although these suggestions refer only to catalase, Blain (57) has found similar behaviour with cytochrome, and it seems likely from the observations of Banks and Tappel that similar considerations would apply to other haem proteins.

SECTION III

THE UNSATURATED-FAT OXIDASES IN RELATION TO THE BLEACHING AND IMPROVING OF WHEAT FLOUR.

SECTION III

THE UNSATURATED-FAT OXIDASES IN RELATION TO THE BLEACHING AND IMPROVING OF WHEAT FLOUR

Preliminary Considerations

Bread of generally acceptable quality cannot be economically baked without the use of bleaching agents to remove the carotenoid tint of raw flour, and improving agents to impart strength to the wheat proteins. In order to rise well in baking and to give a light loaf of large volume and good texture, flour proteins must have certain elastic properties known to millers and bakers as "strength". According to the behaviour of their proteins, flours are classified as "weak" or "strong", although bakers and millers use the terms "winters" and "springs" in a synonomous fashion, presumably through associating weak flours with winter-sown wheat and strong flours

with spring-sown wheat. While different wheat varieties differ in the baking characteristics of their flours, the climatic condition under which the wheat is grown is the most important factor in determining the strength of the flour (99). In round figures Great Britain produces about two million tons of wheat per annum, and imports about five and a half million tons of which the principal supplier is Canada, followed by Australia (99). Canadian wheats are predominantly strong and very suitable for bread-making, while British and Australian wheats are weak and more suited to the manufacture of other cereal products.

In practice, bread flours in this country are milled from mixed grists containing a fairly high proportion of Canadian wheat, blended with varying percentages of British, Australian and sometimes other wheats. From the point of view of national economics such a procedure is a necessity, but the flours resulting from the milling of such grists

are seldom of sufficient strength to give loaves of large volume and good texture without the use of a chemical improving agent. The difficulties are made more acute in mechanised bread factories where processing conditions are less flexible and less easily adjusted to variations in the flour baking quality. A skilled craftsman baker, working on a small scale without mechanised processing, can bake good bread from rather inferior and weak flour by the use of extended fermentation procedures and manipulative measures which are not practicable on a larger scale. Baking in this fashion is no solution to the problem, since even if a sufficient number of skilled craftsmen were available, the labour and other costs would be so high as to raise the price of bread in a marked degree.

As the quotation at the beginning of this thesis indicates, the sophistication of flour is of long standing. The desired effects may be produced by a wide range of chemical additives, but in practice only

Treatment	Level Used (p.p.m.)	Bleaches.	Improves
Nitrogen trichloride	60	+	+
Chlorine dioxide	30	+	+
Benzoyl peroxide	15	+	-
Potassium bromate	20	-	+
Ascorbic acid	20	-	+
Ammonium persulphate	160	-	+

TABLE VI

a few of the possible substances are actually used. Most of these substances are oxidising agents, and it is found that fat-soluble oxidising agents bring about bleaching of the flour pigments, while water-soluble agents bring about improvement (114). Agene and chlorine dioxide are soluble in both fat and water and thus have the advantage of acting both as bleachers and improvers.

Table VI, adapted from a paper by Coppock (100), lists some examples of commonly used bleaching and improving agents and gives an indication of the level of concentration at which they are effective. Ascorbic acid is an apparent exception to the rule that improving agents are also oxidising agents, but it has been shown that this substance is rapidly reduced to dehydroascorbic acid in bread doughs and that the reduced material is the active improver (100).

The work of Sir Edward Mellanby (101, 102, 103)

to which reference was made in the preface has focussed public attention on possible dangers attendant on the use of such substances. Mellanby fed dogs on Agenized bread and the animals developed canine hysteria. The toxic agent was isolated by Bentley et al. (103, 104, 111, 112) and found to be methionine sulphoximine. Rabbits, dogs, ferrets, mice, rats and monkeys have all been found to be affected by this toxin. In ordinary commercial flour about 2 p.p.m. of methionine sulphoximine are present. It can thus be calculated that in the course of a year a man consumes about 2 mg. per kg. of body-weight. By comparison, a monkey requires 200 times this amount to produce toxic symptoms, and this amount must be administered in a single dose. In 1948, Pollock (106) and Erickson and Gibson (105) reported that man is unaffected by Agenized flour, but from a recent report by Sheldon and Yorke (107), it now appears that sensitive individuals do develop toxic symptoms traceable beyond any doubt to bread or other flour products which have been treated by Agene

or chlorine dioxide.

This very brief account of the present position is perhaps sufficient to indicate that Agene and chlorine dioxide must be regarded as being, at best, substances under suspicion on grounds of public health. It is interesting to note that chlorine dioxide is a permitted flour additive in the United States, while Agene is not permitted in either Germany or the United States.

A suitable alternative to the Agene process has been difficult to find. Ascorbic acid would appear to be free from serious objections but even if it were to be produced on a sufficiently large scale, its manufacture would be a heavy national cost. In addition, it has no bleaching effect, and bread prepared by its use is cream-coloured. Objections of one sort and another may be raised in connection with the other chemicals listed and it is not

satisfactory to move from a known evil to an unknown and perhaps greater evil.

For these reasons, a "batter" or "aeration" process of breadbaking, which can be used with untreated flour, has attracted considerable interest. In this process, which was invented by Rank and Hay (108), half of the required flour is added to all the water normally used in doughing, and the resulting slurry beaten at high speed for five to eight minutes in a specially designed machine. After high-speed mixing, the remainder of the flour is added to the batter and mixed at slow speed to produce a dough which is thereafter processed in a conventional fashion.

During high-speed mixing, changes take place which result in strengthening of the flour gluten and partial bleaching of the carotenoid pigments. In most flours the bleach can be noticeably increased by the addition of a small quantity (about 0.1%) of

unprocessed soya-flour to the mix before treating in the high-speed machine. The "chemical-free" bread produced in this fashion is equal in quality to that made from Agene-treated flour, but the machinery is costly to install and operate. Thus, the enforcement of this process would limit bread manufacture to large-scale producers.

The batter process was discovered and developed on an ad hoc basis. The bleach was ascribed to the action of soya and wheat lipoxidases, while improvement was variously believed to be due to "work strengthening" of the gluten or to the action of oxidising enzymes. The possibility of improvement being brought about by some oxygen transfer mechanism through the action of lipoxidase was not excluded.

The work described in the previous Sections of this thesis was carried out with a view to making a study of the reactions involved in this process and the conditions under which they might be most effectively

carried out. Arising from this work, a simplified process which gives a better bleach and an equal degree of improvement is suggested.(109).

In the remainder of this Section, a shortened account of the experimental results is given with a view to relating these to the work on carotenoid bleaching already described.

The Approach to the Problem

Consideration of the batter process of Rank and Hay led to the conclusion that the primary effects of the high-speed machine and method of mixing were air incorporation and the performance of work on the thin flour-water mixture previously mentioned. The work performed on this mixture is considerable, the temperature rising at the rate of 1°F. per minute during operation, from the internal friction developed.

The incorporation of air would be a pre-requisite were lipoxidase bleaching involved. The

oxygen relationships of lipoxidase are briefly discussed in Appendix II, p. 548, and the solubility of oxygen in the reaction system has been implicit in all the work described previously in this thesis. Due to the presence of glutathione in wheat germ, doughs are reducing systems, and without some form of active oxygenation a lipoxidase system would not be free to react.

In planning experimental work, the problem was resolved as follows:-

1. Evidence was required as to whether the unsaturated-fat oxidases were involved in the observed bleaching effect.

2. Evidence was required as to whether the work performed on the batter was related to the improvement.

3. In the event of it being shown that improvement was not related to expenditure of work on the batter,

evidence on the effects of incorporation of air into doughs would be obtained.

It was clear at the outset that a number of explanations for the observed effects were possible. The suggestion that the expenditure of work on the flour proteins might change their physical properties, in a similar fashion to the effect of work on metals, had its origin in the well-known fact that the rheological properties of doughs are affected by work done on the dough, although in previously observed cases the dough recovered its original properties after a rest period. Without discussing other possible theories of the batter process effect, it was also clear that experiments must be carried out on the actual process, and for this purpose a standard test-baking procedure would be required. The procedure adopted is described in Appendix IV.

In order to determine whether atmospheric oxygen was involved in the improving effect, it was

desirable that some study of the oxygen relationships of wheat flour should be made. A polarographic technique was evolved for this purpose by Hawthorn and Young.

Finally, a rapid method for the determination of carotenoid pigments in flour and bread was required. A suitable method was developed by Styles (113) and is described in Appendix III.

Experimental:-

1. The Distribution of Unsaturated-Fat Oxidases in the Wheat Berry

Lipoxidase activity has been reported in wheat and wheat flour by several investigators. Sumner (37) reported the presence of lipoxidase in wheat germ. Miller and Kummerow (115) studied the fate of wheat lipoxidase during the baking of bread and found that the enzyme was completely destroyed during the baking process. Irvine and Winkler (116, 117) ascribed losses of pigment in macaroni doughs to lipoxidase action, and Irvine and Anderson (41) recently carried out kinetic studies on the lipoxidase system of wheat. This last paper forms the most complete account of lipoxidase activity in wheat so far published. A manometric procedure was used in conjunction with a linoleic acid substrate and the characteristics of the system were evaluated at different levels of oxygen tension, enzyme concentration, substrate concentration, temperature and pH. On the basis of these data, an assay method for wheat lipoxidase was proposed. Using this method, the

activity of a range of wheats was investigated (119) and results varying from 12 to 40 units per gram were obtained.

The system described by Irvine and Anderson appears to be flexible and sensitive. They unfortunately found it necessary to use an emulsion of linoleic acid which was stabilised with a surface-active agent of commercial origin. Although they claim that this agent has no effect on their system, they offer no supporting evidence for this. The dangers of basing kinetic data on systems containing surface-active agents have already been pointed out in this thesis, and the figures they quote for the Michaelis constant of their reaction as well as their figures for activation energy are subject to this criticism.

Moreover, the reported data were obtained using a crude substrate containing 60% linoleic acid. The competitive effect of substrate impurities has already been illustrated in this work in Fig. 5. On the other hand, their substrate concentration curves as

published give no indications of competitive inhibition, and the occurrence of this phenomenon will depend on the nature of the linoleic acid impurities. In support of their use of a crude linoleic acid substrate, they used a sample of pure linoleic acid for reference purposes. In this connection they report that the pure acid showed an induction period while the crude material showed little evidence of this.

In the four papers mentioned Irvine and Anderson have assumed that the enzyme responsible for the phenomena observed was lipoxidase. They make no reference to the possibility of haem compounds being concerned in the reactions. The observation of this induction period with presumably peroxide-free linoleic acid (the acid was supplied by the Hormel Foundation who specialise in the production of this material in a highly purified form), coupled with their previous observation of marked inhibition with 0.001 M cyanide, strongly suggests that haem catalysis is also involved.

Sample	H_2O_2 destruction. Reaction Velocity Constant.	Relative Activity.
Purified Wheat Endosperm	0.0070	1
Bran (Spring Flour)	0.0320	4.6
Bran (Winter Flour)	0.0294	4.2
Purified Wheat Germ	0.7200	101
Commercial Wheat Germ	0.5800	83
Winter Flour (80% Extraction)		
Sample a.	0.0190	2.7
b.	0.0152	2.2
Blended Flour (80% Extraction)		
Sample a.	0.0175	2.5
b.	0.0127	1.8
Spring Flour (80% Extraction)	0.0175	2.5
Defatted Raw Soya Meal		
Sample a.	0.1300	18.6
b.	0.1500	21.5

TABLE VII.

To date, the most systematic survey of unsaturated-fat oxidase activity in the wheat berry has been carried out by Blain (57) who has used novel electrostatic methods of separation to obtain very highly purified samples of wheat germ, bran and endosperm. He tentatively finds the activity in the germ to be of the order of one twentieth that of unprocessed soya-meal, the endosperm to contain about one fiftieth of the activity of the germ, while the bran, although somewhat more active than the endosperm, is of low activity. He takes the provisional view that the observed unsaturated-fat oxidase activity in wheat is partly due to true lipoxidase and partly to haem catalysis.

So far as haem catalysis by wheat flour is concerned, the writer has carried out a survey of catalase distribution in the wheat berry using purified milling fractions supplied by Blain. The catalase was determined directly by von Euler and Josephson's method (96) and the results are shown in Table VII along with figures for commercial flours and soya. For convenience in making comparisons, the relative

Catalase added (µg/ml. of aqueous phase)	Pigment destruction (%)
None	56
1.2	58
6.2	65
12.4	66

Lipoxidase added (mg. soya-meal/ml. aqueous phase)	Pigment destruction (%)
None	45
1.3	46
2.6	51
6.5	53

TABLE VIII.

The effect of additions of catalase and lipoxidase to batter process doughs.

activities of the various materials in the table are shown in the third column calculated on a basis of unit activity for endosperm. Blain (120) reports a very similar distribution of unsaturated-fat oxidase activity in the same samples. It is not suggested from this that the apparent lipoxidase activity of wheat fractions is entirely due to catalase, but it is believed to be unlikely that the whole of the unsaturated-fat oxidase activity of wheat can be ascribed to a true lipoxidase.

In this connection the effect of added lipoxidase and catalase to the high-speed mixer of the batter process is of interest. The results shown in Table VIII were obtained under the standard conditions of baking described in Appendix IV, while the method of carotenoid analysis is that of Appendix III. All results are calculated to a moisture-free basis and expressed as a percentage of the pigment originally present in the flour. The same procedures were used in the other baking tests reported in this section.

The two tests were carried out on different flours and the pigment destruction in the absence of added enzyme is therefore not the same in both cases. These tests clearly establish that both lipoxidase and catalase are capable of carotenoid destruction under the conditions of the batter process, the flour fat acting as a substrate for the enzymes.

The bleach obtained in the absence of added enzyme is comparatively large, and it is of interest that increasingly large additions of enzyme give diminishing returns in the form of pigment destroyed. As the table shows, a tenfold increase in catalase added gives only a fivefold increase in carotene destroyed, and similar considerations apply to lipoxidase. This behaviour is very similar to that already reported for catalase (page 83).

Before leaving this consideration of the occurrence of unsaturated-fat oxidases in the wheat berry, it might be mentioned that their appearance

in flour is, within limits, in the control of the miller, who can adjust his procedures to include a greater or lesser proportion of the germ.

Experimental:-

2. The Effect of Hydrogen Peroxide and Antioxidants on Unsaturated-Fat Oxidase Activity

From the behaviour of unsaturated-fat oxidase systems in the laboratory, it became clear early in this work that these systems required an abundant supply of oxygen for their proper functioning. Bread doughs are characteristically reducing systems, having rH values of about 15-19 (121), and the intimate introduction of molecular oxygen into large cohesive masses of dough presents difficulties. Thinking to take advantage of the presence of catalase in flour, a number of experiments were carried out in which hydrogen peroxide was added to doughs. For the purposes of comparison studies were also made on the effect of hydrogen peroxide on lipoxidase activity using the Appendix II system.

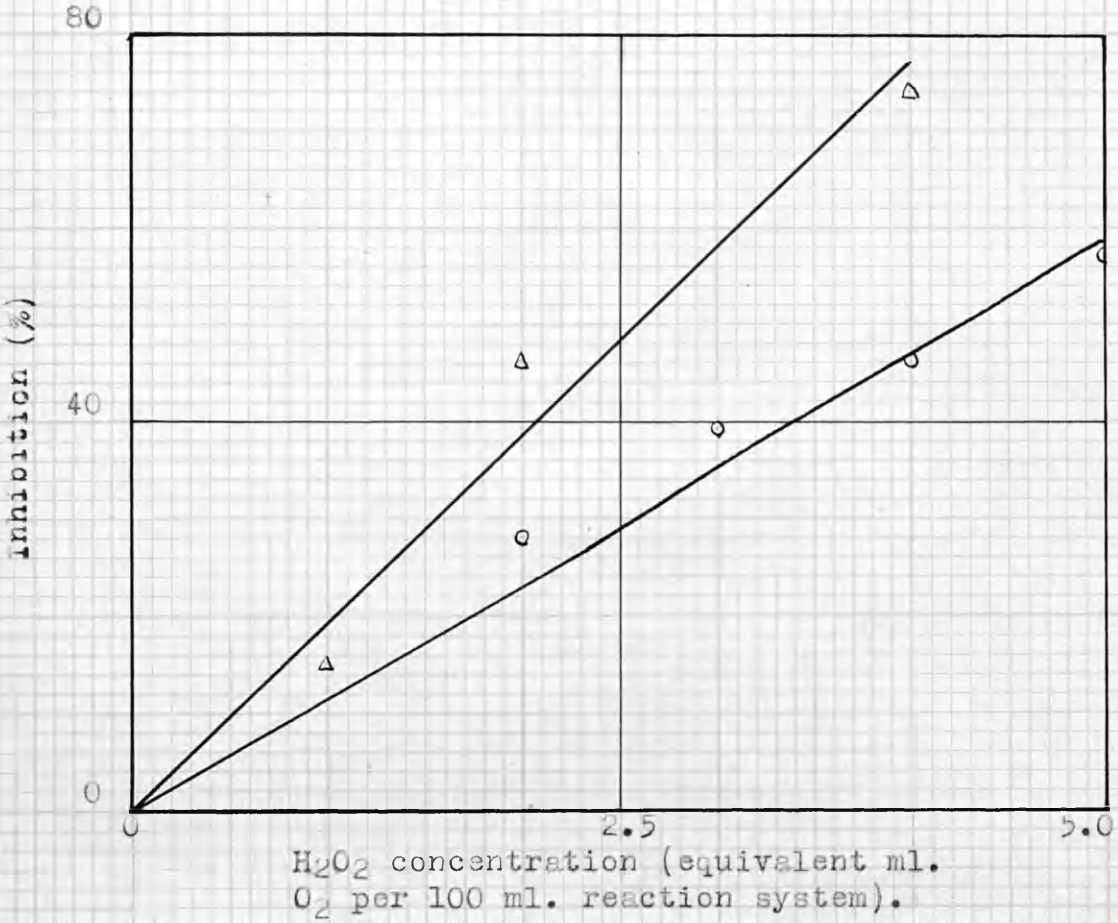


Fig. 20

Inhibition of lipoxidase by hydrogen peroxide as measured by

upper curve: carotene bleaching

lower curve: diene conjugation.

Flour added to hydrogen peroxide readily decomposes it with visible evolution of oxygen. Elion (122) has used a manometric method to determine catalase activity in flour, and he found that small quantities of reducing agents greatly retarded the decomposition of hydrogen peroxide. It can be calculated from the data given in Table VII that the catalase activity to be expected in 1 g. of a normal bread flour is equivalent to about 0.5×10^{-6} g. of pure crystalline ox-liver catalase of Kat. f. value 32,000. In other words, flour can be considered to contain about 0.5 p.p.m. of catalase.

Fig. 20 shows the inhibition of the lipoxidase action of crude soya extracts in presence of varying levels of hydrogen peroxide as measured in the reaction system of Appendix II. Inhibition of both diene conjugation and carotene bleaching was observed. When hydrogen peroxide in similar levels in the aqueous phase was added to bread doughs prepared by conventional procedures, inhibition of bleach was

observed in a similar fashion at higher levels of peroxide. In the following table the results of this baking test are shown. The hydrogen peroxide additions are expressed as equivalent ml. of oxygen at N.T.P. per hundred ml. water in the dough.

H_2O_2 Added (ml. Avail. O_2)	Pigment Destruction (%)
None	26
2.7	35
5.4	40
8.0	32

TABLE IX.

Table IX and Fig. 20 taken together suggest that in doughs hydrogen peroxide is decomposed to a considerable extent and thus provides some oxygen for unsaturated-fat oxidase action. At higher levels of addition sufficient unchanged hydrogen peroxide is present to inhibit unsaturated-fat oxidases. The fact that hydrogen peroxide inhibition of bleach is observed in doughs to

which no unsaturated-fat oxidase has been added, does suggest that the bleaching of such doughs is due to inherent unsaturated-fat oxidase activity in the flour.

It may be felt that the fact that unsaturated-fat oxidases are known to be present in flour is sufficient to explain why the bleach obtained by the batter process is significant in the absence of added enzyme. However, this bleach is seldom less than 45% of the total pigment present, and the addition of even massive doses of lipoxidase or catalase rarely increases the figure above 60%. In view of the comparatively low activity obtained by direct assay by Blain, the possibility that some other system may be involved is not ruled out.

Direct evidence to the contrary is found in the behaviour of batter process doughs in the presence of chain-breaking antioxidants. Table X shows the effect of nordihydroguairetic acid (N.D.G.A.) and

Process	N.D.G.A.added (mg.%)	Pigment Destruction (%)
Batter	None	55
"	26	46
"	65	27
"	130	25
"	260	15

Process	∝ -tocopherol added (mg.%)	Pigment Destruction (%)
Conventional	None	33
Batter	None	47
"	58	37
"	232	36

TABLE X.

The effect of N.D.G.A. and ∝-tocopherol acetate on the batter process.

α-tocopherol acetate on the bleaching of carotene in this process. The concentrations of antioxidant are expressed as mg. % in the aqueous phase of the dough in conformity with the previous table. There is no basic justification for expressing the concentrations of these additions in this way, but it is impossible to know the concentration of the antioxidant in the fat itself while the experiment is proceeding.

The fact that the addition of a chain-breaking antioxidant such as N.D.G.A. gave such a high degree of inhibition in spite of the difficulties of its incorporation in the dough, clearly confirms that carotene destruction is coupled with fat oxidation. Since the time intervals are short, the reaction must be catalysed in some fashion by a lipoxidase-like system.

As is well known, wheat germ oil is a rich source of α-tocopherol, and of course, the natural fat in the flour is mainly derived from the germ. It would therefore be expected that flour fat would not be a very

good substrate for the unsaturated-fat oxidases. Nevertheless, as the lower half of Table X shows, relatively massive additions of α-tocopherol were required before much inhibition of bleaching occurred. The relative size of the additions is indicated by the fact that the α-tocopherol content of whole wheat is of the order of 5 mg.% (123), and since it originates in the germ which is partially removed by milling, the concentration in flour will be lower. The possible argument that the tocopherol content of the flour is sufficient to seriously inhibit unsaturated-fat oxidase action is thus disposed of.

On the other hand, there can be little doubt that the vitamin E content of bread will be adversely affected by the batter process. It is interesting to note that in 1945, the use of nitrogen trichloride was discontinued in Germany on the grounds that about 80% of the vitamin E present in the flour is destroyed by this treatment (124). In a private communication, Hay has informed the writer that about 35% of the

vitamin E present is destroyed by the batter process.

The behaviour of haem proteins and lipoxidase in linoleate systems containing α-tocopherol or N.D.G.A. has been described by a number of investigators, the most recent and extensive studies being those of Tappel and his co-workers (77, 50). Both of these substances were found to inhibit unsaturated-fat oxidase action, although so far as soya bean lipoxidase is concerned, N.D.G.A. was found to be an effective inhibitor at one-tenth of the concentration required for effective inhibition by α-tocopherol. Thus the behaviour of the test-baking system is in broad conformity with published data on the in vitro behaviour of lipoxidase and haem systems.

From the evidence cited it can be concluded that the bleaching observed in the batter process is due to the action of the unsaturated-fat oxidases.

Experimental:-

3. The Oxygen Relationships of the Unsaturated-Fat Oxidases in Flour and Bread Doughs.

Thus far the behaviour of the unsaturated-fat oxidases in doughs has been considered from the point of view of bleaching carotenoid pigments. The relatively anaerobic conditions prevailing in bread doughs, as evidenced, for example, by their rH values, give rise to the question of whether the degree of bleach is limited by oxygen availability. In experiments not detailed here, it has been shown that in the batter process the degree of bleach increases progressively with mixing time in the high-speed machine.

The phenomena involved are more complex than would appear from this type of experiment. As bleaching progresses, the dough is simultaneously improved, and this process also requires oxygen. Baker and Mize (126, 127) and Freilich and Frey (128, 129, 130) have observed that bread doughs are improved

by mixing in an atmosphere of oxygen, the degree of improvement being generally less on strong flours. Smith and Andrews (39), in rheological studies of doughs using the Brabender Extensograph, found that doughs mixed in an atmosphere of oxygen developed greater resistance to deforming forces, and at the same time lost extensibility.

Direct evidence that oxygen reacts with some factor present in flour has been obtained by Hawthorn and Young (44). In these experiments a sensitive polarographic method has been used to study the oxygen uptake of wheat flours suspended in sealed buffer solutions containing dissolved oxygen. Evidence has been obtained that

(1) Flours rapidly absorb oxygen on wetting.

(2) The absorption takes place in two stages, the first of which is a very rapid initial uptake and the second a slow steady uptake over several hours. The initial uptake

is so rapid that it was missed in the early experiments.

(3) The rate of the initial uptake is tentatively concluded to be proportional to the logarithm of the oxygen concentration.

(4) Flours which have been treated with chemical improvers, or flours which have been stored for a considerable period after milling have a much lower rate of oxygen absorption than freshly milled untreated flours.

(5) Defatted flours tend to have a higher initial rate of oxygen uptake than that before the removal of fat.

It is thus established that flour contains a system, which is capable of rapidly absorbing oxygen. Since the system operates after removal of fat it does not involve the unsaturated-fat oxidases.

Fig. 21.

This evidence taken in conjunction with Freilich and Frey's work strongly suggested that the improvement associated with the batter process was due to incorporation of oxygen in the batter during high-speed mixing. A re-examination of this process was therefore undertaken with this suggestion in mind.

Fig. 21 shows the results of a test-baking experiment. The loaf on the left of the photograph was prepared by the batter process, that in the centre was prepared by a conventional straight-dough process, while the loaf on the right was mixed under identical batter process conditions to that on the left except that air was excluded and nitrogen substituted. All the loaves were the same weight and were baked in tins of the same size so that increased volume due to improvement shows as an increase in height, this being the only direction in which the loaf is free to expand. The results, which have been repeated many times and on different flours, leave little doubt as to the importance of oxygen in improvement.

The simultaneous importance of oxygen in the bleach is illustrated in the carotenoid destruction figures obtained on two typical flours by this experiment.

	Pigment Destroyed (%)	
	(a)	(b)
Batter process	52	44
Conventional process	32	28
Batter process in nitrogen	27	25

The destruction of some pigment in presence of nitrogen reflects experimental difficulties in rigorously excluding all traces of oxygen under practical working conditions. Nevertheless the following conclusions can be drawn.

(1) Both the bleach and improvement obtained by high speed mixing are dependent on free access of atmospheric oxygen.

(2) Early suggestions that improvement was due to work-strengthening of the flour proteins are not valid. The nitrogen-mixed loaf illustrated had the same mechanical working as the air-mixed loaf.

(3) Conventional bread-making processes involve the same reactions though in a lesser degree to those obtaining in high-speed mixing. This follows since conventional methods give bleach and improvement intermediate to that obtained with high-speed mixing in air and nitrogen.

The third conclusion is of some importance in considering the public health aspects of the batter process. If this process merely permits reactions which have already commenced to proceed further, and if bread which has been made by this process is suspected of being deleteriously treated, then it follows that

all bread ever made has been subject to the same deleterious treatment although in a lesser degree.

While these results show that oxygen is necessary for both bleach and improvement, and the previous experiments showed that bleaching was due to unsaturated-fat oxidase action, the question remains as to whether these unsaturated-fat oxidases might be involved in improvement. In view of the large range of substances mentioned in Section I, which can be oxidised in a coupled fashion by the unsaturated-fat oxidases, there is no a posteriori reason why readily oxidisable flour proteins should not act as hydrogen donors in an unsaturated-fat oxidase system.

It is true that in the antioxidant experiments, no evidence was obtained that inhibition of unsaturated-fat oxidases gave rise to significant differences in loaf volume. Again, the polarographic evidence with defatted flours suggests that these enzymes are not involved in improvement. Nevertheless, it was felt

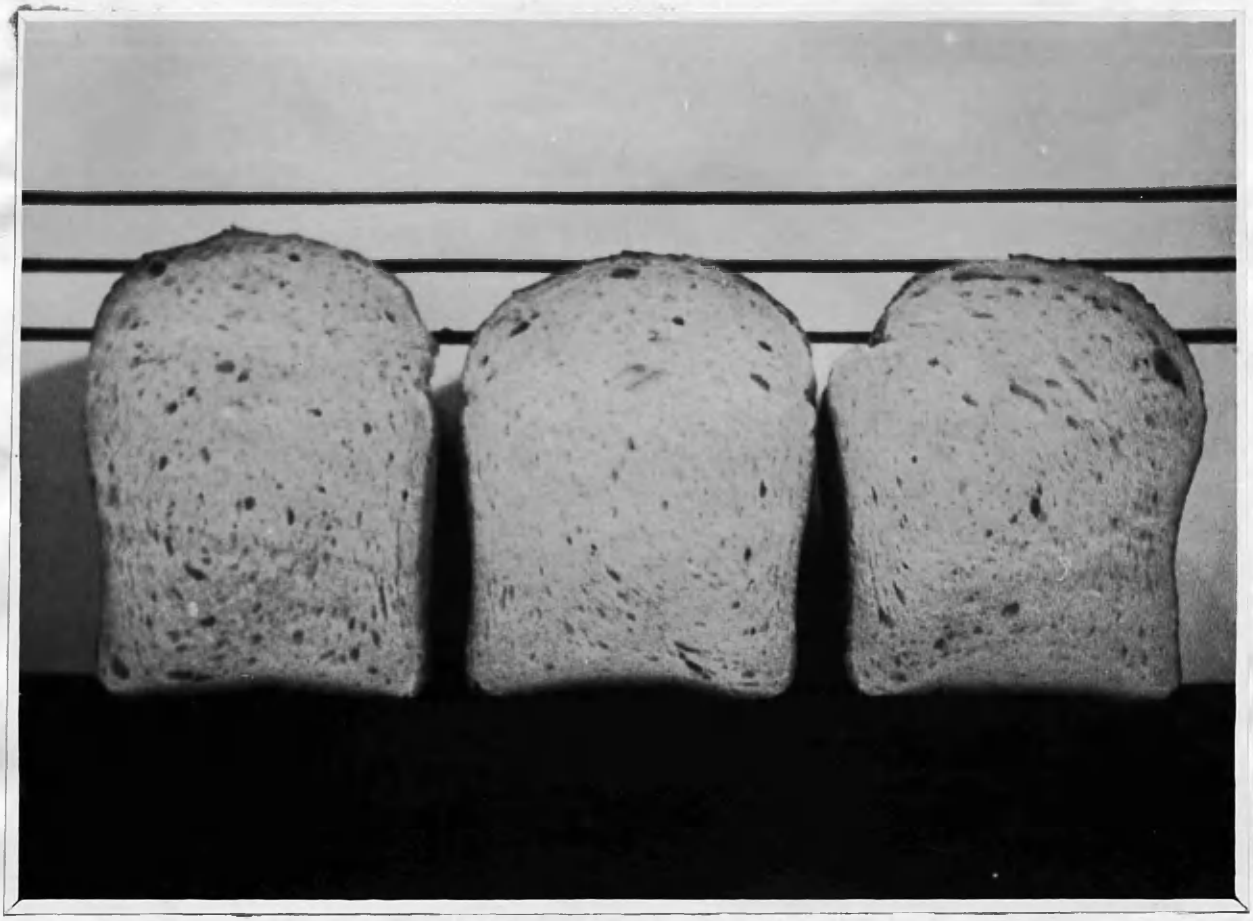


Fig. 22.

desirable to obtain direct evidence on this point.

The most convenient way to stop unsaturated-fat oxidase activity was to remove the substrate. Accordingly a large-scale extraction apparatus was used to remove the fat from sufficient flour to carry out baking tests. The solvent used was a petroleum ether fraction boiling below 40^{o}.

The literature contains a few references on the behaviour of defatted flours on baking. Johnson (78, 118) reported that ether extraction of flours gave increases in loaf volume, the increase being most marked with flours of lower grade. Sullivan and her co-workers (125) found some exceptions to the increases in volume noted by Johnson.

Fig. 22 shows the result of a baking test similar to that illustrated in Fig. 21, except that defatted flour was used. As was anticipated from Johnson's papers, all loaves were of greater than normal

Fig. 23.

volume, but the loaf on the left shows a clear response to the batter process in the absence of unsaturated-fat oxidase activity. The loaf on the right was also made by the batter process but in the presence of nitrogen, and is slightly smaller than the control loaf in the centre prepared on a conventional straight-dough mixing machine. It is clear that direct improvement by molecular oxygen is independent of intermediate fat peroxidation and the action of unsaturated-fat oxidases. This still does not exclude the possibility that an unidentified oxidase system is involved in improvement.

From the polarographic work previously described, it would be expected that by increasing the partial pressure of oxygen during mixing, improvement would be effected more rapidly. Fig. 23 demonstrates this conclusion. As before, the loaf on the left was obtained by the batter process, while that on the right was prepared by a conventional process. The centre loaf shows the effect obtained by applying a conventional

dough-mixing process in an atmosphere of oxygen. The pigment analyses and loaf heights were of the pattern expected, and the results of a typical test are given below.

	Loaf Height (cm.)	Pigment Dest.(%)
Batter process in air	14.6	40
Conventional process in oxygen	15.1	46
Conventional process in air	13.3	29

Since the publication of these experiments, the use of oxygen in conventional machinery has been extensively tested on a commercial scale in a factory which is already operating the batter process. Similar results to these already described are obtained under commercial conditions, the oxygen-treated loaf being generally rather whiter than a corresponding batter-process loaf, and showing evidence of a somewhat greater degree of improvement.

DISCUSSION

DISCUSSION

Experimental evidence on the behaviour of lipoxidase and catalase in the bleaching of carotenoid pigments has been described, and an account of the application of these studies to the evaluation of a breadmaking process using unbleached untreated flour has been given. The significance of the results in terms of hazards to public health arising from the widespread use of strong oxidising agents in wheat flour has been pointed out. Owing to the nature of the evidence presented, discussion of detail has been dealt with in the experimental sections. It is the purpose of this section to consider the future development of the work in relation to what has already been done.

In considering the experimental data, the criticism that no attempt to deal with the kinetics of the bleaching reaction has been made will be raised. The omission is deliberate for the following reasons.

1. It has been shown that the observed reaction velocities are primarily dependent on the dispersion of substrate, and kinetics based on the

combined variables of such a system would be virtually worthless.

2. The coupled reaction involves the four reactants enzyme, substrate, oxygen, and secondary carotenoid substrate. A complex system of this sort does not readily lend itself to kinetic treatment.

3. Inspection of the substrate concentration curves for both catalase and lipoxidase shows that these reactions do not follow Michaelis kinetics.

Soya-bean lipoxidase must now be regarded as a well characterised enzyme. It has been studied in crude extracts and in crystalline form under a sufficiently wide variety of conditions for the general pattern of its behaviour to be clear. Satisfactory methods of assay have been evolved using oxygen uptake, diene conjugation and carotenoid bleaching as criteria of reaction progress. Two major problems remain.

The first and less important of these concerns the natural occurence of lipoxidase. All reports on the existence of lipoxidase in plants other than soya-beans are at least ten years old and were made at a time when the significance of haem catalysis of unsaturated fat was not widely appreciated. Many of these so-called lipoxidases may only bear a superficial resemblance to the soya-bean system. The only closely examined evidence for the existence of a true lipoxidase apart from soya is that of Blain on the behaviour of wheat germ.

The second problem is that of the physiological role of lipoxidase. If a re-investigation of the distribution of plant lipoxidases showed that this enzyme is indeed as widespread as it appears to be from the existing literature, the function of the enzyme becomes a matter of great interest in plant physiology. It is to be regretted that Holman's work on lipoxidase in germinating soya-beans has not so far been followed up. The experimental difficulties are considerable, but even a repetition of his quite simple experiments on other lipoxidase sources would be a valuable contribution to knowledge in this field.

The behaviour of haem compounds as unsaturated-fat oxidases is open to a wide range of investigation. The large number of these compounds and the differences in their behaviour merits an extensive comparative study. Moreover, serious experimental difficulties do not arise since most of the haem compounds of interest may be fairly readily obtained in a pure state, and the lipoxidase system described here is well adapted to following the pro-oxidant behaviour of haem compounds. Particular interest would centre in vegetable catalase if purified material could be obtained. In this connection, attention is drawn to the high catalase content of wheat germ. It can be calculated from Table VII that the concentration of catalase in wheat germ is about 240 p.p.m. on a Kat. f. basis of 30,000. Wheat germ catalase may well have a higher Kat. f. value than this and in that case the concentration in p.p.m. will be correspondingly less, but nevertheless it offers promise as a starting material for the preparation of a vegetable catalase, and is readily obtainable in quantity. Its occurrence with peroxidase may present difficulties in separation.

The possibility of haem catalysis of unsaturated fat oxidation in animal tissues raises an interesting problem. As already pointed out, Tappel has recently obtained *in vitro* indications that α-tocopherol might be involved in the protection of depot fats from haem-catalysed oxidation. On the other hand, there seems to be fairly general agreement among workers in this field that haem catalysis of the oxidation of unsaturated fat proceeds by a chain-reaction mechanism *in vitro*, and it is doubtful whether such mechanisms are operative *in vivo*. The possibility that the unsaturated-fat oxidase activity of haem may be without physiological significance in healthy animals awaits further evidence, but the work of Dam and his colleagues makes it probable that these reactions do occur in vitamin E deficient animals.

The figures reported here on the inhibition of lipoxidase by catalase in very low concentration call for further investigation. Holman, whose work on lipoxidase has been very extensive and who has prepared the only sample of crystalline lipoxidase isolated to date, clearly suspects that the presence of catalalase does

complicate the behaviour of lipoxidase. As shown in Table VII, catalase is present in significant quantities in dormant soya-beans, and as Fig. 14 shows, the catalase content rises very sharply on germination of the beans. The existing evidence is insufficient to suggest that catalase may be concerned in metabolic processes involving lipoxidase action, but the possibility exists. It will be recalled that the observations on catalase inhibition of lipoxidase were obtained with crude preparations of lipoxidase. In view of work in progress on lipoxidase purification, it is hoped that it may be possible to repeat these observations at a later date with a pure or near-pure sample of lipoxidase.

So far as the investigation of the Rank and Hay process is concerned, the role of the unsaturated-fat oxidases in the process is now clear and it is established that the observed improvement is due to the uptake of oxygen. The remaining point of interest in connection with this process is the mechanism involved in this uptake of oxygen by the flour proteins. At the time of writing, work on this point has just commenced and it is hoped

that it will be possible to ascertain whether an enzymic mechanism is involved, and if so, through which oxidase system the reaction operates.

SUMMARY

SUMMARY

The practice of adding chemical oxidising agents to the wheat flours used in breadmaking is of long standing. Recent work has shown that certain hazards to public health arise from these additions, but the introduction of legislation to prevent their use has been delayed by technical difficulties in finding an acceptable alternative. The function of these additions is to bleach the carotenoid pigments in the flour and to modify the wheat proteins in such a way as to render the flour more suitable for making bread of a fine quality. Any breadmaking process which dispenses with chemical treatment without sacrifice to quality is therefore of considerable importance. Details of such a process have been published, but the chemical mechanism of its action has been a matter of conjecture.

In the belief that the process involved the intervention of that group of enzymes known as the unsaturated-fat oxidases, a study of these systems was undertaken. The best characterised member of the group is lipoxidase, an enzyme which is abundantly present in

soya-beans. The development of a suitable system for the study of coupled carotene bleaching by lipoxidase gave rise to difficulties associated with the heterogeneous nature of the reactions involved, but these were eventually circumvented. In the system adopted, the oxidase under investigation was allowed to react in a buffer solution which had been equilibriated with atmospheric oxygen, and which contained a small concentration of pure sodium linoleate as substrate. Arrangements were made to disperse carotene in the solution at the beginning of the reaction, the course of which was followed by observing the degree of bleaching in a suitable instrument.

The unsaturated-fat oxidases may be divided into two classes, namely, the true lipoxidases which are believed to be without a metallic prosthetic group, and the haem proteins, which under appropriate circumstances are able to catalyse the oxidation of fats in a rather similar fashion to the lipoxidases. The enzymes soya lipoxidase and ox-liver catalase were selected as typical representatives of each class, and their behaviour was studied in the carotene-linoleate system with respect to such

variables as reaction time, enzyme concentration, substrate concentration, and pH. The inherent pH optimum of soya lipoxidase received detailed attention because of its importance in distinguishing between true lipoxidase systems and haem catalysis. The optimum pH of this enzyme has been the subject of considerable controversy among workers in this field.

Aided by these studies, attention was given to the behaviour of unsaturated-fat oxidase in flour during the course of its preparation for baking. Coupled with a rapid method of analysis for carotenoid pigments in bread and flour, a test-baking system was used to follow the course of the reactions during the mixing of bread doughs. It was established from these studies that the bleaching of bread by the process under consideration was catalysed by the unsaturated-fat oxidases, and that the "improvement" or modification of the wheat proteins was brought about by the increased access of oxygen which is a feature of the process. Arising out of these investigations an improved method of carrying out this process was suggested and has been tested successfully on a commercial scale.

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APPENDIX I

LIPOXIDASE ASSAY - EMULSIFIED SYSTEM

LIPOXIDASE ASSAY

(Provisional System Using Emulsified Substrate)

Reagents and Apparatus

Reaction buffer:- It is convenient to prepare this solution in quantity. 27.22 g. KH_2PO_4, 7.50 g. NaOH and 0.40 g. of Stergene are dissolved in distilled water and diluted to 4 l. The solution thus prepared is at pH 8.0. Stergene is a proprietry non-ionic detergent of undisclosed constitution, and of a large number of materials tested, it was by far the most effective.

Carotene solution:- A saturated solution of carotene (90% β, 10% α from the British Chlorophyll Co. Ltd.) is prepared by allowing 300 mg. of carotene to stand in 2 l. of a mixture of acetone and ethanol (75/25 V/V) for twenty-four hours with occasional shaking. The undissolved carotene is filtered off and the solution diluted with the same solvent to the required colour value as described below.

Sodium linoleate substrate:- Prepared as described in Appendix II.

Enzyme extraction buffer:- 114 ml. of 0.1 N acetic acid and 86 ml. of 0.1 N sodium acetate. The pH of the mixture is 4.5.

Colour measurements:- The Hilger Spekker Absorptiometer with Ilford spectrum violet filters (No. 601) and 40 mm. cells, is used.

Method

Five to twenty grams (depending on the activity) of the defatted material to be assayed is suspended in 100 ml. of extraction buffer and shaken thoroughly. It is allowed to stand for four hours with occasional shaking and then centrifuged, and if necessary, filtered. The extract thus obtained is used in the assay.

The reactions are carried out in conical flasks.

To each flask is added 20 ml. of reaction buffer and 80 ml. of distilled water. The flasks are allowed to stand for at least three hours and preferably overnight to equilibriate with atmospheric oxygen. 2 ml. of substrate and 5 ml. of the carotene solution are then added, and an initial absorptiometer reading obtained against a distilled water blank. The strength of the carotene solution is adjusted by dilution during preparation so that this initial reading lies between 0.32 and 0.36 on the instrument scale. The observed reading is then halved, and the instrument set at this value. The solution is returned to the flask and a suitable volume of enzyme solution added, a stop-watch being started simultaneously. The bleaching of the solution is followed on the instrument and the time to half bleach noted. The procedure is repeated at several levels of enzyme concentration, and a graph prepared of time to half bleach against enzyme addition. A unit of enzyme is defined as being contained in that volume of extract which bleaches half of the carotene present in 300 seconds.

APPENDIX II

LIPOXIDASE ASSAY - IMPROVED SYSTEM

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THE BLEACHING OF CAROTENE BY A LIPOXIDASE–LINOLEATE SYSTEM

By J. A. BLAIN, J. HAWTHORN and J. P. TODD

A system for studying the coupled oxidation of carotene by lipoxidase and similar fatty acid oxidases is described. The experimental characteristics of the system are illustrated, and particular attention is paid to the effect of pH on the observed reaction velocities. The system presents certain advantages over previous systems of a similar nature.

Introduction

A recent review by Holman & Bergstrom[1] surveys the fairly extensive literature of lipoxidase and other unsaturated-fat oxidase systems. The technical importance of the lipoxidase-coupled bleaching of carotenoids has been known since 1934, when Haas & Bohn[2] patented their process for the bleaching of the yellow pigments in bread doughs by the use of soya preparations. The reactions of these unsaturated-fat oxidases have been shown to be responsible for such diverse and undesirable effects as losses of vitamin A in dried lucerne[3] and in butter,[4] the development of rancidity in herring muscle,[5] and peroxide formation on low-temperature storage of hog adipose tissue.[6, 7] Additional technical interest is given to carotenoid bleaching by such recent publications as those of Irvine & Winkler[8, 9] on colour losses in macaroni doughs, and that of Rank & Hay[10] on a bread-baking process with unbleached untreated flour.

The only unsaturated-fat oxidase so far characterized and isolated in a pure form is soya-bean lipoxidase,[11] although it is known that haem proteins are associated with similar reactions in animal tissues.[5, 7]

In early studies of lipoxidase activity, [12–15] the bleaching of carotenoids was used for assay purposes. These methods have been largely superseded by the spectrophotometric assay of Theorell, Bergstrom & Åkeson,[16] which utilizes the absorption band at 234 mμ to measure the degree of diene conjugation produced by the enzyme in an unsaturated fatty acid substrate. This method is not subject to certain errors of the carotenoid bleaching methods which are associated with variations in the degree of dispersion of the substrate and the carotenoid. Recently, Irvine & Anderson[17] proposed a manometric technique for the estimation of lipoxidase activity in wheat.

Nevertheless, the assessment of technical processes involving the coupled enzymic bleaching of carotenoids, and the study of their reaction conditions and mechanisms, cannot be carried out solely by the spectrophotometric method. In previous carotenoid assay systems little systematic attention was given to some of the factors. The work described in the present paper was carried out in an attempt to establish valid conditions for the quantitative study of these bleaching processes.

The unusual feature of the lipoxidase reaction is that it takes place in two phases, the coupled reactant and the fatty substrate being insoluble in the aqueous enzyme phase. The observed reaction velocities will therefore reflect the degree of dispersion of the reactants, and consequently any method must seek to standardize the reactant dispersion.

The enzyme is known to have three substrates, namely linoleic, linolenic and arachidonic acids, which are water-soluble only at high pH values. The spectrophotometric method overcomes the dispersion difficulty by the use of sodium soaps as substrate at pH 9, where an apparent activity optimum is obtained and where dispersion is reproducible. In the carotene-bleaching method, stabilizers of various kinds,[13] as well as surface-active agents, have been used, but these may introduce activating or inhibiting effects which vary with concentration and pH.[18] The method to be described avoids the use of such agents.

In previous measurements of carotenoid bleaching, and particularly at acid pH values, observations were made on solutions which were turbid to a greater or less degree unless a stabilizer was used. Alternatively, some workers extracted the pigment with a suitable solvent after carrying out the reaction, but the procedure was tedious. In the proposed system extraction is avoided and measurements are made on clear solutions. As a result of this, a method of activity measurement of crude soya extracts is proposed which may be used in the study of technical processes involving carotene bleaching by lipoxidase or by similar systems.

Reagents and apparatus

The following reagents and apparatus are required:

Extraction buffer, pH 4·5.—114 ml. of 0·1N-acetic acid and 86 ml. of 0·1N-sodium acetate.

Reaction buffer, pH 6·0.—12 ml. of M/15-disodium hydrogen phosphate and 88 ml. of M/15-potassium dihydrogen phosphate, diluted to 500 ml. with distilled water.

Alternative reaction buffers, pH 3·0 *to* 8·0.—Where observations over a fairly wide range of pH are required, McIlvaine's[19] citrate/phosphate buffer system has been found convenient. Five volumes of McIlvaine's mixtures of 0·2M-disodium hydrogen phosphate and 0·1M-citric acid are diluted to fifty volumes. The pH is determined in the diluted buffer in the presence of the appropriate quantities of substrate and carotene solutions. Buffer strengths generally should be about M/75, since higher concentrations tend to cause precipitation of carotene.

Carotene solution.—1·5 mg. of carotene (98% β-carotene from The British Chlorophyll Co. Ltd.) from a freshly opened ampoule is dissolved in 100 ml. of a mixture of acetone/ethanol (75/25 v/v).

Aqueous caustic soda.—A 20% solution in distilled water is used to stop the enzyme reaction.

Alcoholic caustic soda.—4 g. of caustic soda pellets are dissolved in 5 ml. of warm distilled water and diluted to 100 ml. with ethanol. The solution is standardized in the usual way.

Sodium linoleate substrate.—Ethyl linoleate is prepared from cottonseed oil[20] and stored in evacuated ampoules at − 20°, each ampoule containing 0·44 g. of ethyl linoleate, which is equivalent to 0·40 g. of linoleic acid. A slight excess of the alcoholic N-caustic soda solution is added to an opened ampoule and mixed. The solution is allowed to saponify overnight and diluted to 400 ml. with distilled water. One ml. of this solution contains 1 mg. of linoleic acid. The substrate thus prepared must be kept at 0° to 4° when not in use, and should be used within 14 days of preparation.

Colour measurements.—The Hilger Spekker absorptiometer, with Ilford spectrum violet filters (No. 601) and 40-mm. cells, is used.

Reaction flasks.—The reaction is carried out in 200-ml. conical flasks, which are set aside for this purpose after being soaked overnight in a strong solution of caustic soda. They are thoroughly rinsed with distilled water before drying for use. After use, rinsing and drying are repeated before re-use. Soaps and detergents are to be avoided for cleaning glassware, since the presence of traces of such surface-active agents has been found to give rise to inaccuracies. After repeated use there is a slight tendency for carotene to be precipitated on the surfaces of the flasks. This is removed from time to time by rinsing with acetone before preparing the flasks in the usual way.

Flask shaker.—In carrying out the additions to the reaction buffer, it has been found that reproducibility is enhanced by a simple mechanical-shaking device as shown in Fig. 1. This consists of a circular horizontal plate, of the same diameter as the base of the flask, fitted with a raised edge and mounted 5 mm. off centre to the vertical driving shaft. The flask is located on the plate by means of an annulus of rubber tubing stiffened by passing a length of thick wire solder through it. It is held in position by means of two wires soldered to the plate and diametrically opposite one another. A small motor drives the plate at about 300 r.p.m. When additions are made, the pipette is introduced under the surface of the liquid and the violent agitation produced gives very rapid mixing of the reagents and uniformity of dispersion.

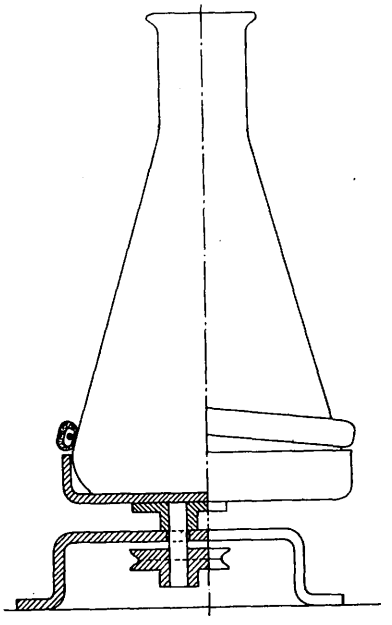

FIG. 1.—*Flask-shaking device*

Method

The extraction of lipoxidase from soya flour.—The coarsely ground material is defatted for six hours in a Soxhlet apparatus with *n*-pentane or a light petroleum fraction boiling below 40°. The defatted material is ground to pass a 60-mesh sieve, and 5 g. suspended in 50 ml. of extraction buffer. The mixture is shaken for one hour, then centrifuged for ten minutes at 2000 r.p.m. and decanted through a No. 1 Whatman filter paper. The solution thus prepared loses little activity during the course of a few weeks when stored at 0–5°.

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

Addition	Reaction flask	Blank flask
1	Reaction buffer, 50 ml.	Reaction buffer, 50 ml.
2	Substrate solution, 1 ml.	Aqueous sodium hydroxide, 2 ml.
3	Carotene solution, 2 ml.	Substrate solution, 1 ml.
4	Enzyme solution, x ml.	Carotene solution, 2 ml.
5	Aqueous sodium hydroxide, 2 ml.	Enzyme solution, x ml.

The reaction buffer is added to the flasks and left overnight to equilibrate with atmospheric oxygen before use. During the reaction it should be at a temperature of 20° (± 2°). The volume of enzyme solution added should be about 0·05 to 0·30 ml., and a microburette to deliver under the surface of the liquid is necessary. In carrying out the reaction there should be no delay between additions after the substrate has been added. As soon as the enzyme has been added a stop-watch is started and after exactly one minute caustic soda is added to stop the reaction. The percentage of carotene destroyed is then read in the absorptiometer with 40-mm. cells. Gas bubbles in the solutions should be allowed to disperse before readings are taken. In bright daylight some slight bleaching of the alkaline solution takes place, and it is therefore desirable to make the observations within an hour of carrying out the treatment. The solution is cloudy while the reaction is proceeding at acid pH values, but becomes clear on the addition of alkali.

Readings are obtained for at least three levels of enzyme concentration, so that the curve joining them (plotted against carotene destruction) passes through the 50% destruction level. From the curve, the volume of enzyme solution required to destroy 50% of the carotene present (i.e. 0·015 mg. of carotene) is read. This volume represents one unit of activity for the system described.

Experimental

The main characteristics of the proposed system are illustrated in Figs. 2 to 10.

Optimum pH.—The optimum pH of lipoxidase has been the subject of controversy. Holman,[21] using crystalline lipoxidase and a sodium linoleate substrate with the spectrophotometric assay, found the optimum to be pH 9, and Smith & Sumner,[22] using an emulsified ester substrate, found an optimum at pH 6·5 and suggested that Holman's figure represented increasing availability of substrate with pH for his system, but direct experimental evidence for this has not been given. Holman[1] doubts the possibility of measuring the inherent optimum of lipoxidase until a substrate, water-soluble over the whole pH range of interest, can be obtained. More recently, Fukuba[23] has studied the substrate properties of polyoxyethylene linoleate, whose interfacial tension against xylene was found to be independent of pH, and observed an optimum between pH 6·5 and 7·0.

In the system described here, the omission of carotene and the measurement of diene conjugation by the band at 234 mμ gave a curve that was essentially similar to that of Holman, activity increasing with pH towards an optimum at about pH 9, although the overall substrate concentration (1 mg. of sodium linoleate in 53 ml. of reaction mixture) was about one-eightieth of that of Holman (2 mg. in 1·2 ml. of reaction mixture). When carotene was introduced into this system the curve shown in Fig. 2 was obtained. Similar curves were observed whether the treatment was carried out in citrate/phosphate or phosphate buffers.

On this evidence it appeared that the carotene, and the acetone/ethanol solvent introduced with it, must alter the characteristics of the system. A study of the carotene solution in the quantities used added to the phosphate/citrate buffer showed that the absorption, and hence presumably the dispersion of the carotene, did not change significantly with pH. The data plotted in the upper part of Fig. 3 show the results obtained, and it may be noted at this stage that carotene dispersion in buffer in the absence of substrate tends to be unstable. Strict attention to technique is necessary to obtain these observations. Fig. 4 shows a spectrophotometric comparison, on an $E^{1\%}_{1cm.}$ basis, of the absorption of a specimen of carotene in a chloroform solution and in the aqueous reaction system at pH 8, appropriate corrections having been made for the absorption of the acetone. Clearly, the carotene in the aqueous solution is in a different state of dispersion from that of carotene in organic solvents.

At acid pH values the solution of substrate in the reaction buffer becomes turbid almost immediately owing to precipitation of linoleate. With the appropriate buffer blanks, measurements of Spekker absorptiometer readings showed that the turbidity increased very rapidly

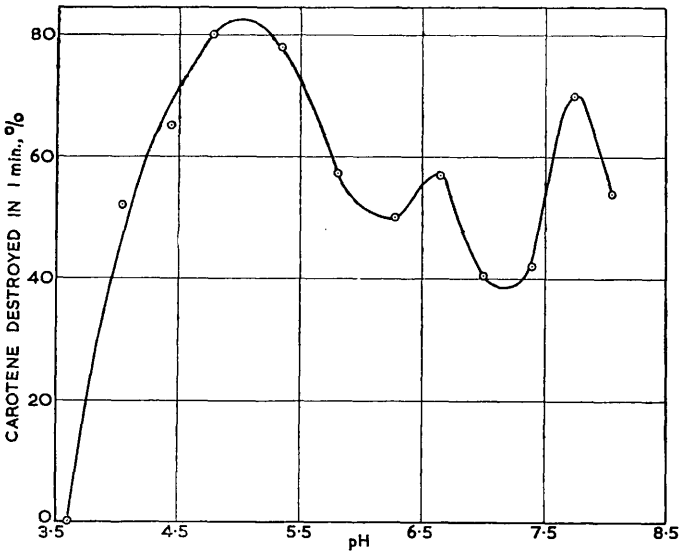

FIG. 2.—*Activity curve of the system*

during the first minute after the addition of the substrate and afterwards continued to increase gradually. The lower curve in Fig. 3 shows this development of turbidity with pH in the buffer, in the absence of enzyme and carotene, two minutes after the addition of substrate. It would appear that precipitation of linoleate with decreasing pH value begins at about pH 7·7, but the general form of the curve, although indicative of unexpected complexities, offers no explanation of the peaks on the pH–activity curve.

When, as shown in Fig. 5, the absorption of the complete system in the absence of enzyme is studied, a curve is obtained with maxima and minima related inversely to those of the pH–activity curve. As Fig. 3 shows, the carotene readings do not change appreciably with pH,

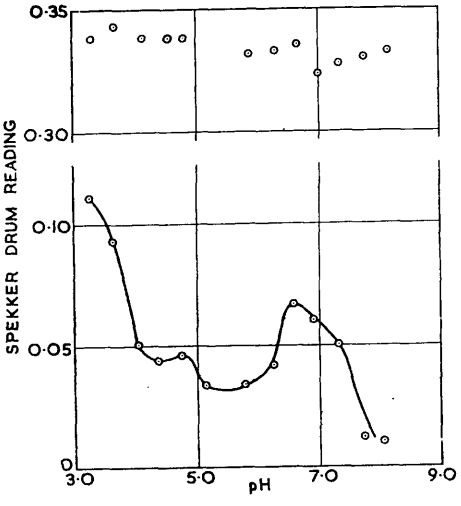

FIG. 3.—*Absorption characteristics of the components of the system*
Upper plot: Variation in optical density of carotene in phosphate/citrate buffer with pH
Lower curve: Variation in substrate turbidity in phosphate/citrate buffer with pH

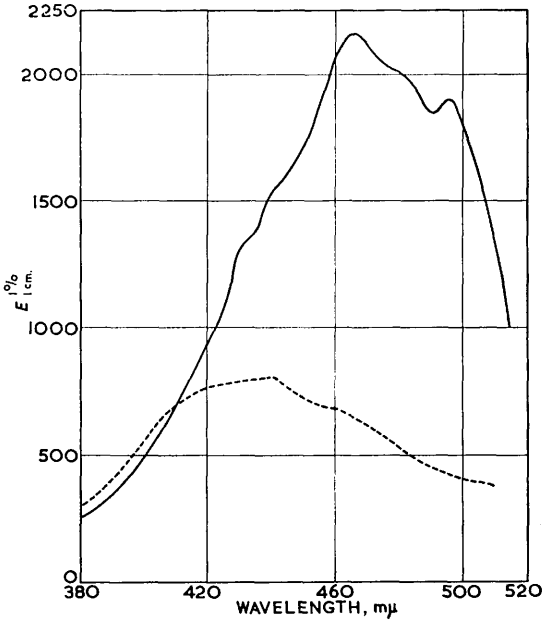

FIG. 4.—*Absorption curves of carotene*
Upper curve: In chloroform solution
Lower curve: In phosphate buffer dispersion, pH 8

but the substrate-turbidity readings change markedly. It would therefore be expected that the absorption of the complete system (i.e. buffer with substrate and carotene in absence of enzyme) will parallel the substrate-turbidity curve. This, however, does not take place, and it follows that the presence of substrate modifies the state of dispersion of the carotene. Moreover, the inverse relationship of the curves shown in Figs. 2 and 5 indicates that this modification is related to the observed activity of the system. It appears, therefore, that the pH effects observed reflect the mutual availability of enzyme, substrate and carotene. It will be noted that the maxima and minima in Fig. 5 show a slight pH shift with respect to the minima and maxima in Fig. 2, but this would be expected, since the inherent activity of the enzyme will vary over the pH range. The precise position of any single peak on the pH–activity curve will reflect availability of substrate and carotene, modified by inherent activity of enzyme.

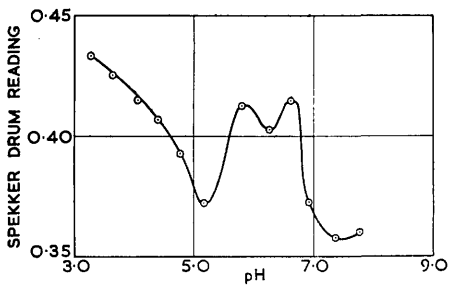

FIG. 5.—*Variation in optical density of the complete system (enzyme absent) with pH*

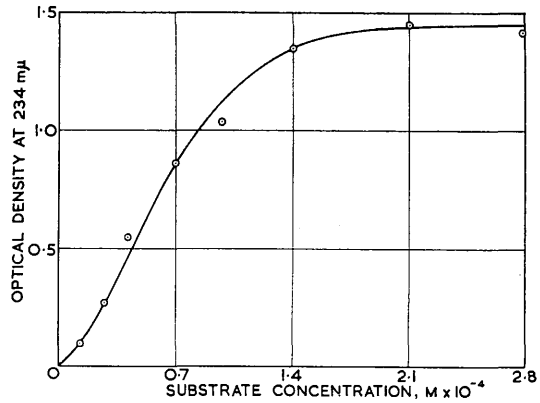

FIG. 6.—*Substrate concentration/velocity curve for the reaction system in absence of carotene at pH 8*

Effect of substrate concentration.—Fig. 6 shows the diene conjugation (as measured spectrophotometrically by the peak at 234 mμ) produced by the interaction for one minute of a constant quantity of enzyme with increasing quantities of substrate, in the absence of carotene, in the proposed reaction system at pH 8. The form of the curve is as would be expected—the activity increases to a maximum and afterwards remains constant.

Fig. 7 shows the destruction of carotene under the same conditions of substrate concentration, enzyme concentration, pH and time as those in Fig. 6. A maximum is reached at a substrate concentration of approximately 0·01M × 10^{-2}, but the carotene destruction at this substrate concentration is only very slightly greater than at 0·007M × 10^{-2}, which is the proposed level for the system. In the region of this level, variations in substrate concentration of up to 20% have only a minor effect on the carotene destruction. The existence of an apparent substrate optimum in carotene-bleaching systems has been noted by previous workers, and was related by Balls, Axelrod & Kies[15] to the presence of an activator in crude soya extracts. A study by the present workers of the substrate relationships of lipoxidase-active wheat-germ extracts did not show such an optimum in the presence of carotene.

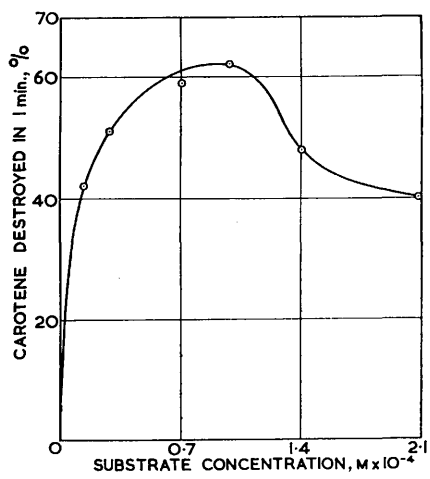

FIG. 7.—*Substrate concentration/carotene destruction curve for the system at pH 8 with a one-minute reaction time*

Holman[1] gives a figure of 160 mm. for the oxygen saturation pressure of lipoxidase and it was found that, under the assay conditions described here, bleaching rates were almost doubled when the reaction mixture was saturated with oxygen. However, the passage of a current of oxygen through the reaction mixture has the disadvantage of selectively removing acetone/ethanol from the system and thus rendering the carotene dispersion less stable. Moreover,

conditions of active oxygenation are not easy to reproduce. In the assay system described, sub-optimal but reproducible conditions are easily obtained if the reaction buffers are left overnight in the flasks to equilibrate with atmospheric oxygen. This procedure was therefore adopted as being convenient and reliable.

Effect of temperature.—Fig. 8 shows the effect of temperature on the carotene system at pH 6·3. The optimum of 25° is in agreement with that of other studies on carotene bleaching. The reaction rate is only slightly decreased at room temperature, and it is within the accuracy of the method to work at 20 ± 2°.

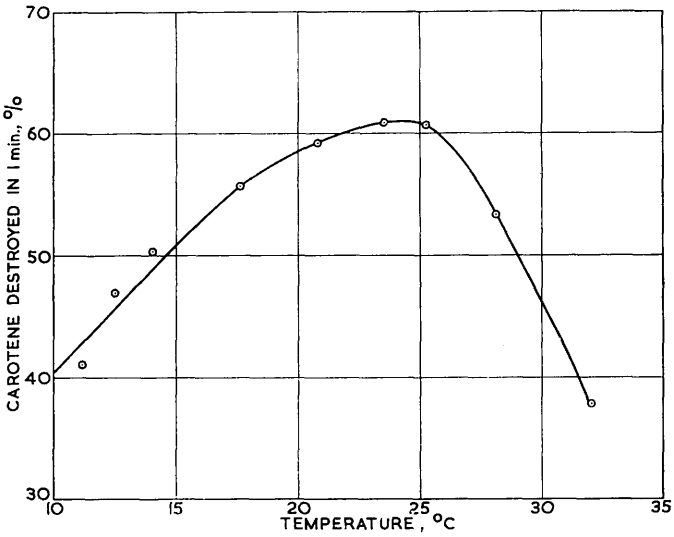

FIG. 8.—*Temperature/velocity curve at pH 6*

Effect of reaction time.—Fig. 9 shows the course of carotene destruction with time in the presence of about one unit of enzyme at pH 6. The flattish appearance of this curve indicates that small errors in timing the reaction would bear an almost linear relationship to carotene destruction.

Effect of enzyme concentration.—Fig. 10 illustrates the effect of concentration of enzyme on the reaction system under the conditions of the assay at pH 6. In the region of about 50% destruction of carotene the relationship is again almost linear.

Discussion

In the method described, an effort was made to achieve a system having adequate stability without the use of surface-active or dispersing agents as employed in previous assays. As shown by results not quoted here, such compounds, apart from their function of improving dispersion and stability, may have an inhibitory effect on bleaching, and this effect varies with their ratio to substrate. Strict adherence to procedure, coupled with short reaction times, gave reproducible results, despite the probability that substrate dispersion varies during the reaction period.

The use of caustic soda to stop the reaction eliminated troubles associated with those systems that use the measurement of time to half-bleach as an index

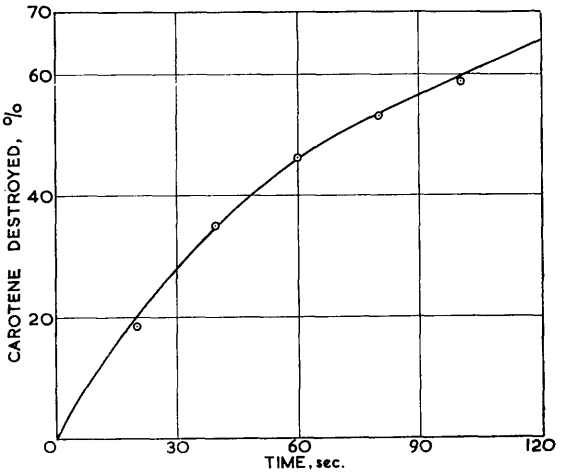

FIG. 9.—*Carotene destruction/time curve at pH 6*

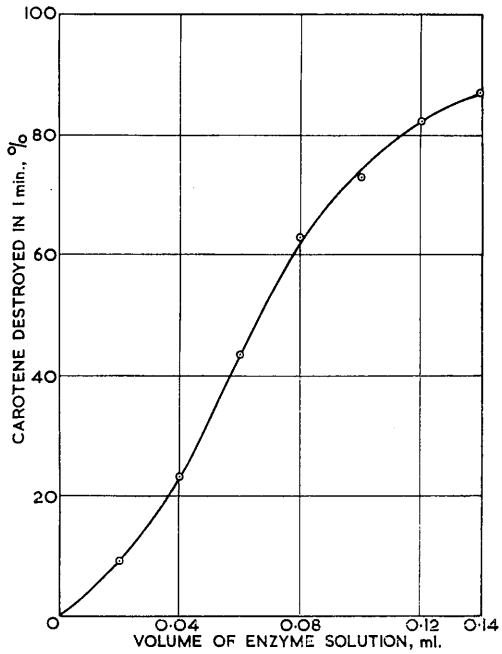

FIG. 10.—*Carotene destruction/enzyme concentration curve at pH 6*

of enzyme activity. Although short reaction times are desirable, in such systems bubbles disperse only slowly and give trouble with colour readings, and, in addition, there are turbidities due to protein or linoleate.

The necessity of having precise information on the effect of pH on the system is stressed by the recent work of Tappel.[24] In this work the restriction of fatty acid peroxidation by haem proteins to low pH values has been made a criterion of distinction between the fatty acid oxidase action of haem proteins and that of lipoxidase.

The use of carotene in lipoxidase assays has been criticized by Sumner & Smith[14] on the grounds that carotene solutions are unstable and that carotene soon separates from aqueous dispersions. Carotene solutions prepared by the method described above are stable over a period of months in their original solvent, and in the reaction system for many times the period required for the reaction. Difficulties do arise if the buffer strengths are exceeded and if the order of mixing of reactants is not strictly adhered to.

With due allowance for its limitations, the method has the advantage of speed where large numbers of analyses are required, and has been used not only for the analysis of sova extracts but also, with minor modifications, for the assay of lipoxidase activity in wheat germ and wheat flour. It may be of wider applicability than the recent manometric technique of Irvine & Anderson.[17] A future paper will report work on the application of this assay system to sources of lipoxidase other than soya.

Conclusions

1. A system for the study of the lipoxidase-catalysed bleaching of carotene in the presence of linoleate is described.

2. The effects of temperature, enzyme concentration, substrate concentration, time and pH on bleaching rates are discussed.

3. Evidence is presented that the interaction of linoleate and carotene dispersions is the principal factor determining the form of the pH–activity curve.

4. The system as described may be used to assay soya-bean lipoxidase. Its use, with minor modifications, may be extended to the measurement of lipoxidase activity in wheat products.

5. The rapidity of the method, the wide range of pH over which it may be used, and the simplicity of the equipment required renders the method particularly suitable for industrial control work.

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Food Science Section
The Royal Technical College
Glasgow

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APPENDIX III

THE ESTIMATION OF CAROTENOID PIGMENT IN FLOUR & BREAD

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THE ESTIMATION OF CAROTENOID PIGMENT IN FLOUR AND BREAD

Reagents and Apparatus

Water-saturated butanol:- Redistilled n-butanol is shaken with distilled water until a saturated solution is obtained. The temperature of the butanol should be 18° or higher when carrying out the extraction.

Shaker:- A shaker capable of taking the centrifuge bottles used is required. The Griffin & Tatlock Microid shaker run at its highest speed has been found suitable.

Colour Measurement:- The Hilger Spekker absorptiometer with Ilford spectrum violet filters (No.601) and 40 mm. cells is used.

Method

A suitable weight of the material to be tested (normally 25 g.) is added to a 200 ml. centrifuge bottle and covered with 100 ml. of water-saturated butanol.

The bottle is stoppered, shaken, and allowed to stand for 30 minutes with occasional shaking. It is now placed on the mechanical shaker and vigourously shaken for twenty minutes. It is then centrifuged and the supernatant decanted.

The procedure is repeated with a fresh 100 ml. portion of butanol and the combined supernatants are made up to 200 ml. before reading on the Spekker against a butanol blank.

The moisture content of the materials under test is determined by any of the standard methods (in this work the Carter-Simon moisture oven was used), and the readings are calculated to a moisture-free basis. From these figures the pigment destroyed in a baking process may be expressed as a percentage of the pigment originally present in the flour.

APPENDIX IV

TEST-BAKING PROCEDURES AND EQUIPMENT

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TEST BAKING PROCEDURES AND EQUIPMENT

Apparatus

Mixing machines:- A Morton No. 0 experimental mixer is used. This machine is constructed with the mixing chamber and blades of stainless steel, and the chamber is fitted with a water-jacket to permit temperature control during mixing. The machine is supplied with two mixing blades geared to run at a differential ratio of 3:5 revolutions, the blades revolving in opposite directions. A special gear train is fitted to permit speeds of 425 r.p.m. on the faster arm for use in the batter process. An alternative gear to allow the machine to operate at half this speed is provided to simulate the conditions of conventional doughing.

Proofing cabinet:- This consists essentially of a cabinet of about 8 cu. ft. capacity, fitted with electric heaters and provided with means for maintaining a water-saturated atmosphere under temperature control at any desired temperature between 70°F and 110°F.

Test-baking oven:- A Simon electrically heated oven, capable of baking eight full-sized loaves is used. The heating arrangements are under thermostatic control.

Method

Mixing procedure:- The following basic recipe is used-

Flour	: 2½ lb. (1140 g.)
Salt	: 17 g.
Compressed yeast (D.C.L.)	: 15 g.
Water	: 600 ml.

The water added is varied according to the water absorbing capacity of the flour used, and for flours deficient in amylase activity, 5 g. of malt flour are added. Depending on the requirements of the experiment, enzyme extracts or antioxidants etc. are added. The dough prepared from these ingredients is sufficient to bake two 28 oz. loaves.

Mixing procedures:- Where conventional doughs are required, the ingredients are simply added to the machine, and the machine run for 40 secs. with the slow-speed gear engaged.

For batter process tests, half the flour and all the other ingredients are added to the machine and mixed at high speed for six minutes. The remainder of the flour is then added and a further 40 secs. mixing given at slow speed.

For experiments in different gaseous atmospheres, a cover is provided for the mixing chamber of the machine through which the gas is introduced in a steady stream from a cylinder. When mixing under nitrogen is required, the flour is stored for two days prior to the test in a sealed container which has been evacuated and filled with nitrogen. This procedure is adopted with a view to displacing oxygen adsorbed on the flour particles or held between the particles. The nitrogen from the cylinder is stripped of remaining traces of oxygen

by passing it through an alkaline pyrogallol absorption train before use.

Procedure:- The temperature of the mixing water is adjusted to give a temperature of 80°F in the completed dough, which is then allowed to ferment at this temperature for exactly three hours timed from the commencement of mixing. The dough is then "knocked-back" (i.e. subjected to mechanical handling to expel most of the carbon dioxide produced during fermentation) and weighed into 28 oz. pieces. The weighed pieces are then manually manipulated to spherical shapes ("chaffing" is the baker's term), and set aside for fifteen minutes to relax. This relaxation period is necessary since the operations of "knocking-back" and cutting for weighing produce internal stresses in the dough mass which becomes difficult to handle. After resting for this period, it regains its original plastic properties and may now be moulded. Moulding is a further manipulative process which forms the shape of the loaf, and requires a certain skill for its proper performance. (In the experiments

described, a laboratory technician was specially trained to carry out these manipulations and a high degree of skill and reproducibility was attained).

After moulding, the formed dough piece is put in a standard baking tin and given a further fermentation in the proofing cabinet. The conditions are arranged so that proofing takes one hour and during that time the fermenting loaf expands and reaches the top of the tin. After proof, the resulting loaf is baked for 45 mins. at 450°F.

Suppliers of Equipment

Mixer:- The Morton Machine Co., Wishaw, Scotland.

Proofing cabinet and oven:- Henry Simon, Ltd., Manchester.

APPENDIX V

COMMUNICATION ON THE RANK AND HAY PROCESS