MARY D. CAMERON.

Summary

During a study of the oxygen uptake of wheat-flour doughs, it was found difficult to distinguish between reactions catalysed by lipoxidase and similar reactions catalysed by haematin compounds. All these systems are capable of catalysing the oxidation of unsaturated fats and may also cause secondary bleaching of carotenoids and other pigments. Valid comparisons between the two systems required the preparation of lipoxidase in a purified form and this purification was the purpose of the present work.

The isolation of lipoxidase has been achieved in only one recorded case, by a complex combination of precipitive and electrophoretic methods. This thesis describes the application of the use of ethanol as a precipitant in a purification procedure for lipoxidase.

A study of precipitation conditions is reported, and arising from the data obtained, routes of separation are described on an experimental basis. From these studies and the experience accumulated, a simple procedure for obtaining a useful degree of concentration of the enzyme is described.

The procedure calls for experimental arrangements to permit

working at controlled temperatures between 0^{o} and -5^{o} with a sufficient reserve of refrigerative capacity to rapidly dissipitate the heat of solution of ethanol. The bulk of the activity is removed in the fraction between 30 - 45% ethanol and the subsequent precipitate, which is readily obtainable in quantity, may be further concentrated by continuous electrophoresis at pH 6·0.

The conditions, required to prevent undue losses, are shown to be somewhat critical but given these conditions, the procedure evolved is simple.

During the course of this investigation, a study was also made of the effect of ionising radiations on fats as these radiations appeared to show analogous reactions to these associated with lipoxidase. A comparison was made of the effect of β-rays on a linoleic acid - carotene system and on butter. It was shown that bleaching of carotene did not require the simultaneous oxidation of dienoic fats, and thus there is no fundamental similarity between radiation effects and the lipoxidase catalysed oxidation of unsaturated fats.

THE PURIFICATION OF SOYA LIPOXIDASE

by

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A thesis (with an additional paper) submitted in accordance with the requirements of the Faculty of Science of the University of Glasgow for the Degree of Doctor Philosophy.

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PREFACE

PREFACE

The work described here is part of a general programme of research into problems associated with the uptake of oxygen by wheat-flour doughs. One of the objectives of this programme is to increase our knowledge of the enzyme lipoxidase, and in particular to distinguish between reactions catalysed by it and similar reactions catalysed by haematin compounds.

Valid comparisons between the two systems require the preparation of lipoxidase in a purified form, and this purification was the purpose of the present work. As progress was made, it became increasingly clear that the critical conditions of temperature required for the separation of this enzyme could be achieved only with difficulty and on a very small scale using the normal sources of a biochemical laboratory. This thesis presents evidence demonstrating the feasibility and advantages of the method proposed, and reports a larger-scale application of the method using special facilities provided by a commercial cold-storage organisation.

During the course of the investigation the writer

had the opportunity to study for a short period in the United States of America under Professor Bernard E. Proctor of The Massachusetts Institute of Technology, who is engaged on extensive studies of the effects of ionising radiations on biological materials. Published reports on the effects of these radiations on fats had shown analogous reactions to those associated with lipoxidase. The writer carried out a comparative study of the reactions involved and a report of the work is included in this thesis.

INTRODUCTION

INTRODUCTION

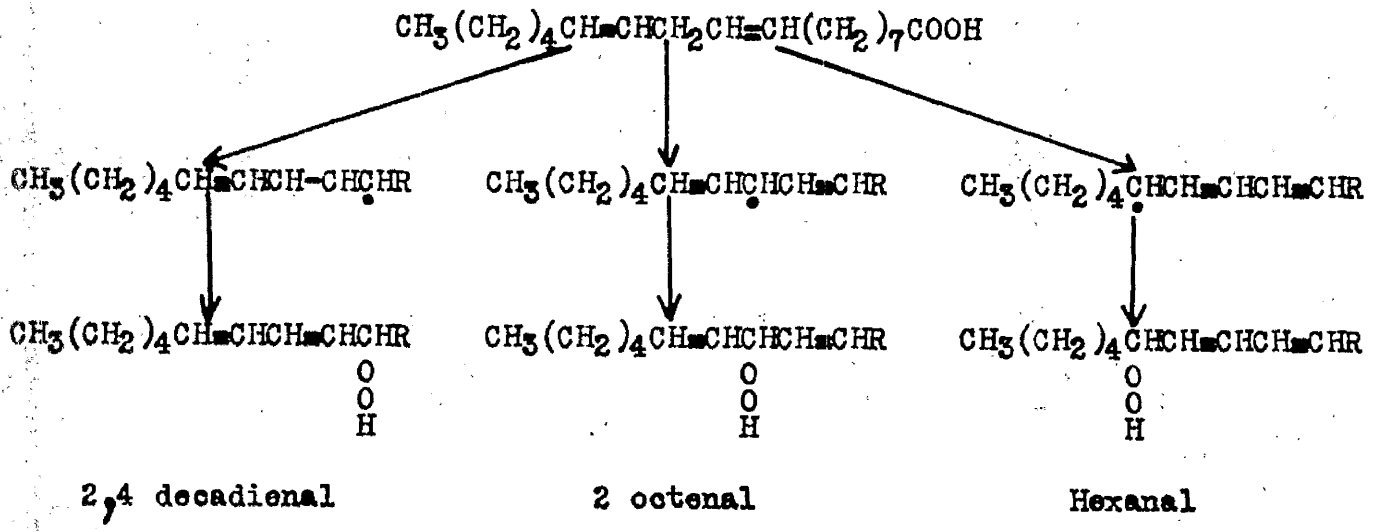
Lipoxidase is the principal member of a group of enzymes collectively known as the unsaturated-fat oxidases. While there have been numerous reports of its occurrence in a variety of plant tissues, the lipoxidase of the soya-bean is the most active source so far reported. There have been several accounts of "lipoxidase" systems in animal tissues, but recent critical studies have assigned the activity of these systems to haematins, in particular to haemoglobin, the cytochromes and catalase.

While the fat oxidations catalysed by haematins show remarkable similarities to those catalysed by soya lipoxidase, there are certain essential differences between the two systems when examined under identical reaction conditions, and there is no evidence to show that soya lipoxidase contains the characteristic iron-porphyrin grouping of the haematins. There are thus at least two classes of substances capable of catalysing the oxidation of unsaturated fats, and the wide-spread occurrence of haematins in plant tissues raises the question as to whether many of the effects attributed to lipoxidase in plant tissues are in fact due to haematins.

The question is of more than academic interest, since problems associated with the use of soya in wheat flour necessitate a distinction between haematin and true lipoxidase activity in wheat which can, at present only be made with great difficulty and with some doubt as to the validity of the results.

An adequate comparison of lipoxidase and haematin activity is dependent on observations made on pure preparations. While purified haematins can be fairly readily prepared, the isolation of lipoxidase presents major difficulties. It is true that one report of a successful isolation has been published, but the quantities prepared were not sufficient for further study, nor has the isolation been repeated. This thesis reports an examination of the possibilities of using ethanol as a fractionating agent for the purification of lipoxidase, and proposes a simple procedure for obtaining a useful degree of concentration of the enzyme.

In the literature review which follows, particular account is taken of work published during the past six years, since two authoritative surveys have given a detailed account of the field up to 1950.[1,2].



Holman's Chain Reaction Theory

Fig. 1

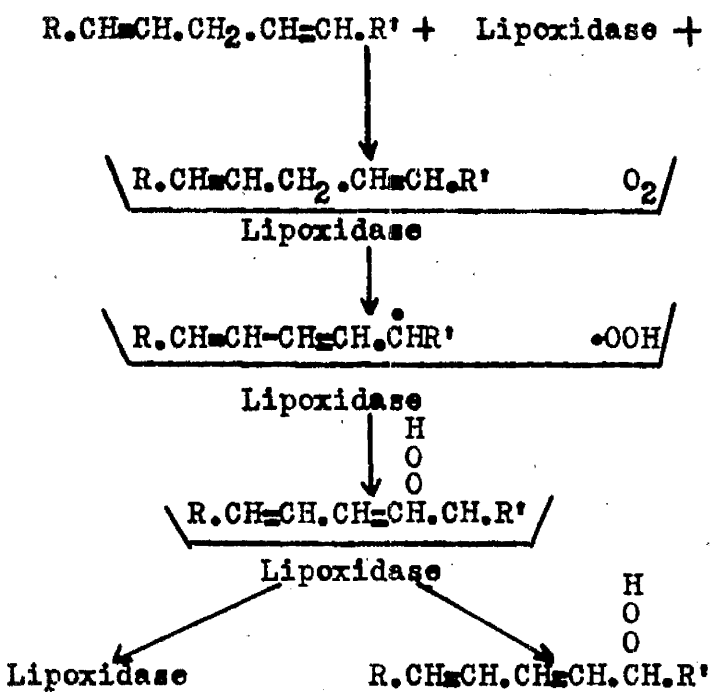
1. The Properties of Lipoxidase Enzyme Systems

The Soya-bean Lipoxidase Enzyme System

Soya-bean lipxidase oxidises unsaturated acids containing a cis-cis pentadiene grouping with the double bonds in the 9, 10 and 12, 13 positions to the carboxy group[3,4,5,6,7,8]. The substrates for lipoxidase are, therefore, linoleic, linolenic and arachidonic acids and their esters. It is not known whether physiological significance can be attached to the specificity of lipoxidase for these "essential" fatty acids.

On oxidation by lipoxidase, linoleic acid gives absorption peaks at 234 mμ which have been shown to be indicative of the presence of conjugated hydroperoxides[8,9,10,11]. Infra red studies showed that these hydroperoxides consisted of two isomers, cis-trans conjugated and trans-trans conjugated[12]. On allowing the reaction to continue further, carbonyl compounds are formed[3] and breaks occur in the chain of the unsaturated acid[13].

Due to this formation of conjugated hydroperoxides and on further oxidation of carbonyl compounds, Holman[14] and Bergstrom[9,15] suggested that a chain reaction similar to autoxidation was taking place. The suggested



Tappel's Reaction Mechanism

Fig. 2

mechanism is shown in Fig. 1. Further evidence of the similarity of the action to autoxidation was found when it was discovered that antioxidants had a strong inhibiting effect on the oxidation[16]. However the molecular extinction coefficient of the conjugated diene hydroperoxide at 234 mμ is not the same as that of the autoxidation product but corresponds to complete conjugation of the double bonds. This could be explained if the lipoxidase system only adds oxygen at the outside carbon atoms of the unsaturated group of the linoleic acid yielding conjugated products.

On the other hand, as the rate of oxidation is proportional to the square root of the concentration[17], the reaction mechanism seems more like that of a simple enzyme system than a chain reaction. Keppel[18] therefore suggested that a complex is formed between the unsaturated acid and the lipoxidase on which the oxygen is absorbed and activated, the unsaturated acid being in such a position as to be easily oxidized at the unsaturated centres. The mechanism proposed is shown in Fig. 2. This mechanism appears to be confirmed by the action of antioxidants on the reaction. On addition of H.D.G.A. to the system there

is complete inhibition of the reaction at high concentrations, but although the antioxidant is being destroyed there is no linoleate oxidation[18]. If the reaction was a chain reaction, it would be expected to give conjugated products in the presence of anti-oxidants.

The similarity of the lipoxidase reaction to haematin-catalysed oxidation[51,56,57] has suggested that the reaction may be due to the presence of a metallic group. However, cyanide, fluoride, pyrophosphate, azide, pp-chloro-mercuribenzoate and diethyldithio-carbamate do not inhibit lipoxidase[10] and no metal has been found in the analysis of the purified enzyme which could be acting as an active centre in the protein[19]. Therefore, although both haematins and lipoxidase are able to oxidise unsaturated fats, the mechanisms of these reactions are not similar.

Lipoxidase systems, as well as oxidising unsaturated fatty acids, can cause secondary reactions such as bleaching of vegetable pigments. As has already been reported, both carotene and Vitamin A are oxidised by this system but other carotenoids, bixin, xanthophylls and chlorophylls are also bleached[5,20]. These pigments are oxidised by some intermediate

formed during the lipoxidase-catalysed oxidation of linoleic and linolenic acids[6,20,21,22]. When pigments are present the degree of peroxidation of fatty acid is reduced under comparable reaction conditions[23,24,25]. The lipoxidase/linoleic oxidising system has also been reported to oxidise other systems, for example amino acids[26] and glutathione[27].

Kinetics of Lipoxidase Oxidation

For soya lipoxidase, in the oxidation of linoleic acid at pH 9.0 the amount of diene conjugated was found to be both proportional to enzyme concentration and time[23]. The rate of product formation was linear with time within the limits of experimental methods from the instant the enzyme was introduced into the substrate[17,28]. The Michaelis constant for combination with linoleic acid was 1.35×10^{-3}M for soya lipoxidase[10].

In the coupled oxidation of carotene by lipoxidase in the presence of linoleic acid the amount of carotene destroyed was proportional to enzyme concentration and to time[23]. The increase in absorption at 234 mμ was proportional to decreased absorption of carotene[14]. This has also been shown with bixin[29] but Kunkel[17] found that the presence of bixin initially inhibited diene conjugation, but as the bixin was destroyed the

rate of diene conjugation increased above that in the control samples. The destruction of bixin was logarithmically related to the reaction time and to the square root of the lipoxidase concentration.

Using a manometric method, Irvine and Anderson[30] found that with wheat lipoxidase the rate of the reaction was linear with respect to enzyme concentration. They showed that the reaction went in two stages, a rapid initial rate followed by a steady state where the rate is constant. The initial rate follows Michaelis and Menten's kinetics and K is 5×10^{-6} at pH 6.5 and 30°.

There has been a variation in the pH optima of the system as reported by various workers. For maximum enzyme reactivity pH values of 6.5 and 9.0 have been reported[13,22,29,30,31,32]. This variation in pH optimum is due to the different systems used in the assay and to different solubilities of substrates under various conditions. Reaction rates vary with substrate availability which in turn is a function of pH in the heterogeneous systems used. With pure lipoxidase and using a pure linoleate substrate, Holman found that the optimum was 9.0[32].

Raising the temperature of the system above 30°

resulted in a gradual loss of lipoxidase activity[30,31]. The optimum temperature has been variously reported as 30° and 25° [18,29,31] but Holman has reported it as 30° for pure lipoxidase with a pure linoleate substrate in the spectrophotometric assay[32]. Irvine and Anderson[30] found that the wheat enzyme was not inactivated below 60° and in the range between 10° and 50° there was very marked effect of temperature on the relative rates of the two stages of the reaction.

It has already been shown that the lipoxidase system is inhibited by antioxidants, such as tocopherol, ethyl gallate and N.D.G.A.[10,16,18,33], but other substances have an activating effect. The principal activators which have been reported are all surface active agents which stabilise the system in such a manner that the substrate is more available to the enzyme. When sodium linoleate at pH 9.0 is used the reactive system is in a more homogeneous state and these substances have no activating effect[10]. A crystalline substance was obtained from soya-beans[7, 34,35] which had an activating effect on the system but this was again proved to be acting on the availability of the substrate as all the coupled reactions of lipoxidase are not activated by it equally[35].

The action of lipoxidase can therefore be summed up as the oxidation of linoleic acid, linolenic acid and arachidonic acid at their double bonds and the coupled oxidation with these acids of natural pigments. This could be due to a chain reaction initiated by the enzyme or to the more usual "lock and key" mechanism attributed to enzymes in general, in which an active centre in the protein forms a complex with the substrate. Results in support of both these theories have been obtained but they have not been conclusive enough to confirm one or the other. This disparity in results has been due to the great differences in the experimental techniques of the various workers. In many cases no effort was made to obtain a pure enzyme or a homogeneous substrate system so that other oxidising systems, for example haematins, may be playing a part. Therefore it is imperative if any advance is to be made in the knowledge of the reaction that a sample of enzyme be obtained which is free from haematins.

Other Lipoxidase Sources

Although lipoxidase has been reported mostly in the root, stem, leaves and root nodules of legumes with

soya-bean being the most concentrated source[5,36,37,38,39] it has been detected in many other vegetable tissues. Potato, potato juice, radish and asparagus have all been reported to contain it[5,39,40,41].

Its presence in flour has been known since the early days and Miller and Kummerow[42] showed from examination of mill stocks the greatest activity was found in the germ fraction, but that the branny stream also showed activity. Recently, Blain and Todd[43] showed that the scutellum and the embryo were the most active centres in the wheat berry. They also found that the activity in the bran was associated with the aleurone layer. Environment appears to have little effect on the lipoxidase activity of wheat but there is a large variation in the different varieties[35,44].

The unsaturated-fat oxidases are generally associated with regions of the plant that are alkaline or neutral, where water loss is taking place, beneath wound surfaces or where inhibitols or antioxidants are absent or inactivated[45,46]. Lipoxidase is associated with the soluble parts of the cytoplasm and is not associated with the mitochondria[27].

Lipoxidase appears to play a part in the early stages of germination of soya-beans[47], but the actual function is not known. Holman[48] has suggested that

lipoxidase initiates the oxidation of unsaturated acids in the seed and therefore initiates the metabolism of these substances for energy or synthetic purposes. On the other hand, according to Teppel's theory[18], lipoxidase might oxidise the antioxidants in the seed, using linoleic acid as a prosthetic group, and producing hydrogen peroxide which could be used by catalase in further oxidations. This would appear to have corroboration from the association of haematin with lipoxidase in the seeds but seems to be in disagreement with Van Fleet's finding that oxidative enzymes are never associated with antioxidants in the tissue[46]. It has also been suggested that before the lipoxidase acts in the seed, physical changes occur in the fatty acid substrate which make it more susceptible to attack[27].

In corn seedlings it has also been found that lipoxidase takes part in the early stages of germination, the greatest oxygen uptake being during the second and third days[49].

Lipoxidase has not been identified in animal or microbiological materials[48,50,51,52]. It has been reported in animal tissues several times[53,54,55] but recently with improved assay techniques this unsaturated

fat oxidation has been shown to be due to haemoglobin and myoglobin[50,56]. Tappel's method[51,56,57] of differentiating between haematin oxidation and lipoxidase oxidation in animal tissues is based on the fact that haemoglobin catalyses linoleate oxidation only in emulsions, whereas soya-bean lipoxidase catalyses oxidation both in emulsion and in homogeneous substrate.

"Lipoxidase" activity has been reported in fish tissues[58,59] but the reaction described is sensitive to cyanide[60] and it may well be due to haematins rather than lipoxidase. Again the importance of adequate methods of distinguishing between haematin and lipoxidase catalysis is apparent. While it is comparatively easy to distinguish between the action of haematin-free lipoxidase and lipoxidase-free haematin with existing methods, plant extracts may contain mixtures of the two systems and give rise to confusion. The characteristics of such mixtures can only be adequately studied if haematin-free lipoxidase is available.

2. The Purification of Lipoxidase

General Methods of Enzyme Purification

The purification of enzymes is extremely difficult because of their low concentrations in plant and animal materials, their instability and their colloidal nature. Some of the methods used have been reviewed by Northrop[61] and Sumner and Somers[62]. In 1926, Sumner succeeded in crystallising urease[63], and since then some thirty-five enzymes have been separated as crystalline proteins. From the experience of these separations it has been shown that no one method can be relied upon to lead to the isolation of an enzyme. Any successful process must be worked out largely by repeated trial with gradual and systematic variation of the conditions of fractionation. The general rule is that enzymes are handled by the techniques of protein separation. But it is most important that in using these techniques, solutions of as high a concentration as possible must be used so that weighable solid precipitates are available and not simply dilute solutions. In addition, concentrated solutions of proteins are more stable and yield more clean-cut fractions[61].

The entire process of protein fractionation depends on differences in the solubility of the different proteins. This solubility bears a relationship to the chemical structure of the protein and therefore can be highly specific. The solubility of proteins, as well as depending on the inherent characteristics of the molecule such as crystal lattice energy and the presence of different alkyl or ionic groups, is also affected by its surroundings. Thus protein solubility is affected by intermolecular forces in the solid in equilibrium with the solution, the ionic strength of the solutions and the presence of salts.

Salts affect the solubilities of proteins in two ways, namely a "salting-in" and a "salting-out" effect[64]. Proteins commonly become more soluble in the presence of added salts due to attractive inter-ionic forces between the salt and the protein. This "salting-in" effect is found to increase with salts commonly used for this purpose in the order sodium sulphate, ammonium sulphate, sodium chloride and calcium chloride.

However, if neutral salts are added to any solution of a non-electrolyte in water, where the

solute has a dielectric constent less than that of water, the solubility of the solute is decreased. The relative "salting-out" effects of different salts are very nearly the same no matter what the nature of the substance being salted out. Ammonium sulphate has the greatest effect followed by sodium sulphate. For most proteins in dilute salt solutions, the "salting-out" effect is far more than counterbalanced by the "salting-in" effect. In more concentrated salt solution, the solubility of the proteins passes through a maximum with increasing ionic strength and at still higher ionic strengths there is a rapid decrease in the protein solubility. For example, at ionic strength 1.0 using sodium sulphate horse carboxyhaemoglobin has its maximum solubility and then the solubility decreases[63]. Ammonium sulphate on the other hand does not show the maximum solubility peak until 1.5. The use of neutral salts in protein separations thus offers a wide range of protein solubilities at varying salt concentrations.

The above is true of aqueous solutions but if salts are added to an aqueous solution containing alcohol the solvent effect of the salts increases and will increase with raising the alcohol concentration[66].

Of course if alcohol or acetone are added to an aqueous solution of proteins the decrease in the dielectric constant of the solvents leads to a great decrease in the solubility of the protein. It also however leads to denaturation of the protein unless the precipitation is carried out at low temperatures, preferably below 0^{o} [67].

Protein solubility is also affected by hydrogen ion concentration because the number of charged groups in a protein varies with pH. In strongly acid solutions the NH, H and NH_2 groups show positive charges while in alkaline solutions the OH, COOH and SH groups show a negative charge[68]. At pH values between 4 and 8, at which most fractionations are carried out, the net charge on the protein molecule is much smaller than in the extreme acid or alkaline ranges, but the total number of positive and negative groups reaches a maximum somewhere within this range. Commonly at or near this isoelectric point of the protein where the concentration of anions and cations is equal the solubility reaches a minimum and increases with change of pH to either side of this point. As the ionic strength of the solution increases the minimum solubility increases rapidly and moves more to the acid side[64]. The wide difference

between different types of proteins in the form of their solubility curves at different pH values and different ionic strengths provides a very great range of possible conditions which may be chosen in order to obtain separation of the proteins.

Temperature also affects protein solubility. Increase in temperature decreases solubility of certain proteins especially in the "salting-out" range[69,70] but usually there is an increase of solubility with rising temperature[64].

Other factors which influence protein solubility are interaction between anions and cations of different proteins and interaction between proteins and other organic non-protein anions and cations[68]. The former must be guarded against by carrying out fractionations under conditions at which all the protein components present carry a net charge of the same sign. Little is known of either effect and they have found no general use in protein separations.

Therefore, from the above considerations of protein solubility the methods of enzyme purification have developed along the following lines:

1. "Salting-out", by the addition of such a salt as will reduce the solubility of specific

proteins, has perhaps been the most widely used. The salt most commonly used is ammonium sulphate but sodium sulphate, phosphates and magnesium sulphate have also been used. Ammonium sulphate is generally preferred because a much greater range of salting out is attainable than with any other salts. It has the drawback of being a poor buffer. The potassium phosphate precipitants introduced by Cohn[71] are sometimes preferred due to the fact that the pH is well defined and can be stabilised at any given value over a wide range of ionic strengths. Sodium chloride can be used to separate proteins which can be readily salted out.

2. Acidification may be employed to change the pH to the isoelectric point to precipitate the protein.

3. Alcohol, acetone, dioxane and other related substances can be used to separate proteins by precipitation but for enzyme purification this must be done at low temperatures. Otherwise there will be denaturation of the

protein and inactivation of the enzyme.

4. Temperature difference effects can be used either by a cooling fractionation, e.g. in the purification of pepsin[72] or by heating, e.g. the removal of albumins by heating to 63° [34].

5. As well as these methods influencing the solubility of the protein, other physical methods of separation have been used. For example adsorption at a suitable pH on tricalcium phosphate, aluminium hydroxide, kaolin or some other adsorbent followed by centrifuging down the adsorption complex and eluting the enzyme from the adsorbent[73,74]. This is not a good method as a considerable amount of inactivation takes place. It can be useful in enzyme purification to adsorb inert material and colouring matter from the enzyme solution.

6. Crystallisation, by causing a concentrated and partly purified enzyme solution to become supersaturated by cooling, has also proved useful in certain cases. Crystallisation can also be induced by dialysing off certain salts which may be in solution.

7. The more recent techniques of ultracentrifuging and electrophoresis are used to separate the proteins in a concentrated solution of a highly purified enzyme. It is not useful to use these methods until the later stages of purification but they are very valuable tools in the ultimate refinement of the enzyme. The purity of the final concentrates can also be examined by these methods.

In all these methods of purification it is important that, where possible, several grams of material be obtained in each step, since concentrated solutions (1-10%) are essential in protein separations. Proteins in dilute solutions can be separated only with difficulty, if at all, while the same proteins in concentrated solutions may sometimes be separated with ease[61]. Also it is technically impossible to adjust conditions for precipitation, such as pH, salt concentration and protein concentration, with the sharp definition necessary when working with small volumes of solution.

Early Attempts at Purification of Lipoxidase

Most of the methods used by workers in this field have been based on the well known techniques used for the separation of protein fractions. These have included salting-out techniques, precipitation with organic solvents, crystallisation and electrophoresis. The success of these efforts has varied and the results are not always comparable due to differences in the methods of assessment of activity and in the units employed as standards. This makes accurate interpretation of the various results somewhat difficult.

Sullman[75] was able to isolate a product with a 66-fold concentration by fractional precipitation with acetone of a water extract of soya-beans but a great deal of activity was lost in the process.

In the same year, 1943, Balls, Axelrod and Kies[7] used ammonium sulphate at pH 6.8, experiments which eventually led to the preparation of a pure sample of lipoxidase by Holman[19]. Balls and co-workers found that most of the activity precipitated between 0.47 and 0.5 saturation. The concentrate so obtained had an activity 115-fold that of a 2.5% water extract of soya, on a nitrogen basis. By calculation from the author's figures

it appears that 80% or more of the activity of the original solution is lost during this preparation.

A loss of only 10% was claimed by Franke, Monch, Kibat and Hamm[13] for a similar ammonium sulphate fractionation. Unfortunately these workers only obtained a 32-fold concentration of the activity.

A slightly simpler method of ammonium sulphate fractionation was used by Cosby and Sumner in America[76]. They extracted the soya with a potassium alum solution, thus avoiding ballast material, at pH 6.5 and precipitated the enzyme out with 66% saturated ammonium sulphate. A solution of this active precipitate was then fractionated up to 25% saturated ammonium sulphate to remove inactive material and dialysed to give the concentrate. A 60-fold concentration on a water extract was obtained by this method. Later Sumner prepared with Smith[77] a 50-fold concentration of the enzyme by a very simple method involving precipitation in the cold using 0.5 N sodium phosphate as the precipitating agent. Neither of these methods produced a highly active concentrate.

However, the really effective methods of concentration of the enzyme have been developed from fractionation processes based on the original ammonium

sulphate method of Balls and his colleagues[7]. Using such a method Theorell, Bergstrom and Akeson[34] prepared a 388-fold concentrate on a dry weight basis. This product was identified as a globulin of molecular weight 75-80,000, but on electrophoresis it was not homogeneous. They started with a water extract which after acidifying to pH 5.0 was treated with basic lead acetate to precipitate the active protein. A solution of the precipitate in M/5 sodium phosphate at pH 5.8 was brought to 60% saturation with ammonium sulphate which precipitated out an active fraction. This active fraction was dissolved in water, those proteins with solubility ranges differing from the lipoxidase proteins removed, and the albumins precipitated out by heating to 63° for five minutes. The active fraction was reprecipitated with 55% saturated ammonium sulphate and after being redissolved in water it was dialysed to free it from sulphate ions. After a further precipitation by means of carbon dioxide at 0° the active precipitate was again dissolved in sodium carbonate solution and dialysed. This solution was finally separated by electrophoresis in the large Tiselius apparatus at 55 mA and pH 5.8. Unfortunately the yield was very low as much inactivation

Purification of Lipoxidase

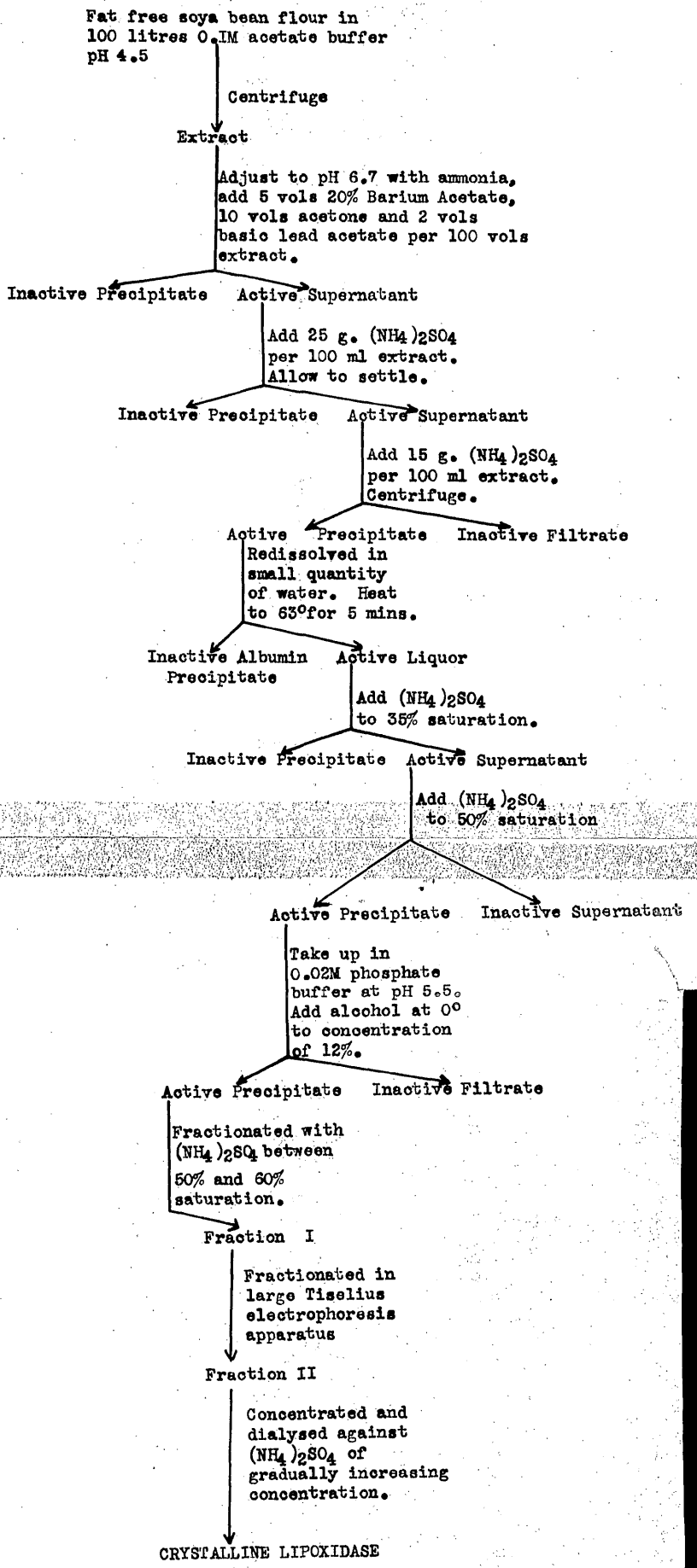


Fig. 3

of the enzyme had taken place during the various manipulations. A calculation of the activities recorded shows that despite the highly active product approximately 83% of the original activity was lost.

Holman's Preparation of Pure Lipoxidase

The most active concentrate yet isolated was produced in 1947 by Theorell, Holman and Akeson[19,79] who prepared a lipoxidase concentrate which was electrophoretically homogeneous and ultracentrifuge sedimentation values confirmed that this was a sample of pure crystalline lipoxidase. The scheme of purification used was based on the previous work of Theorell, Bergstrom and Akeson[34,78], ammonium sulphate being the main precipitating agent. An outline of the purification is shown on the opposite page and a detailed copy of the experimental procedure is given in Appendix I.

The enzyme was extracted from soya-beans with acetate buffer at pH 4.5, chosen because although the total activity extracted was greater at higher pH values, the authors found that the amount of extraneous protein was also greater.

The first step in the purification involves

precipitation with lead and barium acetate at pH 6.7 to remove a gummy material extracted from the soya-beans. In previous purifications it had always been found difficult to remove this material from the final concentrates.

As the enzyme solution was very dilute the active material was precipitated out with ammonium sulphate and redissolved in a small amount of water and the albumins were removed from this solution by heating at 63°.

The solution was then fractionated with ammonium sulphate, the active material being precipitated between 35% and 50% saturation. The concentration of the enzyme at this stage varies in successive preparations, in one it was reported as 230 units/mg., i.e. a concentration of 31-fold on the original solution and in another as 45 units/mg., a concentration of 6-fold. The reason for this variation in the activity of the precipitates may be due to lack of pH control during the precipitation. There is also a variation in the amount of activity lost in the fractionation. By calculation from the authors' figures, when using a small amount of active solution, the inactivation is 17.3% but on using a large volume

the inactivation is 32%.

Following the ammonium sulphate fractionation, the active precipitate was taken up in phosphate buffer as shown in Fig. 3. In the subsequent alcohol precipitation a variation was also found when using different volumes of active solution. When using a smaller volume the active material precipitates out at 3% alcohol, but 12% alcohol was needed to precipitate out the active material from a larger volume of solution. There was again a variation in concentration of the enzyme and inactivation of the enzyme in the different preparations. Concentrations of 5.6- and 2.2-fold appear to be obtained in different fractionations. These variations are not due to a pH difference as the solutions were all controlled at 5.5 but may have been due to a slight change in temperature, which is known to have an effect on alcohol precipitations. The total inactivation was less than with ammonium sulphate, being 2.5% when a large volume of material was used, but in a small precipitation it was as high as 64%. It appears from these experiments that precipitation with alcohol could be a more effective method of precipitation than ammonium sulphate if the volume of material being handled was kept large.

The final product obtained after a further

fractionation with ammonium sulphate and then electrophoresis had an activity of 850 units/mg., i.e. a 115-fold concentration from the crude soya on a dry matter basis. On the basis of Theorell's published figures, the present writer has calculated that 97% of the original activity was lost during the course of this purification.

The product was judged to be a homogeneous protein from sedimentation and diffusion measurements. The molecular weight was 102,400.

The authors report that isolation of the enzyme has been found not to be strictly reproducible with different batches of soya-beans, and suggest that pilot experiments and continued assay of the fractions are necessary. Since no reports of a successful repetition of this experiment have been published it is likely that there are other difficulties which have not been so far reported.

Kunkel's Method of Concentration

Holman suggested[1] that the use of mixed phosphates as an alternative to ammonium sulphate in the precipitation of lipoxidase might be an improvement. Their main advantage was the ease of pH control offered by their use. Kunkel[17] in 1953 followed this

Kunkel's Method of Concentration

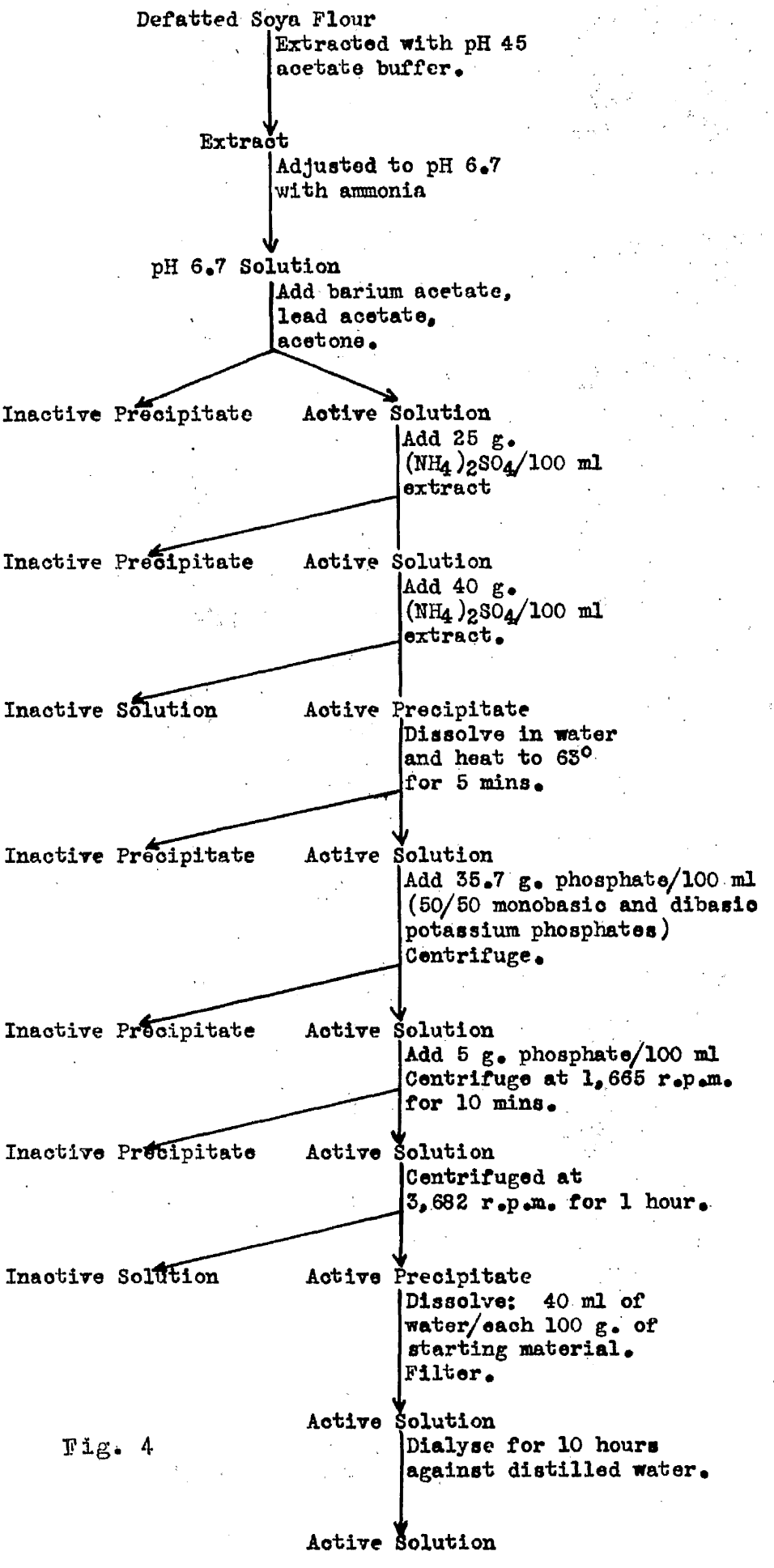


Fig. 4

suggestion and introduced a preparation which was based on that of Holman[19,79]. The method of purification was similar up to the precipitation of the albumins by heat, but instead of fractionating the active solution with ammonium sulphate an equimolecular mixture of monobasic and dibasic potassium phosphates was used. The fractionation is shown in Fig. 4.

To complete the preparation, differential centrifugation was used.

There is no indication of the concentration obtained by this method or what percentage of the enzyme activity is lost by denaturation.

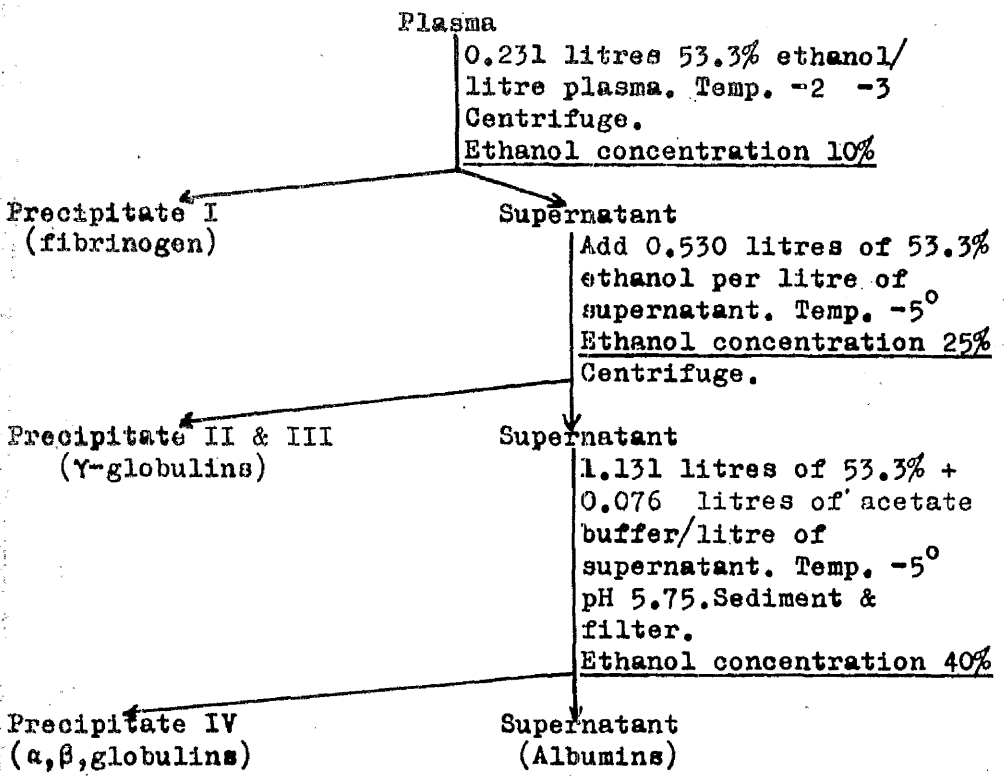
Use of Alcohol in Protein Purification

Alcohol, acetone, ether and other related reagents have been used since early in the nineteenth century to precipitate proteins, to wash them free from impurities and to prepare them for analytical study. The proteins were always denatured in these processes and therefore if these reagents are to be used in separating proteins without changing their properties more careful precautions have to be taken.

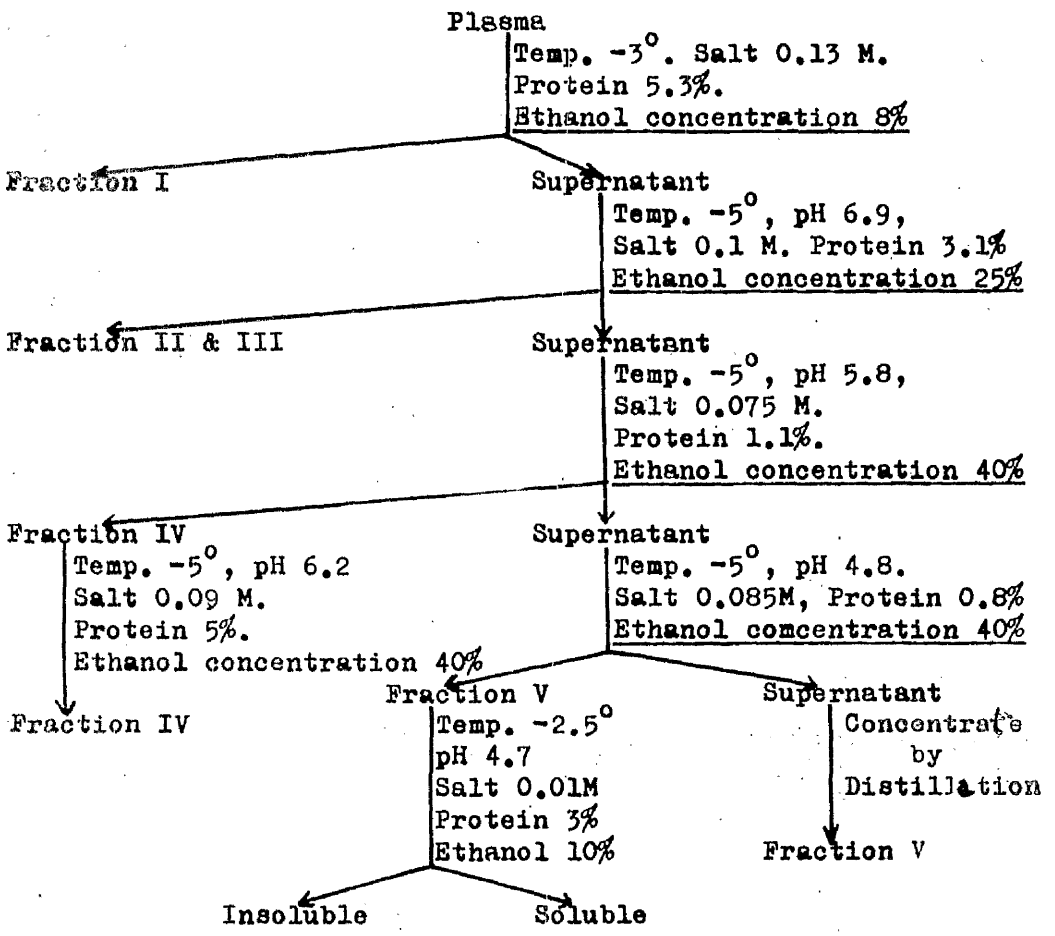
One of the first concentrations of proteins using

Fractionation of Plasma Proteins

Method I



Method II



F

Fig. 5

alcohol was that of Mellanby[80] in 1908 when he separated diphtheria toxin. However, although it was used several times in the intervening years, it was not until 1940 that Cohn[67] reported a systematic fractionation of blood plasma by alcohol at a low temperature. In this fractionation were separated plasma fractions in a concentrated and undenatured form. This was a tremendous advance on any alcohol separation done hitherto when a large amount of denaturation had always taken place.

In the methods introduced by Cohn and his co-workers there was a strict control of all the variants in the system, e.g. pH, temperature and salt concentration. An outline of their fractionation of blood plasma is shown opposite[64,81,82]. Although the actual fractions separated are not of interest in the present discussion a review of the experimental procedures used gives an indication of the precautions which must be taken in using this method.

All the operations were carried out in a cold room at -5° so that the temperatures of the solutions were always kept below 0°, the upper limit of the temperature range. As soon as sufficient alcohol had been added the temperature was kept at 0° or less and if the alcohol concentration was greater

than 20% the temperature was -5° or lower. As the mixing of alcohol and water evolved heat, careful cooling was used. The solution was stirred vigorously so that the temperature equilibrium was rapidly attained. Since there is a considerable evolution of heat on mixing alcohol and water, alcohol additions must be made very slowly.

These precautions of vigorous agitation and addition of alcohol through a capillary also prevented any previous precipitation of protein through a part of the solution attaining, even temporarily, an unduly high ethanol concentration.

A careful control of the pH was maintained either by bringing the plasma to the desired pH or by the addition of an ethanol solution containing buffer during the experiments. Cohn[63] later even effected improved separations by adding controlled amounts of metal ions to the solution.

This method of fractionation has been used successfully many times for plasma separations and has also been attempted in other fields. Edsall[64] has reviewed these but some mention of the use that has been made of this type of fractionation in the separation of lipoxidase might be of interest.

As already indicated, Holman[19,79] used alcohol fractionation in his purification of the enzyme lipoxidase. He found that at 0° the activity in a soya-bean concentrate could be precipitated out with 3-12% alcohol where the pH of the system was 5.5. The results of his fractionations varied but he did find that with larger volumes of material, alcohol fractionation gave as narrow a separation as ammonium sulphate with less inactivation. Kahn[59] also used precipitation with alcohol at 0° in the separation of "lipoxidase" from fish. His method consisted of removing an inactive material by raising the alcohol concentration to 18%. Then the alcohol content was raised to 60% and a precipitate containing a lipoxidase "activator" obtained. The enzyme activity was still in the filtrate and was recovered by vacuum evaporation. It appears unlikely that this material is similar to soya-bean lipoxidase since denaturation would be expected to occur at this concentration of alcohol at as high a temperature as 0°.

In conclusion, the main advantage of using alcohol fractionation is the increase of specificity over "salting out" procedures. The precipitation of proteins by alcohol depends upon the chemical

characteristics of the protein and not just on the size and shape of the molecule. Therefore it is an improvement on "salting-out" in concentrated solutions which depends on the physical properties of the molecules. Alcohol also gives wider conditions of precipitation. "Salting-out" depends on four variables - salt concentration, protein concentration, pH and temperature but the addition of alcohol in such a complex system increases the range of possible conditions which can be chosen for the separation of any given component.

Thus in the purification of lipoxidase, it appears that if the proper precautions were taken, the large amount of inactivation of the enzyme which occurs in other fractionations could be reduced by using alcohol at a temperature below 0^{o}.

EXPERIMENTAL

EXPERIMENTAL

1. Control Methods and Preliminary Studies

Rationale of the Experiments

To follow the course of successive stages of separation of the enzyme a suitable assay system was required. It seemed desirable to choose a method which measured the direct effect of the enzyme on the fatty substrate rather than a coupled reaction such as the carotene bleaching method described by previous workers in this laboratory[84]. Coupled reactions are more sensitive to the presence of inhibitors[7,33] and are linear over a restricted range of enzyme concentrations[76]. In addition Tappel has reported that spectrophotometric methods are less sensitive to haematin interference[28]. For these reasons a modified form of previous spectrophotometric methods[28,78] was adopted.

Some form of electrophoresis also seemed desirable as an aid to following the course of the separations, and a filter paper apparatus was constructed to this end.

So far as can be discerned from published reports, previous separations have been based on empirical methods. It was decided that a systematic

study of the variables should be made in the preliminary stages of the separation, although it was appreciated that at later stages more arbitrary methods might be required.

Lipoxidase Assay

The methods which have been used to determine the activity of lipoxidase are:-

1. The iodimetric measurements of the peroxides formed[62].
2. The measurement of the oxygen uptake of the system using the Warburg apparatus[18,21, 34,60,75,85,86].
3. The measurement of the coupled oxidation of a suitable pigment, usually either carotene or bixin, by following its bleaching rate[7, 23,29,38,76,87].
4. The measurement of the coupled oxidation of an easily oxidised inorganic salt such as ferrous iron, in this case the ferric salt produced being determined as thiocyanate[6,20,88].
5. The spectrophotometric measurement of the diene conjugation developed in pure sodium linoleate by the action of the enzyme[8,15, 18,20,28,78,79].

The last of these methods was preferred for the reasons already given.

The method depends on the fact that during the lipoxidase catalysed oxidation of linoleate, conjugated hydroperoxides are formed on the site of the original methylene interrupted double bond system of the linoleate. The degree of conjugation is thus proportional to the amount of oxidation which has taken place and the process can be readily followed from the ultra-violet absorption band of the conjugated system which shows a maximum in the region 232-235 mμ. The method has been subject to modification in the hands of various workers[28,78], but all these modifications have given proportionality over a reasonable range of time and enzyme concentration.

In adapting this method to the present purpose, which called for speed and convenience of operation, the oxygen saturated solutions used by previous workers were abandoned in favour of air saturated solutions, the reaction volumes were increased to facilitate handling and a new reaction stopping reagent was used to replace the ethanol used in earlier methods. Some sacrifice of precision was justified by the increased number of observations which could be made in a given time. Speed of

assay was found to be an essential requirement in attempting to follow successive stages of a separation.

The substrate used was sodium linoleate and this was placed in a pH 9.0 ammonia/ammonium chloride buffer so that its final concentration in the assay system was 1.4×10^{-4}M. After thorough mixing of the substrate/buffer mixture the enzyme active solution was added through a microburette with a capillary attachment. The enzyme was allowed to act for a suitable time, the solution being mixed continuously during this time. To stop the reaction 20% aqueous sodium hydroxide was added.

The solution was compared on the Uvispek spectrophotometer at 234 mμ against a control solution prepared as follows. To the substrate/buffer mixture was added 20% sodium hydroxide. After thorough mixing the enzyme active solution was added, the volume being the same as added to the test solution.

This assay is described in detail in Appendix II.

In terms of this system a unit of lipoxidase activity is defined as that amount of lipoxidase which on acting on sodium linoleate in the assay system described above for one minute increases the optical

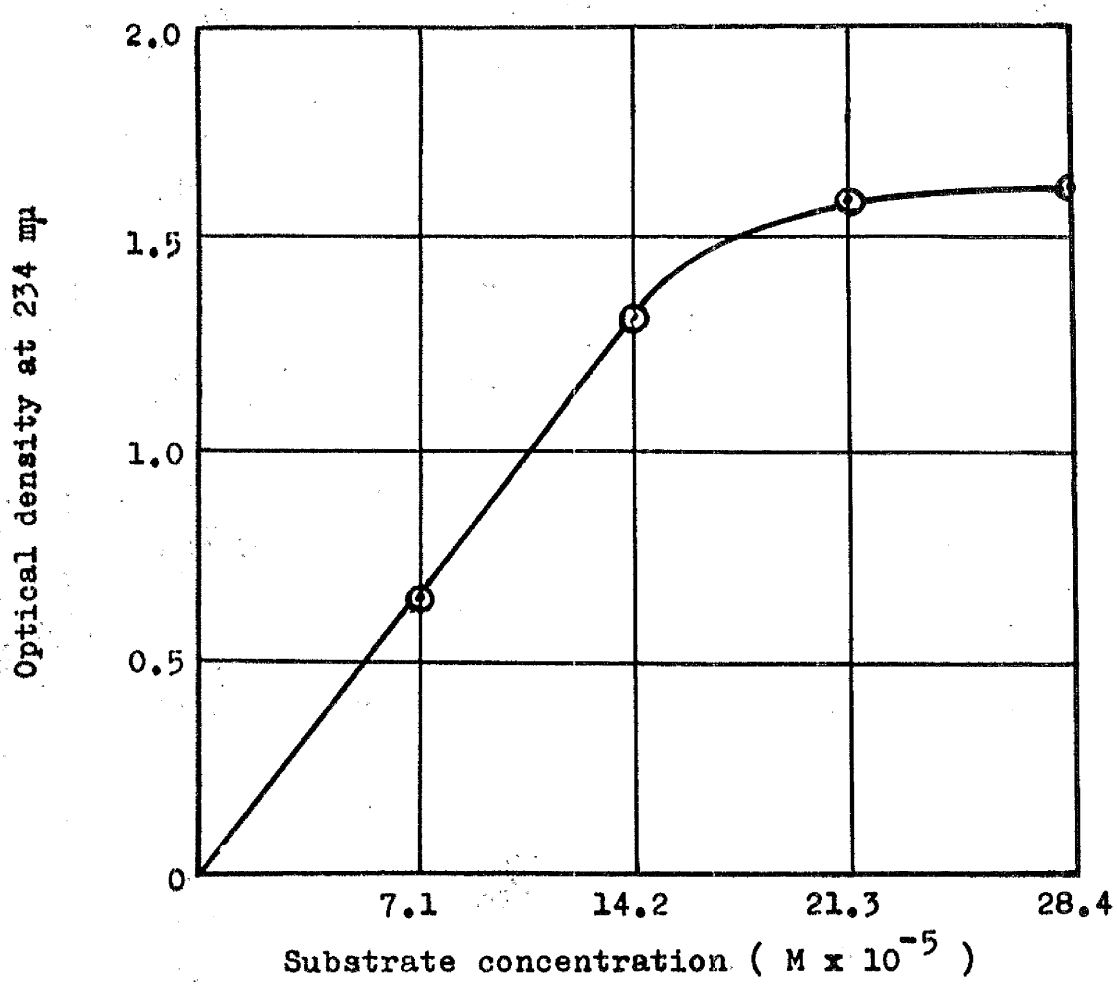


Fig. 6

Variation of optical density
with substrate concentration

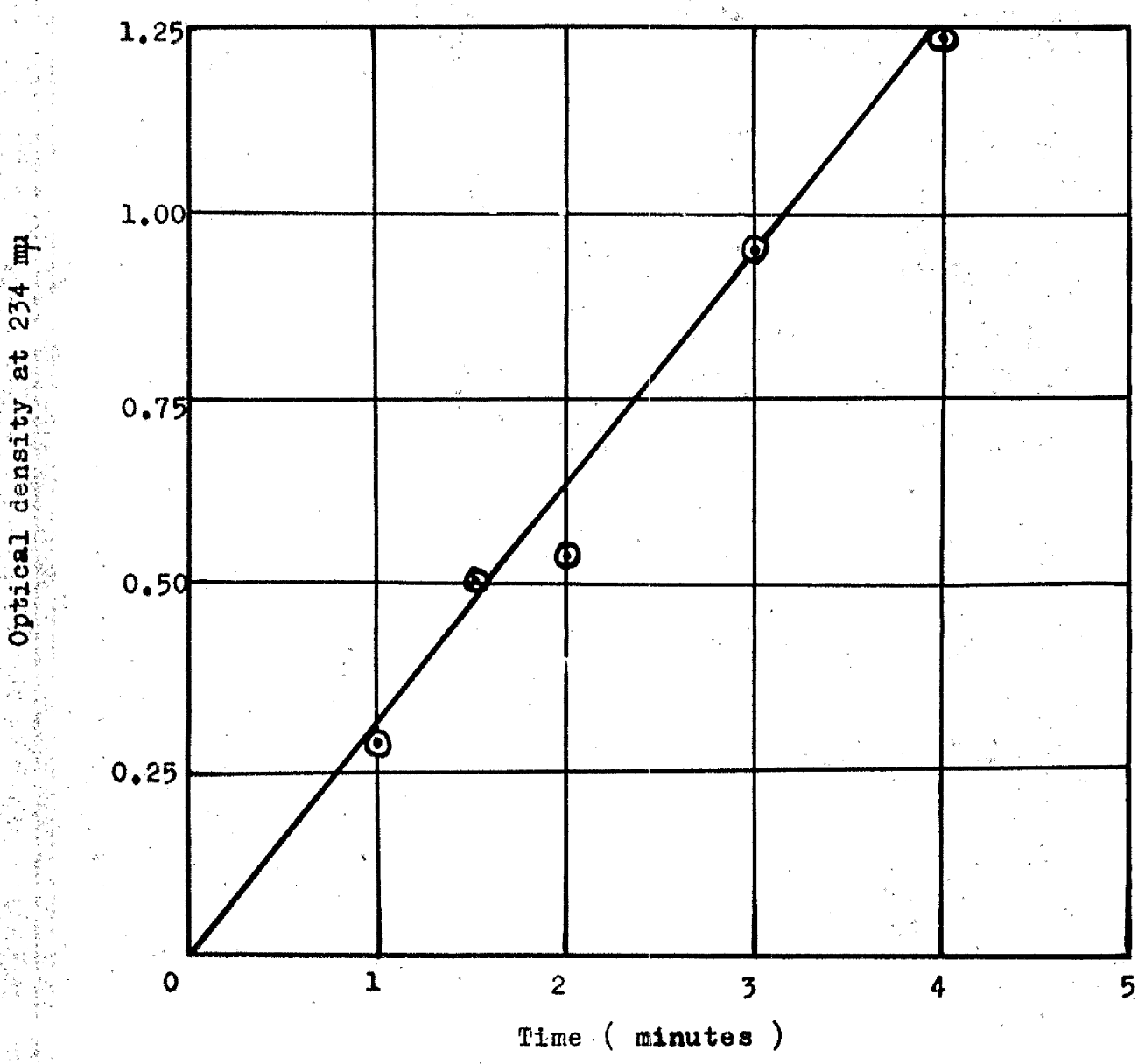


Fig. 7

Variation of optical density

with time of reaction

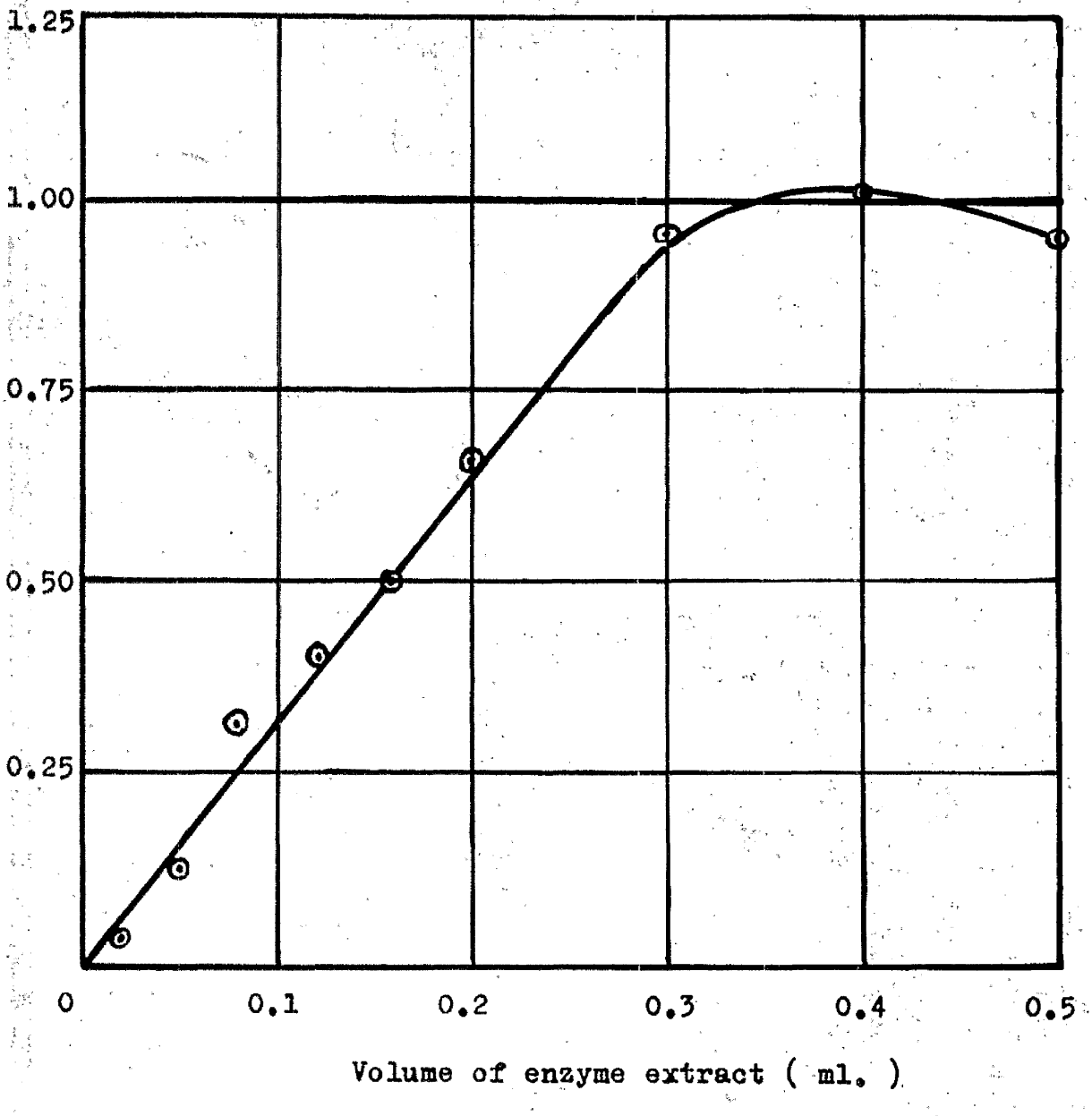


Fig. 8

Variation of optical density
with enzyme concentration

density of the solution at 234 mu by 0.1 unit.

The characteristics of the system are shown in Figs. 6, 7, 8. Fig. 6 which shows the effect of substrate concentration on the reaction at pH 9, is in agreement with a curve obtained under similar conditions but at pH 8.0 by Blain et al[84]. On the basis of this curve and on Blain's data a substrate concentration of 1.4×10^{-4}M was chosen for the reaction system. At this level and with enzyme solutions suitably diluted so that the observed increase in optical density during the reaction does not exceed a numerical value of 1, small errors in substrate additions have a neglible effect on the reaction velocity. In practice it was desired to avoid the observation of optical densities greater than 1 owing to noticeable decrease in sensitivity of the instrument.

Fig. 7 shows the expected linear relationship of optical density with time of reaction as reported by Tappel et al[28].

Fig. 8 indicates the range of linearity of the system with enzyme concentration. The relationship is linear up to an enzyme concentration which gives an optical density of 1.0.

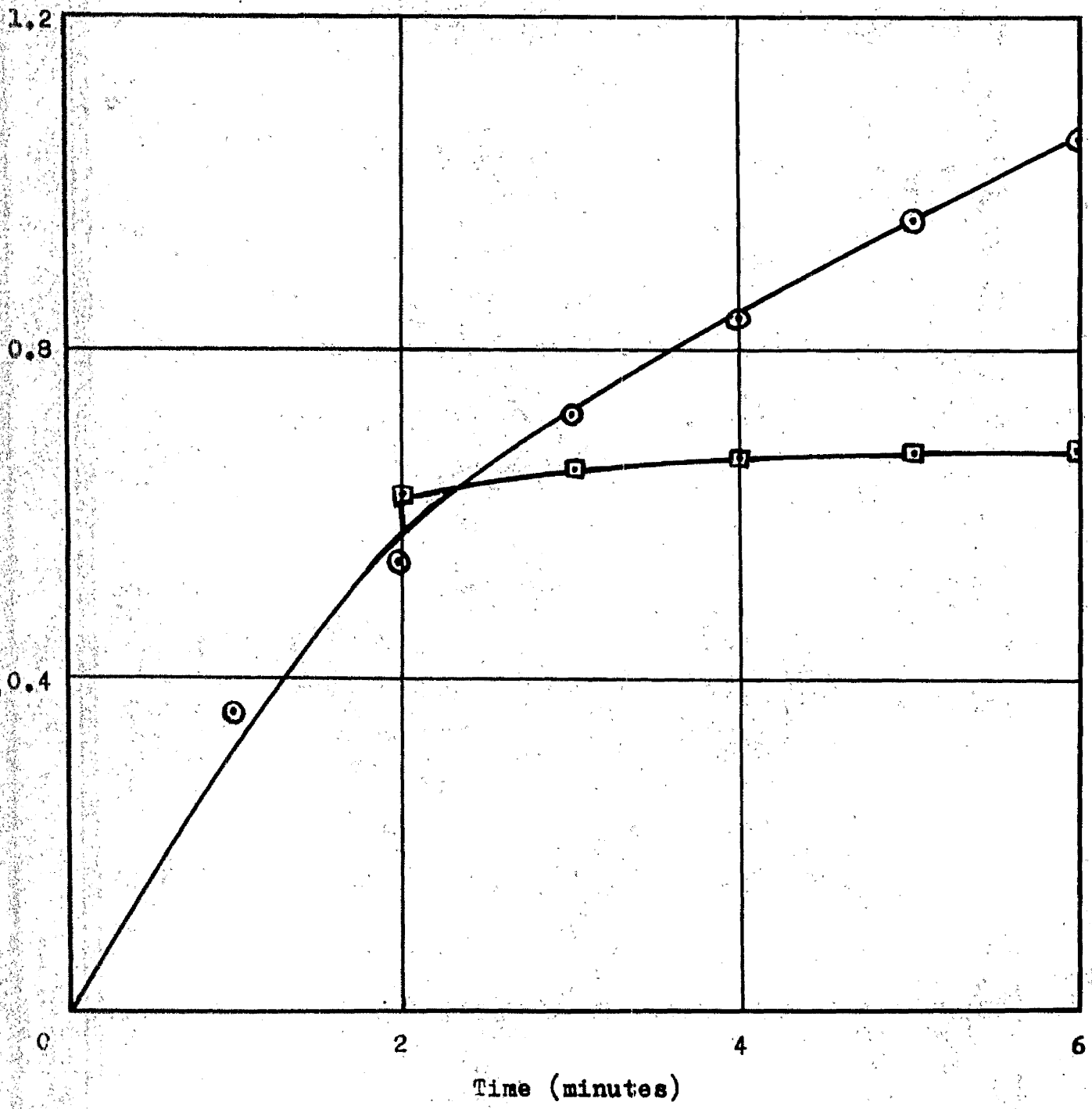


Fig. 9

—⊙— 1 ml. substrate and 0.1 ml. enzyme in buffer

—⊡— 1 ml. substrate and 0.1 ml, enzyme in buffer after the addition of the stopping reagent (20% NaOH) at 2 minutes

From these data it is clear that the proposed system gives a reasonable range of conditions under which proportionality between time and enzyme concentration may be obtained.

The advantage of using a homogeneous system such as this instead of a heterogeneous system e.g. the carotene assay, is two-fold. The activating substances present in soya-bean extracts will have no effect on the enzyme measurements. They are acting as surface active agents and therefore cannot improve an already homogeneous system. Also it has been reported that haematins do not have an oxidising effect on such a system[51].

In carrying out early work with this system a drift in spectrophotometric readings was observed after the addition of the sodium hydroxide (Fig. 9).

TABLE I

The effect of assay reagents on optical density drift

Reagent added	Optical density observed at 0 mins.	Optical density observed at 5 mins.
1 ml. substrate in buffer	0.160	0.158
1 ml. substrate in buffer 1 ml. sodium hydroxide	0.173	0.169
1 ml. substrate in buffer 1 ml. sodium hydroxide 0.1 ml. enzyme extract	0.330	0.425
1 ml. substrate in buffer 1 ml. sodium hydroxide 0.1 ml. enzyme extract 1 ml. 0.1% D.C.P.	0.704	0.802

Table I shows the effect of various combinations of assay reagents on the optical density of the reaction mixture when observed against a water blank. It is clear that the drift only takes place in the presence of enzyme extract, and that it takes place even at pH values at which no enzyme action would be expected. Moreover, it was found that when previously boiled enzyme extract was used, no drift took place.

Haematin compounds are capable of catalysing this reaction even under very alkaline conditions[89], and the fact that the drift was still found in presence

of dichlorophenol (D.C.P.) which is a specific inhibitor of catalase[90], did not eliminate the possibility of interference from other haematins. Accordingly, 5% potassium cyanide was added to the alkaline stopping reagent and this was found to eliminate the drift. Since lipoxidase is relatively insensitive to the presence of cyanide, while haematins are readily inhibited by it[91], it seems at least possible that the drift was due to the presence of these substances in the extract.

Paper Electrophoresis

Electrophoresis is defined as the movement of charged particles through a liquid under the influence of an applied potential. Paper electrophoresis has been developed for qualitative purposes and therefore has been useful in these experiments to follow the purification.

Many types of apparatus for paper electrophoresis have been developed. These have varied in the method of suspending the paper, e.g. inclined[92,93], vertical[94], and horizontal[95,96] papers have all been used. This controversy over the method of suspending the strips is due to the fact that there are difficulties in obtaining clear resolution of the fractions on the

paper because of (a) diffusion, (b) migration of ions due to the electrical field, (c) electric endosmotic flow, (d) evaporation, (e) hydrodynamic equilibrium between capillary and gravity forces, (f) electrical resistance changes due to concentration effects and (g) siphoning.

With horizontal strips there is a danger of flooding on the paper and also of the formation of concentration spots due to slight surface irregularities on the plates holding the strips in position. It has been found in some cases that it is difficult to get well-defined bands using this method.

Siphoning occurs very readily with vertical strips and therefore the proteins will be inclined to be washed off the paper. For these reasons it was decided to use inclined strips and the apparatus used was based on that of Flynn and Mayo[92]. Their apparatus does not have a cooling system but in short runs the difficulties arising from evaporation and condensation are not serious.

With protein and enzyme separations there have always been difficulties of developing an effective procedure for locating the separated bands.

Selective dyes[92] and auto-radiographs[97] using tracer elements have been used, and in the case of the separation of enzymes their reactions with an appropriate substrate have been used.[98]

In this case the methods of development studied were:-

1. Bromophenol blue by the method suggested by Flynn and Mayo[92].
2. Hydrolysis of the protein by trypsin to amino acids and development of the amino acids with ninhydrin[99].
3. Naphthalene black absorption by the proteins[92].
4. A "photographic" method studying the absorption of silver nitrate by the proteins by reduction to metallic silver with hydroquinone and sodium thiosulphate.

Summaries of these methods can be found in Appendix III.

With trypsin the bands were found to be ill-defined and the process was difficult to operate. The naphthalene black gave a very bad background colour although it did give quite clear bands. The washing of the excess dye was very tedious. The "photographic" method did not give distinct bands and the background

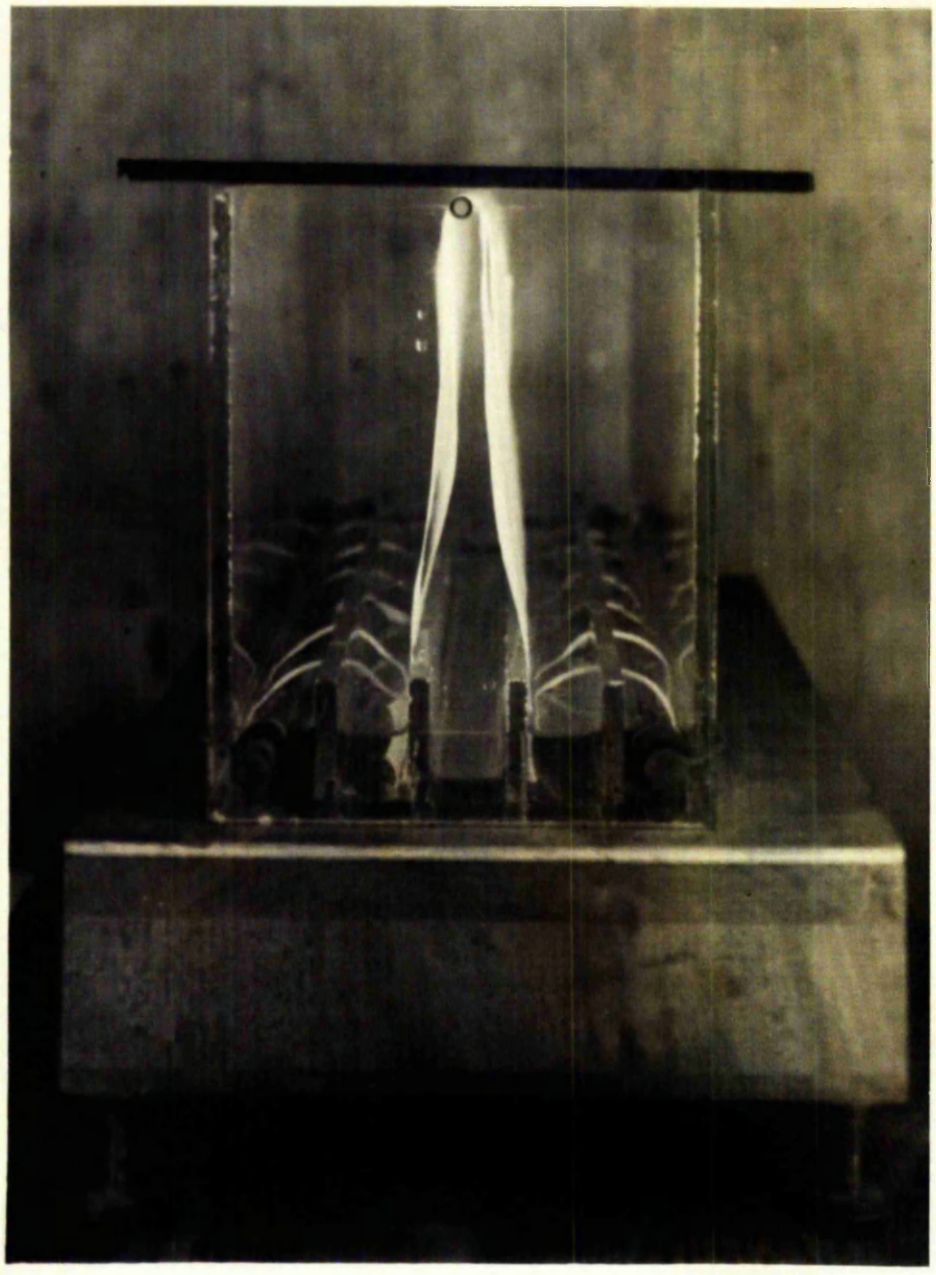


Fig. 10.

Electrophoresis Apparatus

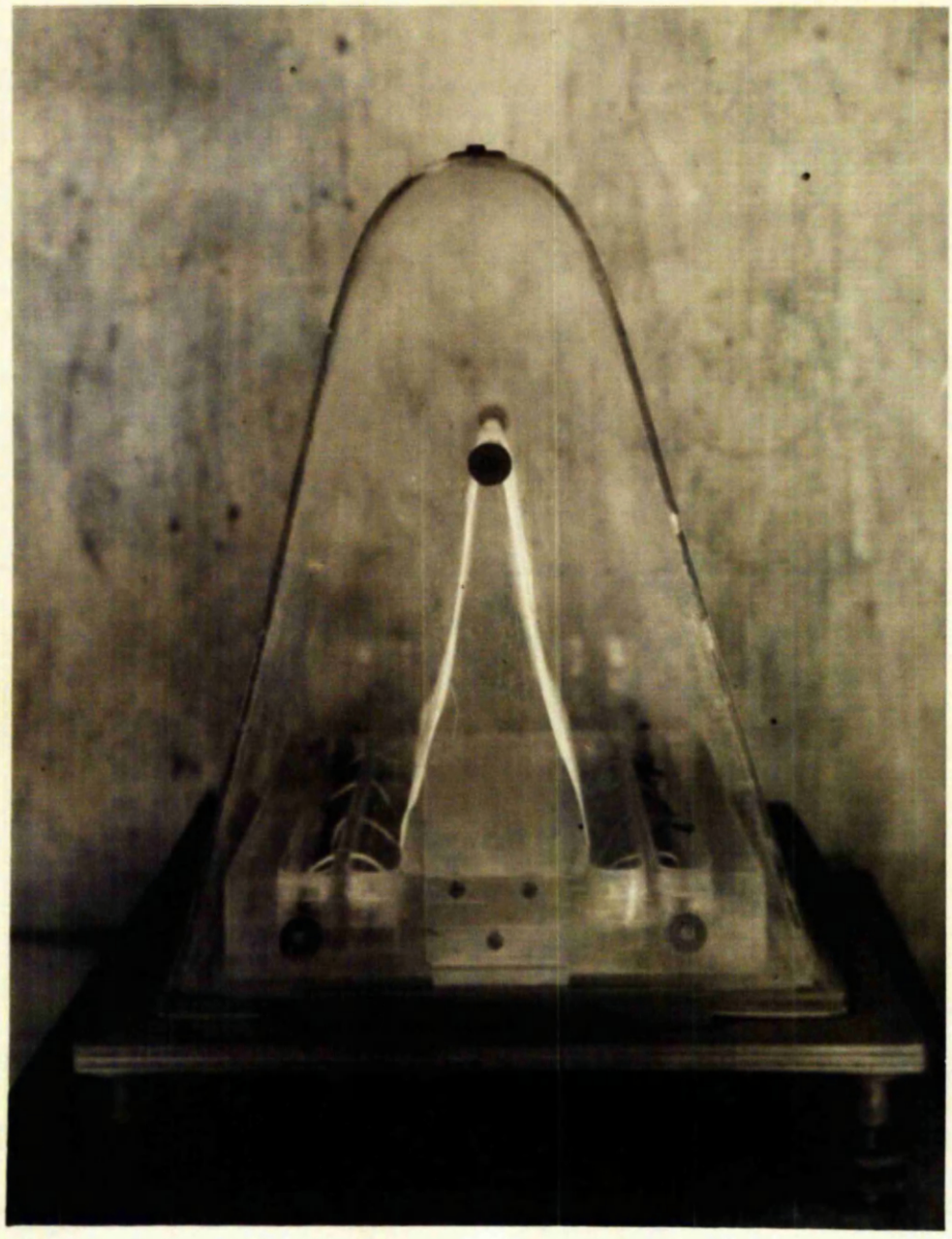


Fig. 11.

Electrophoresis Apparatus

colour was very bad. The bromophenol blue method was found to be the most satisfactory and was the method adopted.

The potential differences used in electrophoresis have varied from 120 to 600 volts. The tendency was formerly to use a high potential difference for a short time but this gives a strong electro-osmotic disturbance of the bands, and a lower potential difference has been more generally preferred. However, it is best to study the system to be used and to vary the potential difference accordingly. In these experiments the separations were found to be clearer if a potential difference between 200-300 volts was used.

The actual apparatus used is shown in the photographs opposite (Figs. 10 & 11). Fig. 11 is a modification of the apparatus, suggested by Thomson[93], to reduce condensation on the paper strips. The source of current used was a D.C. power pack supplying a fully stabilised D.C. output continuously variable from 100-1,000 V.

The method used was as follows:- the four compartments in the tank were filled with an M/15 phosphate pH 6.0 buffer to the levels of the wicks and

strips of Whatman No.1 filter paper were placed in position. After the filter paper had become saturated with buffer, the enzyme solution, which had been dialysed overnight against the phosphate buffer, was applied to the apex of the paper strip by means of a microsyringe.

The apparatus was allowed to run the allotted time and the strips were then dried for 30 minutes at 105°. They were developed using bromophenol blue.

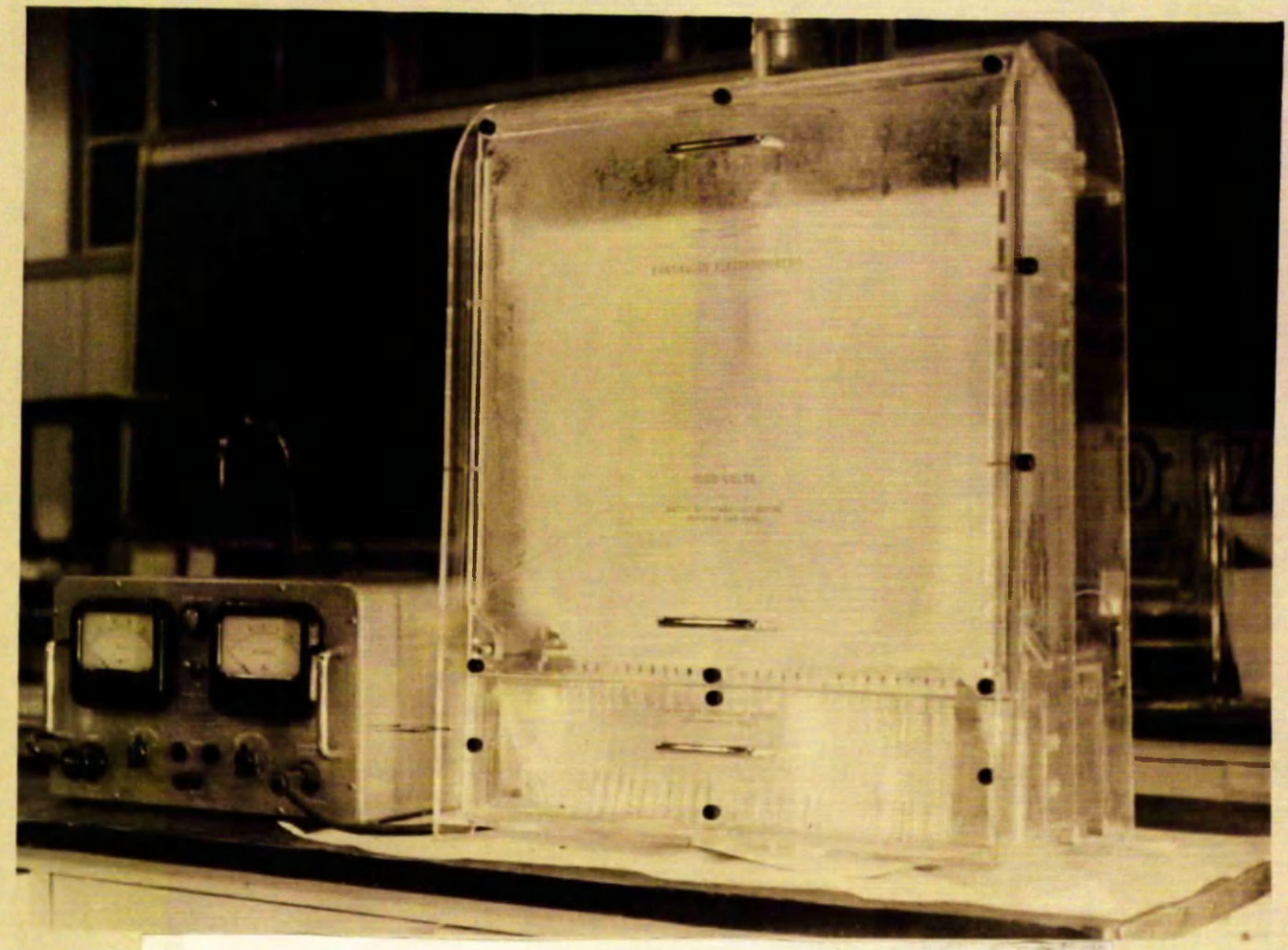
A detailed account of the apparatus and the experimental procedure can be found in Appendix IV.

In later stages of the preparation it was found that to obtain a refined product some method of preparative electrophoresis was necessary. As a Tiselius apparatus was not available a method of continuous paper electrophoresis was adopted.

In 1951, Durrum[100] described an apparatus in which, on a large square of filter paper supported vertically, the horizontal ionophoretic resolution was combined with a steady, non-resolving, vertical downward flow, the horizontally resolved components finally flowing off the bottom edge of the paper into collecting tubes. This is shown diagrammatically in Fig. 12b.

Fig. 12a.

Continuous Electrophoresis Apparatus



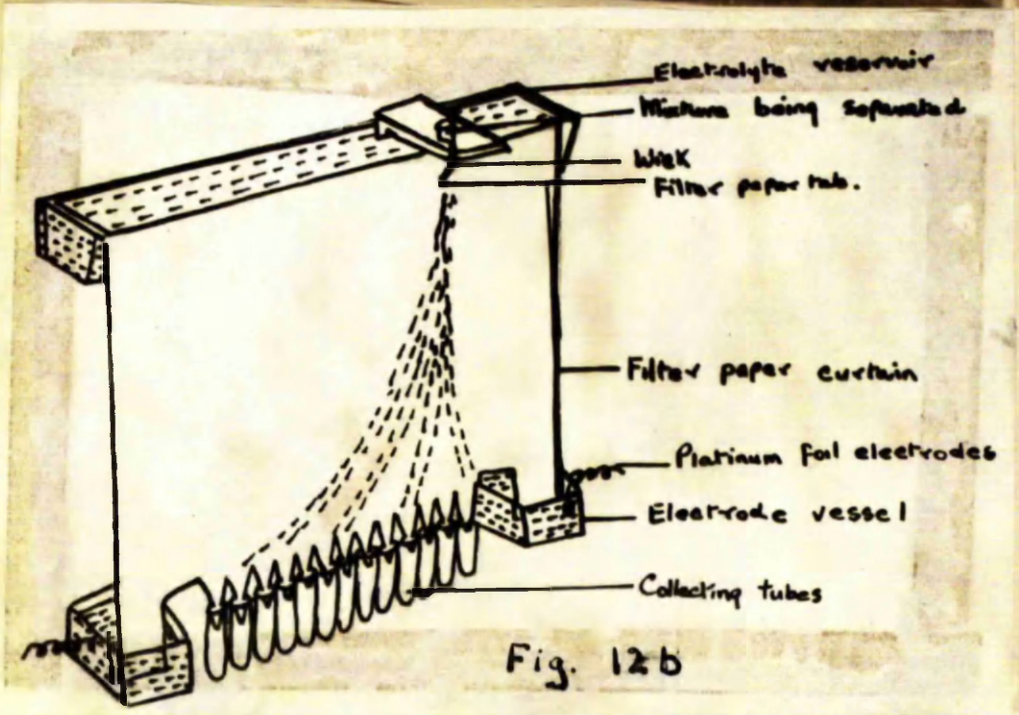


Fig. 12b

The apparatus used was the Shandon Continuous Electrophoresis apparatus which is an adaption of Durrum's design. This is shown in Fig. 12a. The buffer was pH 6.0 M/15 phosphate buffer and the paper was specially cut 4MM Whatman sheets. The voltage used was 6.5 volts/cm.

As it was found difficult to control the rate of addition of the lipoxidase preparation on to the paper by the wick supplied, a motor driven micro-syringe was made. This was led through the front panel of the casing so that the tip of the syringe touched the paper two inches from the top. By means of a rheostat in the circuit it was possible to vary the rate of addition from 1.5 ml. to 4 ml. per 24 hours. The rate of addition used was 3 ml. per 24 hours.

Distribution of Lipoxidase in the Soya-bean

Distribution of Lipoxidase in the Soya-bean

The soya-beans were split up into cotyledons, husk and embryo by manual dissection. The fractions were ground with mortar and pestle to a fine powder and extracted with (40^{o}-60^{o}) petroleum ether in a Soxhlet apparatus for four hours. After drying they were extracted with pH 4.6 acetate buffer for three

hours at room temperature, being shaken fifty times at the beginning of the extraction and fifty times each half hour. After centrifuging at 3,000 r.p.m. for ten minutes the clear liquid was decanted and tested.

TABLE II

Lipoxidase in Soya-bean

Part of Bean	%Composition of Bean	% Fat	Units/g.	Units/100g. Whole Bean
Cotyledon	89.9	17.7	50	4,495
Husk	7.7	0.5	0	0
Embryo	2.5	9.9	34	85
Whole	100.0	115.7	42	4,200

Blain[43] has shown that the unsaturated-fat oxidase activity of wheat, is much higher in the embryo than in other parts of the kernel, but as the table on this page shows, soya lipoxidase is found in greatest concentration in the cotyledons. There is therefore no advantage in using a particular anatomical part of the soya-bean as starting material for a lipoxidase purification.

Factors in the Extraction of Lipoxidase from the Soya-bean

1. Effect of grinding on extraction: in order to discover if the size of the particles of the ground soya had any effect on the amount of the enzyme and extraneous protein extracted by a pH 4.6 buffer, a sample of soya-beans was ground in a laboratory mill to the mesh sizes shown in Table III. "Coarse" consisted of beans which had been just broken but not crushed. After extracting for three hours with petroleum ether (40^{o}-60^{o}) in a Soxhlet apparatus, the defatted meals were extracted for three hours with pH 4.6 0.1M acetate buffer. The supernatant obtained on centrifuging at 3,000 r.p.m. for 10 minutes was assayed for lipoxidase activity. The results are shown in Table III.

TABLE III

Effect of particle size on extraction

B.S. Mesh Size	% Fat	Activity-Units/ml.	Activity-Units/gm. of extracted solids
Coarse	3.1	5	-
20	13.4	12.6	420
24	15.0	13.5	900
30	16.8	23.0	1,353
60	18.2	18.6	845
90	18.8	11.0	550

The loss in activity on the smaller mesh sizes was attributed to heat inactivation on continued grinding.

2. Effect of different fat solvents on extraction: soya-beans, ground to 30 mesh, were defatted with the following solvents - methylated ether, petroleum ether (below 40^{0}), petroleum ether (40^{0}-60^{0}) and carbon tetrachloride. The Soxhlet method of extraction was used and refluxing was continued for 3 hours in each case.

The ground soya was also extracted in the cold with

the following solvents - methylated ether, carbon tetrachloride, petroleum ether (below 40^{o}), petroleum ether (40^{o}-60^{o}) and acetone.

It was found that extraction with 40^{o}-60^{o} petroleum ether and acetone both gave high recoveries of activity in subsequent buffer extracts, while the other solvents used gave varying degrees of denaturation. Acetone gave better results on cold extraction but it was found the petroleum ether could be satisfactorily used in a Soxhlet-type of apparatus.

Subsequently fat removed from soya was therefore carried out with petroleum ether in this type of apparatus.

3. Conditions for extracting lipoxidase from defatted soya meal.

In order to find the best conditions for extracting lipoxidase the effects of pH and temperature on extraction were studied. The soya used was ground to 30 mesh size, and defatted for 3 hours with petroleum ether (40^{o}-60^{o}) in the Soxhlet apparatus. It was then extracted for three hours at room temperature with M/10 acetate buffers at the various pH.

TABLE IV

Effect of variation of pH

pH	4.14	4.24	4.44	4.55	4.58	4.76	5.39
Activity (Units/ ml.)	45.8	56.6	28.5	38.5	52.8	100.0	57.5

Therefore the pH optimum for extraction of this sample of soya lies about 4.7 and not around 4.5 as reported by Holman[19]. It is most likely this pH optimum will vary with different samples of soya.

Using pH 4.76 M/10 acetate buffer a sample of defatted soya meal was extracted at 0^{o}, 6^{o}, 12^{o}, 25^{o} and 35^{o}, for three hours. After centrifuging for 10 mins. at 3,000 r.p.m. the supernatant was assayed for lipoxidase activity.

TABLE V

Effect of variation of temperature on extraction

Temperature, ^{o}C.	0	6	12	25	35
Activity units/ml.	18.3	30.1	24.9	22.9	24.2
Activity units/g. of extracted solids	620	1260	1060	780	105

The soya was therefore extracted at pH 4.7-4.9 and at a temperature of 6^{0}-12^{0}. From the table below it can be seen that the optimum time for extracting the lipoxidase was 4 hours at this pH and temperature.

TABLE VI

Effect of time on the extraction

Time (mins.)	30	60	120	150	180	210	240	270
Activity/ml.	18.3	18.2	18.3	26.1	23.9	28.9	31.0	24.8
Activity/g. of extracted solids	605	620	600	865	795	970	1030	790

It was also found that the activity per gram of total solid extracted was highest if the mixture was shaken once every half hour during the extraction rather than constant stirring or being left still.

Therefore the method of extraction adopted was as follows:- soya-beans ground to 30 mesh, were extracted for 3 hours in a Soxhlet extractor with petroleum ether (40^{0}-60^{0}). After being thoroughly dried the defatted soya-meal was placed in a plastic bucket containing pH 4.76 M/10 acetate buffer (100 ml. per 10 g. defatted soya). It was thoroughly stirred and left for 4 hours

at 6°-12°, being stirred once every half hour during this time. After 4 hours it was either centrifuged at 3,000 r.p.m. for 10 mins. or filtered through a No.13 Whatman filter paper.

Separation by Holman's Method

The first attempts to purify lipoxidase followed the method of Holman[19,79].

A 10% extract of soya-beans was adjusted to pH 6.7 with 0.88 ammonia. Then 5 volumes of 20% barium acetate, 10 volumes acetone and 2 volumes of 20% lead acetate were added per 100 volumes extract. This was allowed to settle out over night and the inactive precipitate was removed by centrifuging for 5 mins. at 3,000 r.p.m. 25 g. ammonium sulphate were added per 100 ml. extract and the inactive precipitate took 2 days to settle out. It was then centrifuged for 10 mins. at 3,000 r.p.m. to remove the inactive precipitate. The active supernatant liquid was decanted and the concentration of ammonium sulphate made up to 40 g. per 100 ml. The solution was allowed to stand over night and then the active precipitate was separated by centrifuging for 5 mins. at 3,000 r.p.m. The precipitate obtained was dissolved in 1 ml. distilled water per 15 ml. original

extract. The supernatant liquid was still active but another precipitate was formed after 4 days and this active precipitate was added to the first. The active solution was dialysed against 1% sodium chloride for 5 days.

As can be seen there was great difficulty in preparing this active fraction as precipitation was very slow. If the solution was left for less than 48 hours after addition of ammonium sulphate to bring the concentration up to 40 g./100 ml. incomplete precipitation occurred. If the precipitate was allowed to lie over 48 hours in association with the supernatant denaturation occurred and there was no increase in activity of the precipitate. It was therefore found preferable to take the active precipitate off after 16-24 hours although part of the activity was still left in the solution.

On electrophoresis of this concentrate at a potential gradient of 7 volts/cm. and using M/15 phosphate buffer at pH 6 for seven and a half hours, a broad band about 7 cm. from the origin and a narrow band about 1 cm. from the origin, both moving towards the cathode, were obtained. The latter was active but the broad band showed no lipoxidase activity.

The concentrate from the ammonium sulphate precipitation was heated to 63° for 5 mins. to remove the albumins. This active, albumin-free solution was fractionated again with ammonium sulphate but the fraction between 35-50% saturation which Holman[19,79] reported active showed no activity. Moreover, all the activity disappeared from the other fractions during the separation of this stage.

This method of using ammonium sulphate was therefore rejected as it was found so difficult to obtain clean fractions and also because so much inactivation of the enzyme took place.

Mixed phosphates (50/50 monobasic and dibasic potassium phosphates) were compared with ammonium sulphate. 52.5 g. phosphate and 40 g. ammonium sulphate per 100 ml. extract precipitated out the active portion from the crude extract. The precipitate which gave the highest activity was that from the phosphate separation. Therefore it was decided to attempt a separation by the method of Kunkel[17] who had used a phosphate fractionation.

Separation by Kunkel's Method

The original method is shown in Fig. 4.

A concentrate prepared by precipitation with 40 g./100 ml. ammonium sulphate as described previously was used as starting material. This was dissolved in distilled water and then 35.7 g. mixed phosphates were added per 100 ml. of the solution. This was centrifuged at 1,665 r.p.m. for 10 mins. and then the supernatant was removed. The inactive precipitate was discarded and 5 g. mixed phosphates were added to each 100 ml. of the supernatant. This was centrifuged at 1,665 r.p.m. for 10 mins. and then the supernatant was decanted from the inactive precipitate and centrifuged for 3,682 r.p.m. for 1 hour. The precipitate obtained was taken up in water, 40 mls. per 100 g. starting material, but it was inactive. The remaining liquid was also inactive. Attempts were made to vary the centrifuging technique in order to save the activity but an active precipitate could not be obtained.

Therefore instead of centrifuging the solution after the addition of 35.7 g. phosphate per 100 ml., it was left to precipitate for 24 hours and then centrifuged for 15 mins. at 3,000 r.p.m. The

solution was still cloudy but a very slight dark precipitate had separated. The solution was left to settle for another 24 hours and cleared but, when centrifuged again for 15 mins., had become cloudy again and no precipitate had separated. To this liquid were added 5 g. phosphate per 100 ml. solution and it was allowed to settle for 24 hours then centrifuged at 3,000 r.p.m. for 10 mins. A slight precipitate had formed which, when dissolved in 10 mls. water gave a solution of activity only about one quarter of that of the original solution on a volume basis. It was found after further fractionations to be very difficult to precipitate out the activity using phosphate in such a manner as to prevent a large amount of denaturation.

As all these methods had been unsuccessful and had only given separation accompanied with a great deal of denaturation it was decided that a separation using alcohol at a low temperature would be attempted.

2. Alcoholic Fractionation of Soya Lipoxidase

Exploratory Experiments

Cohn's[64,81,82] methods for the protein fractionation of blood plasma by the use of alcohol at low temperatures was followed in the preliminary experiments so that some idea of the effect of this system on soya proteins might be ascertained before a more systematic study was attempted. The method used was as follows:- 4 litres of crude M/10 acetate extract were placed in a 5 litre beaker and the temperature was brought to -5° in a dry ice box. The solution was vigorously stirred, care being taken to avoid frothing. When the temperature had fallen below 0° the addition of 53.3% V/V solution of ethanol in water was started through a capillary tube. All ethanol concentrations are expressed on a volume/volume basis. The temperature was gradually lowered to -5° and the final ethanol concentration adjusted to 10% V/V. The solution was then centrifuged for 10 mins. at 2,000 r.p.m., the centrifuge being kept as cool as possible by means of dry ice. All but 13% of the activity was precipitated out at this concentration.

The precipitate was dissolved in M/15 sodium phosphate buffer, pH 6.0, by thoroughly stirring at room temperature and then gently stirring for 30 mins. Celite was added to the mixture and it was filtered by means of a Buchner funnel. It was found that this liquid contained only 6.5% of the original activity. Therefore there must have been a large loss through denaturation. However, the concentration on a dry weight basis was increased. On electrophoresis of this precipitate it was found to divide into two bands as found with the ammonium sulphate concentrate described previously.

A great deal of the activity was later found to have been lost in the method of taking up the precipitate because the undissolved precipitate was subsequently shown to be active.

The above experiment was repeated within narrower limits and it was found that all the activity precipitated out between 8-12% alcohol. Therefore this narrow fraction at a low alcohol concentration appeared to be more effective than the ill-defined ammonium sulphate fractionation used previously. It was decided to continue using alcohol as it was thought that a study of the variables, e.g. temperature

and pH, might reduce the large amount of denaturation of the protein.

The effect of pH, time and temperature on the actual precipitation was first studied and then the effect of the most suitable pH, time and temperature, from this point of view, on the actual activity of the precipitate obtained.

Effect of pH on Precipitation

To find the effect of pH on the precipitation 200 ml. volumes of the extract were adjusted to the various pH values with 0.88 ammonia and then brought down to -5°. 53.5% alcohol was added to raise the alcohol concentration to 4%. The precipitate was allowed to settle out for half an hour and then the solution was centrifuged at 3,000 r.p.m. for 10 mins. being kept as cool as possible. The temperature of the supernatant was again brought down to -5°, and the alcohol concentration raised to 8% over half an hour. After centrifuging again at 3,000 r.p.m. for 10 mins. the alcohol concentration was raised to 12% and the precipitate allowed to settle out for 30 mins. Then the solution was centrifuged again at 3,000 r.p.m. for 10 mins.

In Table VII are shown the lipoxidase activities and the amount of solid matter present in the supernatant liquids thus obtained.

TABLE VII

The effect of pH on alcohol precipitation

% Ethanol	0		4		8		12	
pH	Activity units /ml.	% Dry Matter	Activity units /ml.	% Dry Matter	Activity units /ml.	% Dry Matter	Activity units /ml.	% Dry Matter
3.48	0	2.73	0	2.03	0	-	0	-
4.05	25.5	3.22	2.5	2.17	1.5	-	0	-
4.43	29.5	3.39	2.3	2.15	3.5	2.65	0	-
5.00	26.5	2.85	0	2.29	0	-	0	-
5.58	27.3	2.53	16.6	2.06	16.3	2.43	14.4	-
6.80	25.2	2.77	15.0	2.52	0	2.41	0	-
8.95	21.9	2.59	-	-	15.8	-	12.0	-
11.00	1.6	2.71	0	-	0	-	0	-

As there was inclined to be inactivation of the enzyme at alcohol concentrations higher than 12% the pH values to be preferred were those at which the

activity precipitated below this alcohol level. Therefore the pH values at which were obtained the highest activities per gram of solid precipitate were 5.0 and 6.8. pH 5.0 appears preferable as all the activity comes down by 4% but less extraneous matter is precipitated at pH 6.8.

Also it was found that on using larger volumes of liquids at pH 5.0 only half the activity precipitated by 8%, the remainder not precipitating until 12%. Therefore because of the variation of results at pH 5.0 it was decided to use pH 6.8 in the first steps at least when large quantities of liquids could be used. These conclusions assume that the precipitated activity is recoverable, and this in fact subsequent experience justified.

Effect of Time on Precipitation

As with all fractionations of proteins, denaturation was occurring along with precipitation and after a certain point there was no increase in the activity of the precipitate although there was still activity in the supernatant solution, inactivation occurring at the same rate as precipitation. Thus it was advisable to remove the precipitate as quickly as

possible, and in order to find the earliest point of removal a study was made of the times of precipitation at the various alcohol concentrations.

200 ml. of the soya extract were adjusted to pH 6.8 and then the temperature was brought to -5° with constant stirring. The alcohol concentration was raised to 4% and 5 ml. portions of supernatant were removed at various intervals of time, centrifuged at 3,000 r.p.m. for 10 mins. and the supernatant tested for lipoxidase activity and the amount of dry matter determined. This was repeated at 8% and 12% alcohol and the results obtained are detailed in Table VIII.

TABLE VIII

Effect of time on precipitation

% Alcohol	Time(mins.)	0	30	60	120	Overnight
0	Activity units/ml. supernatant	37.9	37.9	37.9	37.9	37.9
	% Dry Matter	3.50	3.50	3.50	3.50	3.50
4	Activity units/ml. supernatant	-	19.2	18.9	17.2	19.1
	% Dry Matter	-	1.12	1.36	1.12	1.87
8	Activity units/ml. supernatant	20.6	13.9	14.4	8.8	14.5
	% Dry Matter	2.33	-	-	2.50	2.23
12	Activity units/ml. supernatant	14.5	7.2	10.3	3.2	3.0
	% Dry Matter	2.48	2.78	3.06	-	2.49

In most cases the activity is precipitated during the first half hour. When using 12% alcohol the activity of the supernatant was found to decrease up to 3 hours but this was mostly due to denaturation and not to precipitation. Therefore it is preferable to allow

precipitation with this amount of liquid to take place over 30 mins.

Effect of Temperature on Precipitation

That the temperature of the precipitation has an effect on the precipitation of these proteins has been reported by many writers. Cohn[67] found for plasma proteins that a temperature of -5° is preferable but this may not be true for the system being studied. Therefore it was decided to study the effect of temperature, the temperatures chosen being 5°, 0° and -5°. No temperatures above 5° were studied because proteins are denatured by alcohol at these temperatures.

400 ml. of the crude extract were adjusted to pH 6.8 and then the temperature was brought down to 5° with constant stirring. The alcohol concentration was raised to 8% by addition of 53.3% V/V alcohol and the proteins were allowed to precipitate out for 30 mins. The solution was centrifuged at 3,000 r.p.m. for 10 mins. and the precipitate obtained taken up in 20 ml. pH 4.76 buffer. The supernatant was brought down to 5° again and the alcohol concentration increased to 12%. The precipitate obtained by centrifuging for 10 mins. at 3,000 r.p.m. was taken

up in 20 ml. of pH 4.76 buffer. This was repeated at 0° and -5°.

TABLE IX

Effect of temperature on precipitation

Temp.	Extract Total Activity	8% Alcohol		12% Alcohol	
		Supernatant Total Activity	Precipitate Total Activity	Supernatant Total Activity	Precipitate Total Activity
-5°	16,520	15,420	430	11,410	86
0°	16,520	7,380	130	6,312	208
5°	16,520	16,160	1,180	7,520	1,030

From Table IX it can be seen that the mode of the precipitation and the activity of the precipitate varies greatly with temperature. Also it appears from these results that -5° might not be the best temperature for precipitation. To see if the temperature of fractionation had to be varied according to the volume of extract being used the experiment was repeated with 200 ml. extract. The results are shown in Table X.

TABLE X

Effect of temperature on precipitation

Temperature		-5°	0°	5°	10°
Buffer Extract	Activity/ml.	25.9	25.9	25.9	25.9
	Activity/g.	788	788	788	788
8% Ethanol Supernatant	Activity/ml.	9.1	16.3	21.8	0
	Activity/g.	377	604	820	0
8% Ethanol Precipitate	Activity/ml.	3.8	4.7	4.2	9.4
	Activity/g.	428	604	666	1234

From these results it appears that precipitation at 5° was most satisfactory at this volume. To study further the effect of the volume on the temperature of the fractionation, the experiment was repeated using 1 litre of extract. 1 litre of soya extract was adjusted to pH 6.8 with 0.88 ammonia and filtered at room temperature through a No.1 Whatman filter paper. It was cooled down to 5° with constant stirring and 53.3% ethanol was added under the surface by means of a burette to bring the alcohol concentration up to 8%. The addition took place over half an hour. The

solution was then centrifuged for 10 mins. at 3,000 r.p.m. and the supernatant was brought again to 5° and the alcohol concentration increased to 10% and the solution centrifuged as before. This was repeated up to 22% and, as can be seen in the figures below, the activity was not precipitated by this time.

TABLE XI

Precipitation by alcohol at 5°

% Alcohol	Supernatant Activity per ml.	% Dry Matter in Supernatant
0	31.1	3.42
8	26.2	2.95
10	25.1	2.73
12	26.8	2.62
14	28.0	2.55
16	25.3	-
20	27.8	-
22	25.5	-

This was repeated with 830 ml. extract bringing the alcohol concentration right up to 24%, centrifuging at 3,000 r.p.m., and then continued addition of alcohol up

to 44%. The solution was centrifuged after every 2% addition of alcohol. The activity did not precipitate until between 40-42% alcohol and there was no lipoxidase activity found in this precipitate.

Therefore it is obvious that with larger volumes the temperature effect was different from that found with smaller volumes. Using a litre of extract the precipitation was again studied at 0° and -5°. The method used was exactly as described for 5°.

TABLE XII

Precipitation by alcohol at 0°

% Alcohol	Supernatant			Precipitate	
	Activity per ml.	Total Activity	% Dry Matter	Activity per ml.	Total Activity
0	22.6	22,600	3.23	-	-
4	18.4	20,000	2.72	15.3	153
8	18.2	21,680	2.68	14.5	145
12	16.9	22,400	2.13	9.2	92
16	11.1	16,328	1.90	102.4	1,024
20	9.0	15,000	1.78	96.2	962
24	1.5	2,890	1.68	-	-

TABLE XIII

Precipitation by alcohol at -5^{0}

	Supernatant			Precipitate	
% Alcohol	Activity per ml.	Total Activity	% Dry Matter	Activity per ml.	Total Activity
0	25.5	25,500	3.48	-	-
4	23.1	25,110	2.97	0	0
8	21.2	25,250	2.73	0	0
12	20.4	26,850	2.60	0	0
16	13.9	20,450	2.47	108.2	1,082
20	8.7	14,500	2.10	135.4	1,354
24	1.8	3,460	1.33	76.0	760

The activity in both cases was precipitated between 12-24% alcohol although there was more inactive material precipitated at -5^{0} the precipitate had a higher activity on a comparative basis. It can be seen that at these lower temperatures the difference in temperature does not cause a great change in the precipitations. It is better, however, to precipitate at as low a temperature as possible as there is less inactivation of the enzyme. These two tables illustrate the rapid increase in denaturation rates

above 20% ethanol. The question of ethanol denaturation of lipoxidase is dealt with more fully later.

Method of Separation

While the results in the foregoing section showed that high levels of loss of activity were to be expected, a further examination seemed to be justified. On this basis it was decided, firstly, to remove the active lipoxidase fraction from the soya extract by precipitation with ethanol, the active fraction being removed between 12-24% alcohol. The temperature needed for this fractionation was -5° or below. As this temperature appeared from the preliminary experiments to have a controlling effect upon the precipitation it was decided that a better method of cooling than the dry ice box previously used was necessary.

A cold storage cabinet, whose air temperature was controlled at -5° by a Sunvic relay system attached to a bimetal thermostat was at first used. The temperature of the solution, however, rose on the addition of the alcohol and took time to come down to -5° again. This was due to slow heat transfer from the air in the storage cabinet to the solution.

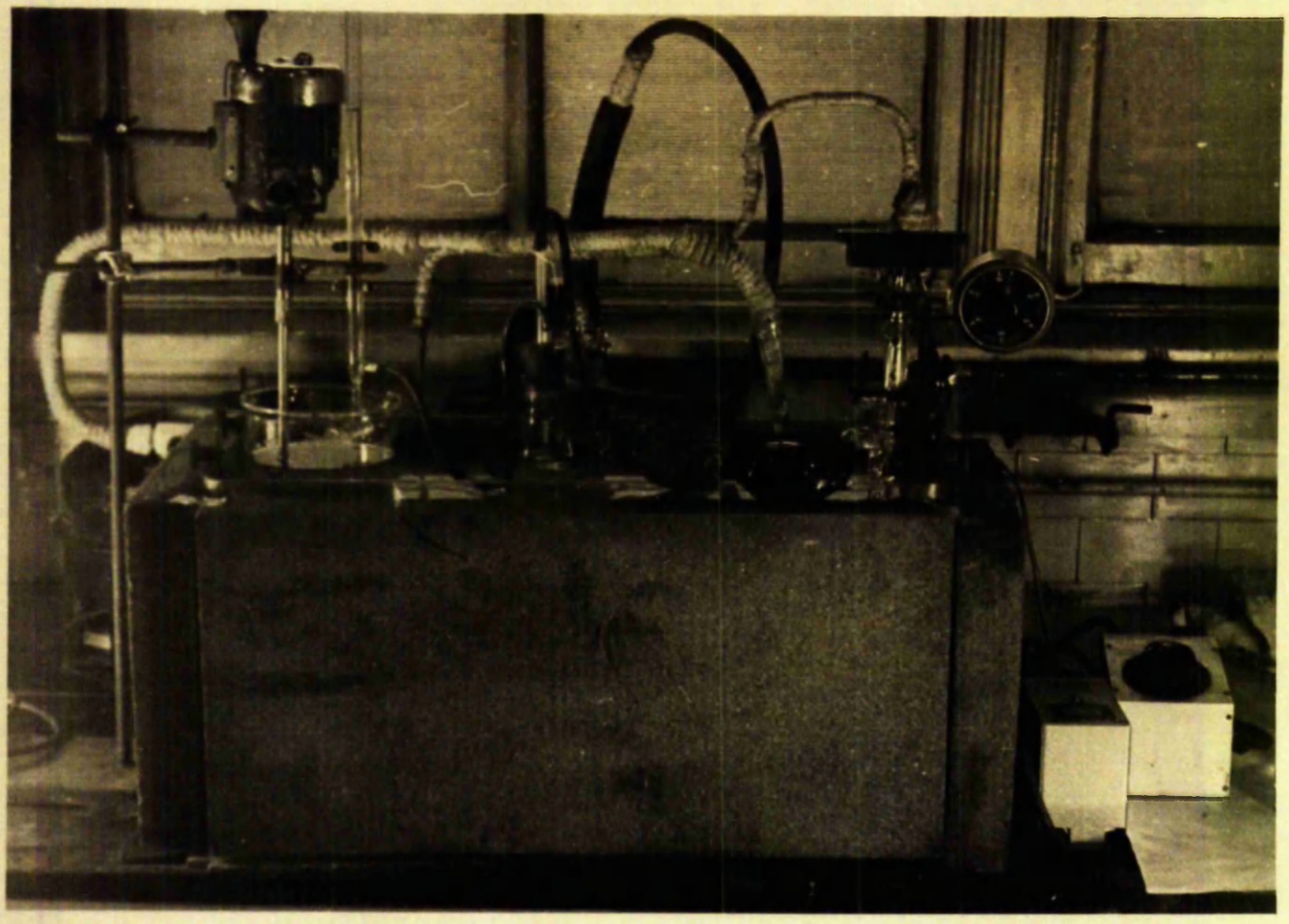


Fig. 13.

Cooling Tank

Because of this need for rapid transfer of heat from the solution a tank was built in which a liquid was used as the cooling medium. An illustration of this apparatus is shown in Fig. 13.

It consisted of a glass tank, two feet long, one foot broad, and one foot deep surrounded by two inch thick cork walls as insulating material. The cooling unit consisted of a copper coil, two inches high, along the floor of the tank, attached to a sealed gas compressor unit. This system was filled with Freon gas so that the coils were cooled by the expansion of the gas compressed by the pump.

The tank was filled with a 50% mixture of industrial spirits and water as this was found to be a medium which retained a low viscosity at the temperature being used. It was essential that this liquid was kept in movement as otherwise a cold layer collected along the pipes and there was not a good heat transfer from the cooling medium to the coils. Also as rapid transfer of heat from the solution being fractionated was necessary it was preferable to keep the cooling medium in motion. This circulation was effected by using two centrifugal pumps with outlets beneath the cooling coils and inlets at the top of the cooling medium.

The energy used by the compressor was roughly controlled by an energy regulator. The temperature of the tank was accurately controlled by means of a toluene regulator attached to a relay system. The latter was in circuit with a 200 watt tank heater and the temperature was controlled by means of this heating cycle. It was considered that a finer control was obtained by this method than if the toluene regulator had been attached to the cooling system.

The fractionations were done with 4,000 ml. volumes of the crude soya extract as larger volumes than this could not be handled in the tank. The method used was as follows:- 4,000 mls. of crude soya extract were brought to pH 6.8 with 0.88 ammonia and filtered through a No.1 Whatman filter paper. The solution was placed in a 5 litre beaker in the cold tank and cooled. It was continuously stirred during cooling. When the temperature had reached 0^{0} the addition of 50% V/V solution of alcohol was started. This was added through a capillary attachment from a 50 ml. burette. The alcohol was pumped into the burette from a storage bottle in the cold tank so that it had a temperature of -5^{0} on being added to the extract.

When the temperature had reached -5° the addition of alcohol was continued until there was a concentration of 12% alcohol on a volume basis in the mixture. The time taken for addition was 30-45 mins.

To remove the inactive precipitate the solution was centrifuged at 3,000 r.p.m. for 10 mins. The centrifuge was cooled with dry ice and the centrifuge bottles and buckets were prechilled. With these precautions the temperature rise in the solution was kept below 2°.

The supernatant was again brought to -5° and ethanol was added as before to bring the ethanol concentration of the solution up to 24%. After the addition of the ethanol the active precipitate was separated from the solution by centrifuging at 3,000 r.p.m. for 10 mins.

The precipitate was dissolved in 250 mls. of pH 4.6 M/10 acetate buffer and filtered through a No.1 Whatman filter paper the temperature being kept below 0°.

The results of a typical fractionation are shown in Table XIV.

TABLE XIV

Fractionation of crude soya extract with ethanol

	Units/ml.	Units/g.	Total Activity
Crude extract	45.5	1,764	177,450
Solution of fraction between 12% and 24% alcohol	28.9	2,429	6,936

This separation was repeatedly carried out with minor variations with essentially similar results to those shown. It is evident that a great loss of activity occurred in this precipitation, only 4.0% of the total activity being found in the precipitate although all of the activity had been removed from the supernatant. The concentration of activity was also very small, being only about 1.2-fold.

It was attempted to purify the concentrate by further alcohol fractionation. The temperature was brought down to -5°, as in the previous fractionation, and the alcohol concentration raised to 8%. The solution was centrifuged for 5 minutes at 1,500 r.p.m. All the activity was found to have been removed but so

Alcohol Fractionation of Soya-Beans

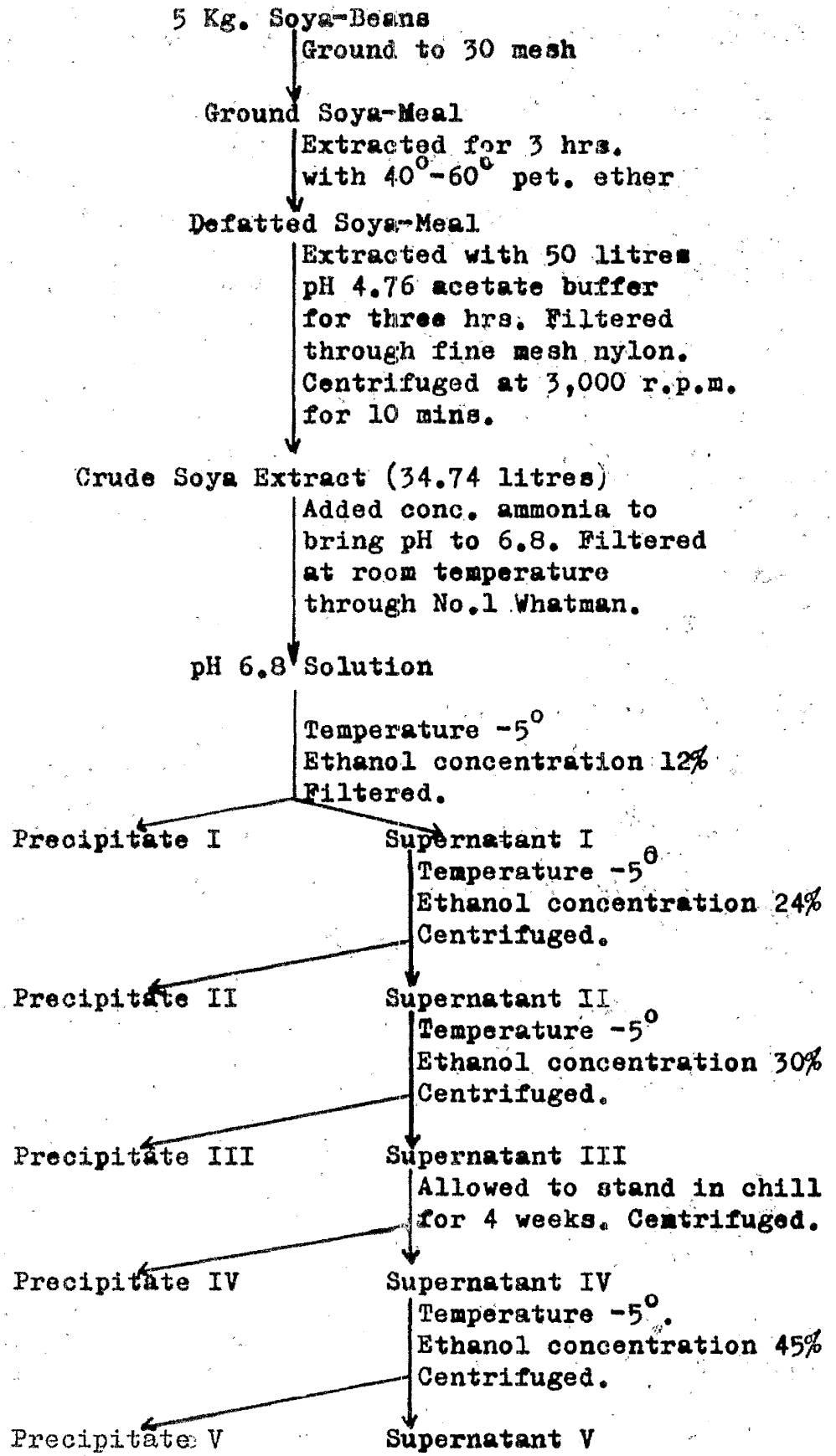


Fig. 14

much denaturation had occurred that the precipitate showed little lipoxidase activity.

Therefore it appeared from these results that this method of fractionation was not going to be successful in purifying lipoxidase as it caused too much denaturation of the enzyme.

However, as a last experiment, a purification on a larger scale was attempted as it was thought that denaturation might be less using larger volumes of solution.

Final Alcohol Fractionation

The proposed scheme of separation was to bring the alcohol concentration up to 12%, centrifuge off the inactive precipitate, and then to raise the alcohol concentration up to 24% to precipitate the active fraction. This precipitate was to be redissolved in distilled water and this solution fractionated with mixed phosphates. The final concentrate thus obtained was to be separated in an electrophoresis apparatus. However, using the larger volume of solution the precipitations did not follow the same pattern as in the previous experiments and the actual precipitation is shown on the opposite page.

5 Kg. of soya-beans were extracted with 50 litres pH 4.76 M/10 acetate buffer to give a crude extract of 34.74 litres. The pH of this solution was brought to 6.8 with 0.88 ammonia and the solution filtered through a No.1 Whatman filter paper. The solution was placed in a large stainless steel container and the temperature of the solution was reduced to -5^{o}.

As the apparatus previously built could not handle such large quantities of liquid this fractionation was carried out in a cold room whose temperature was accurately controlled at -5^{o}. As there was a very good air circulation in the cold room there was found to be no overheating of the solution due to the mixing of the alcohol and water. If the rate of addition of alcohol was carefully controlled it was found that the temperature of the solution varied little from -5^{o}.

As the temperature of the solution was reduced the solution was carefully stirred with a wooden paddle, as no method of mechanical stirring could be obtained in the cold room. As frothing occurred readily with this solution, due to the presence of a surface active agent, stirring had to be very carefully controlled so as to stop the formation of ice crystals without the

formation of froth. Frothing is inclined to cause denaturation of the protein and therefore should be avoided if possible.

As the temperature reached 0^{o} the addition of a 50% alcohol solution was started. The alcohol was added through glass capillaries and stirring was continued during the addition. When the alcohol concentration reached 12% the addition of alcohol was stopped and the solution was filtered in the cold room through No.1 Whatman filter paper. Filtration was completed as rapidly as possible so that the next precipitate would not start to come down before filtration was completed. It was, however, found, on using these larger volumes of solution, that fractionation appeared to be cleaner and there was not a tendency, on standing, for the next precipitate to form or for denaturation to take place.

The solution was replaced in the stainless steel container, and when the temperature had again settled at -5^{o} the addition of ethanol was continued. At 24% alcohol, the solution was centrifuged at 3,000 r.p.m. for 5 mins. It was found that a large quantity of the activity remained in the supernatant.

The solution was therefore returned to the cold room and the temperature brought down to -5^{o}. Alcohol

addition was continued to bring the concentration up to 30% and the solution was then centrifuged at 3,000 r.p.m. for 10 mins. Although some of the activity was precipitated there still remained a large quantity of activity in the supernatant.

From the previous experiments it was thought that increasing the alcohol concentration further would cause increased loss of activity. Therefore an attempt was made to bring out the activity by using other precipitants, namely ammonium sulphate, mixed phosphates, acetone, and basic lead acetate. However, due to the presence of the high concentration of alcohol, the precipitates obtained by these methods showed very little activity.

It was also attempted to precipitate out the activity by lowering the pH to values between 4.2 and 5.9. No precipitation occurred above 5.7 and only incomplete precipitation between 5.4 and 5.7. Only the precipitate obtained by lowering the pH to 5.0 was found to show activity. 12% of the activity in the original 30% alcohol supernatant was found in this precipitate.

As these methods had been unsuccessful the alcohol concentration was increased to 35% keeping the temperature at -5^{0} and using the same precautions as before.

It was found that all the activity had precipitated out and 55% of the activity in the 30% solution was present in the precipitate. This was repeated with the remainder of the solution in 5 litre volumes, as the cold room was not available, and it was necessary to carry out the fractionation in the cold bath. In some cases the activity did not come down until the concentration of alcohol in the solution was 45%. Therefore, this fraction is reported as between 30-45%.

The results of this alcohol fractionation are shown in Table XV. All the precipitates were dissolved in distilled water and the activities shown are of these solutions.

TABLE IV

Alcohol fractionation of a crude buffer extract of defatted soya-bean

	Activity/ml.	Activity/g. of extracted solids	Total Activity
Crude buffer extract at pH 4.76	41.4	1,906	1,440,000
Crude buffer extract adjusted to pH 6.8	34.7	1,542	1,120,000
Supernatant at 12% alcohol (I)	19.2	912	848,000
Precipitate between 0-12% alcohol (I)	13.1	1,149	99,600
Supernatant at 24% alcohol (II)	17.1	1,105	1,110,000
Precipitate between 12-24% alcohol (II)	24.5	3,997	64,410
Supernatant at 30% alcohol (III)	15.9	957	1,029,000
Precipitate between 24-30% alcohol (III)	57.7	11,400	51,300
	-	-	206,000*
Supernatant at 30% alcohol after 4 weeks storage (IV)	7.6	461	347,000
Precipitate obtained at 30% alcohol on 4 weeks storage (IV)	10.4	2,888	5,786
Supernatant at 45% alcohol (V)	0.0	0	0
Precipitate between 30-45% alcohol (V)	67.4	8,892	160,502

* Activity used in tests with other precipitants to remove activity at 30% alcohol.

It can be seen that most of the activity was precipitated between 30-45% alcohol but some activity was present in the earlier precipitates. The 30-45% fraction showed a concentration of 5-fold on the original solution on a dry matter basis.

Inactivation did not appear to play such a great part when using these larger volumes of solution. The loss of activity of the 30% supernatant (i.e. Supernatant III) due to storage was unfortunate and gives a false idea of the denaturation occurring in the last precipitation. This solution was stored for three weeks with only a slight loss in activity but during the fourth week of storage the activity of the solution decreased rapidly.

The percentage of the total activity found in the various precipitates has been tabulated in Table XVI as this gives a clearer idea of the separation obtained and the inactivation occurring during the fractionation.

TABLE XVI

Alcohol fractionation of crude soya

	Total Activity	% of Original Total Activity
Original crude extract	1,440,000	100
Precipitate between 0-12% Alcohol (I)	99,600	6.9
Precipitate between 12-24% Alcohol (II)	64,410	4.5
Precipitates between 24-30% Alcohol (III & IV)	57,086	4.0
Precipitate between 30-45% Alcohol (V)	160,502	11.2
Loss due to storage of 30% solution and to precipitation tests	887,940	61.7
Loss due to denaturation during all precipitations	170,462	11.7

The loss due to denaturation during the actual precipitations was small and is less than that reported in any previous fractionation in purifying soya-bean proteins for lipoxidase.

As it was found that the solution of the precipitate slowly lost its activity on storage it was decided to remove the water by freeze drying and to store the freeze dried product at -20°. Freeze drying was carried out in

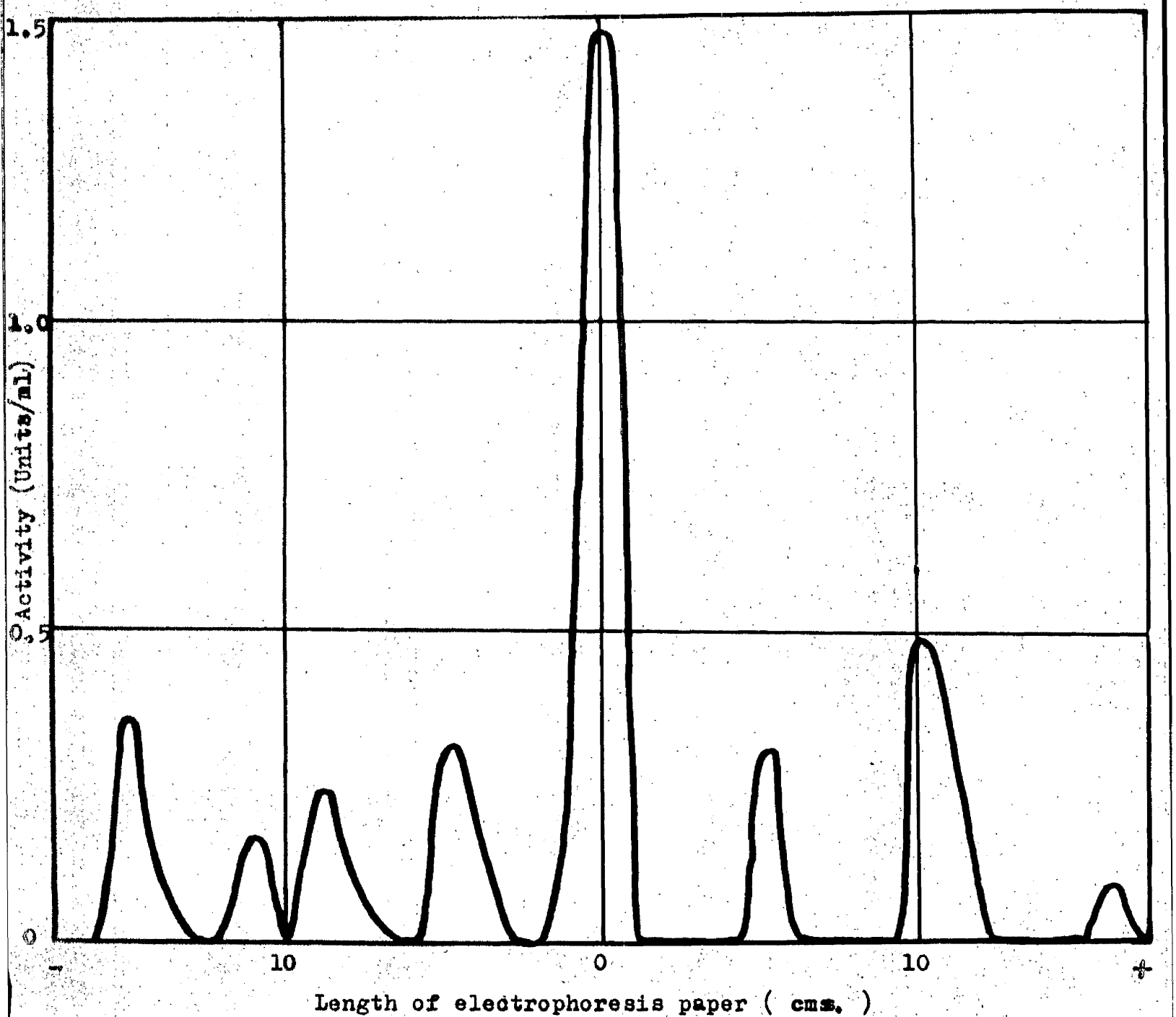


Fig. 15

Electrophoresis of freeze-dried product at pH 6.0

10 ml. ampoules in an Edwards freeze dryer. The activities of the freeze dried product are shown below.

TABLE XVII

Freeze drying of concentrate

	Total Activity	Activity/g.
Original concentrate	160,502	8,892
Freeze dried product	135,580	19,420

Electrophoresis of Concentrate

The freeze dried product was dissolved in distilled water giving a solution of concentration 5.3%. This was dialysed overnight against pH 6 M/15 phosphate buffer. On electrophoresis in the small qualitative apparatus at pH 6.0 and at a voltage gradient of 6.5 V/cm., the concentrate separated into four protein bands - one around the origin, two bands moving towards the cathode and a band moving towards the anode.

On completion of a run on the electrophoresis unit a paper strip was removed and cut into strips 1 cm. wide. Each strip was placed in two mls. distilled water and the solution obtained assayed for lipoxidase activity. The results are shown in Fig. 15. In all runs most of

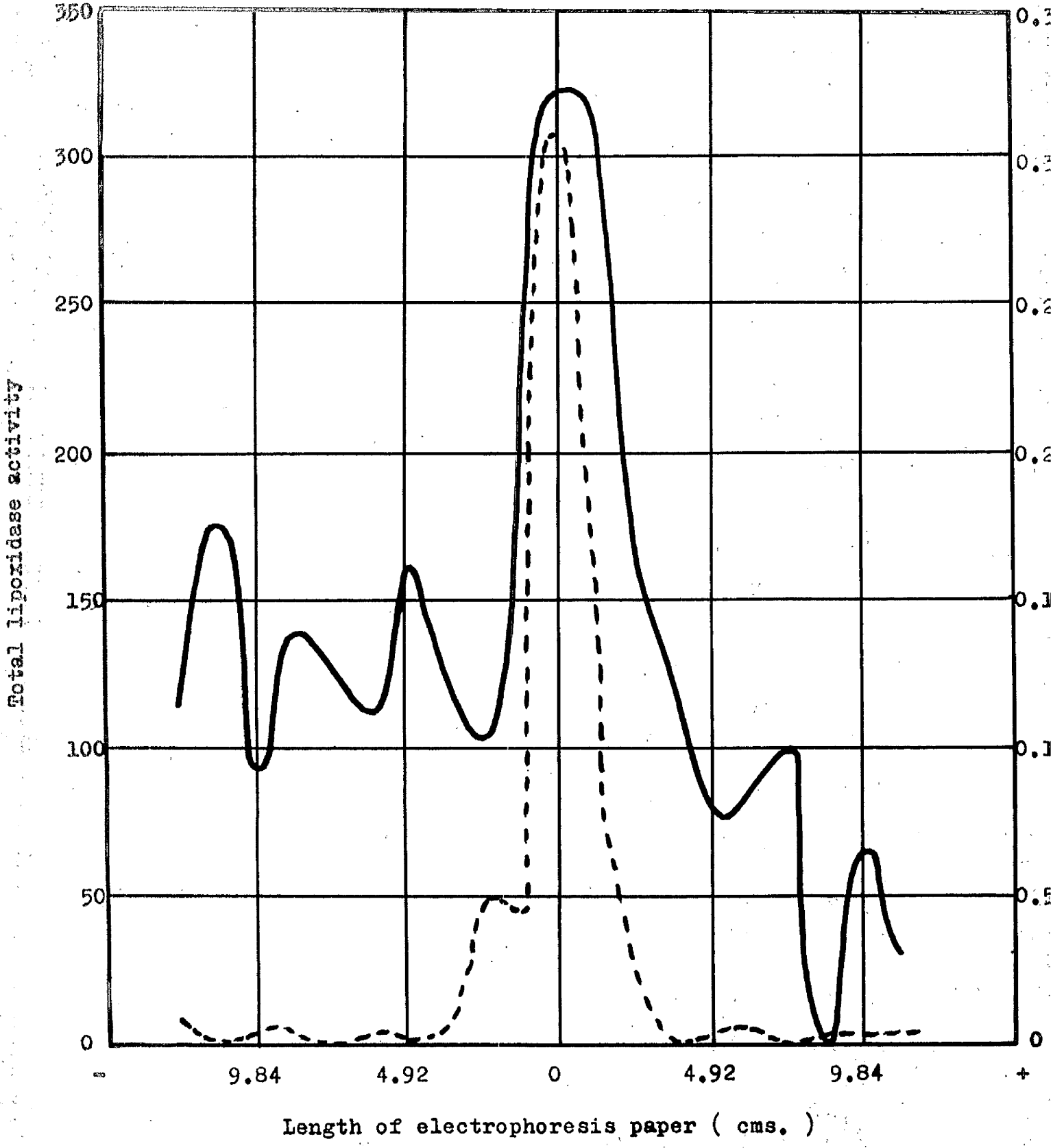


Fig. 16

Comparison of dry matter and lipoxidase activity of solutions from continuous electrophoresis unit

---------- Lipoxidase activity

———————— % Dry matter

the activity was found to be around the origin.

Thus as some of the inactive material was removed by electrophoresis at pH 6.0 the solution of the freeze dried product was separated in the continuous electrophoresis unit described previously. The electrolyte used was again pH 6.0 M/15 phosphate buffer but a higher voltage was used as the separation is horizontal not vertical. The voltage gradient used was 19 V/cm.

The concentrate separated into three protein bands, one which had not moved from the origin, one which had moved 3.5 cm. towards the anode and another band which had moved 6.2 cm. towards the cathode. These bands, shown by development of the paper with bromophenol blue, were confirmed by qualitative biuret tests on the solutions collected in the tubes.

These solutions were also tested, after dialysis, for the amount of dry matter present and for lipoxidase activity. The relationship between these is shown in Fig. 16. Approximately about 25% of the dry matter present in the concentrate was found in the tubes containing most of the lipoxidase activity.

By this separation on the continuous electrophoresis apparatus the concentration of lippxidase activity on a dry matter basis was increased 10-fold. Loss of

activity during the separation varied, being generally about 35%, but in some cases was much lower. It would be an improvement to have some method of cooling in the apparatus as this would both reduce evaporation from the paper and inactivation of the enzyme.

DISCUSSION

DISCUSSION

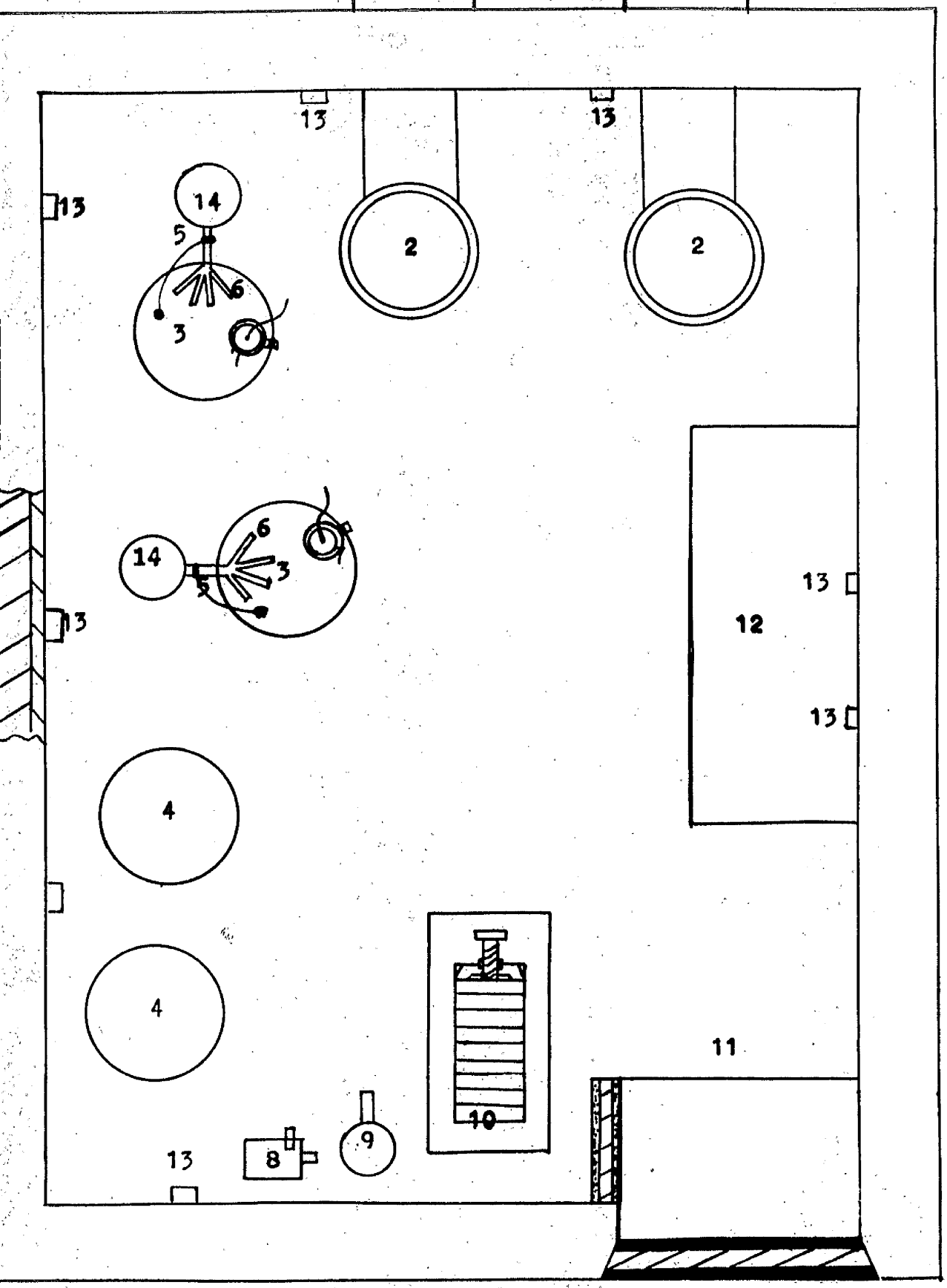
Holman first suggested that the presence of catalase affects the characteristics of the lipoxidase oxidation. This was confirmed in this laboratory when catalase and lipoxidase oxidations were examined in the same assay system and it was found that under certain conditions small concentrations of catalase inhibited lipoxidase. These results were obtained with crude buffer extracts of soya-beans in a heterogeneous assay system and therefore it was thought necessary to obtain a pure sample of lipoxidase and compare this in the assay system with the crystalline catalase already prepared. In addition to the above reasons concerned with the immediate problems of this laboratory, the effect that only one unconfirmed claim of successful isolation of lipoxidase has been made added a general interest to the separation.

While the previous investigators had made numerous attempts to purify lipoxidase with conventional precipitants, the procedures used were arbritary and led to high losses of activity in the course of the separations. It was therefore felt that the use of an unconventional method was justified,

and the success which attended Cohn's alcohol fractionation of blood plasma suggested the possibility of applying his methods.

The text of the experimental section indicates that major difficulties of technique were encountered but the large-scale separation showed that with proper facilities these difficulties could be reduced to comparatively minor proportions by the use of appropriate equipment. The low temperature fractionation with ethanol was found to call for a combination of close control and flexibility. For example, during the initial addition of alcohol to a lipoxidase extract, the extract freezes at a temperature just below 0° and in some, but not all, experiments in which ice crystal formation was allowed to occur loss of activity was found to take place. The experimental requirements are therefore that at the initial stages of alcohol addition, the temperature should be lowered at such a rate so that with the addition of alcohol it is always close to but never at the freezing point. This procedure must be carried out until the alcohol concentration is sufficiently high to prevent freezing at -5°. Once this temperature has been reached without freezing further additions are comparatively simple.

1 inch to 2 feet



Concrete
Plaster board
Wood
Cork

Fig. 17
Cold chamber

1. Refrigerator unit
2. Refrigerated tank
3. Precipitating tank
4. Spare tank
5. Stopcock
6. Glass capillaries
7. Stirrer
8. Pump
9. Centrifuge
10. Filter press
11. Plastic curtain
12. Bench
13. Power points
14. Alcohol container

This controlled rate of heat extraction is greater than can be achieved by allowing the material to stand in still air at -5^{0} and it is therefore necessary to carry out the fractionations in a room at -5^{0}, in a jacketed container with supplementary cooling from a small externally mounted refrigerating unit.

The work which has been described is therefore, in a sense, exploratory as at the outset it was not possible to specify these conditions. During the later stages of the work existing facilities were adequate to make an evaluation of the conditions under which optimum results could be expected. On these grounds it is not irrelevant to put forward proposals for equipment which would be required in a subsequent investigation of the problem.

Special facilities are necessary to carry out such an alcohol separation with large volumes of solution. A cold room whose temperature could be accurately controlled at -5^{0} is sketched in Fig. 17. This only gives an indication of the type of construction that could be used and this could be varied according to the materials available. With this construction a 1 H.P. condensing unit charged with Freon 12/Arcton 6 would be necessary to overcome the heat leak into the chamber.

It is necessary that the room be supplied with electrical points so that centrifuges and other equipment can be run inside the room, as it is essential that the temperatures of the solutions remain as near -5^{o} as possible and therefore facilities should be such that all operations can be performed in the room.

For the actual precipitations cylindrical stainless steel or glass lined tanks which can hold 200 litres of solution are preferable. As 100 litres of solution take a considerable time to fall from room temperature to -5^{o}, two of the tanks should be fitted with jackets through which a refrigerant can be passed. A small $\frac{1}{4}$ H.P. compressor is all that is necessary in this refrigerating system to extract the heat from the solution within a reasonable time.

For stirring purposes portable electric stirrers which can be clipped on to the sides of the tank would be more adaptable than stirrers fixed permanently on the tanks. These must be able to be run at such a speed so that the solution is kept in constant circulation without frothing.

The 50% ethanol solution could be added from a glass lined tank which can hold 50 litres and which is

graduated to 1/10th of a litre. This would have an outlet at the bottom to which are attached several removable capillaries. The rate of flow of the alcohol from this tank should be controlled by means of a stopcock on the main outlet. This stopcock should be attached to a thermometer in the tank, so that if the temperature of the solution goes up the rate of alcohol addition is automatically reduced. This safeguard is necessary if the precipitation is to continue unattended.

For filtration purposes a small stainless steel filter press mounted on a trolley would be suitable. For centrifuging these large volumes of solution a Sharples continuous centrifuge is necessary. This normally runs at a speed higher than necessary, and in fact higher than desirable as these high speeds cause inactivation of the enzyme. Therefore it should be placed in circuit with a resistance so that a speed of about 5,000 r.p.m. is obtained.

While it is not claimed that results achieved gave as high a degree of purification as those of Holman, the data suggest that, up to the stage described, losses from denaturation and the overlapping of fractions were less than in Holman's previous method. Moreover,

from these experiments it is possible to propose a short method of purification which will give at least a 50-fold concentration of the material. This only requires extraction of the original defatted soya with acetate buffer and the removal of the fraction precipitating between 30-45% alcohol. This fraction is of high activity and can be further purified, if required, by electrophoresis. The conditions described in the text are critical, and comparatively small variations may be expected to give rise to difficulties.

An interesting possibility which arises from the work is that of fractionation at a fixed alcohol concentration by altering temperature conditions. It would seem to be a necessary corollary to the experimental results reported that by operating between 5^{0} and -5^{0} at a given alcohol concentration narrow fractions could be precipitated by lowering the temperature in steps. This, in fact, was found to be the case in a few isolated experiments not reported in the general section but the method was not further studied because of the comparatively wide range of alcohol concentration over which this particular enzyme was precipitated. Under other circumstances, and with other systems precipitating over a narrow range of

alcohol concentrations, this method would offer useful possibilities.

With a few notable exceptions plant enzymes are usually found in relatively low concentrations and therefore have been difficult to purify. For example, while catalase is isolated from animal tissues with comparative ease, it has never been prepared from plant tissues although it is an ubiquitous enzyme in plants. The possibilities indicated by this work would, given an appropriate experimental unit, appear to justify the examination of other systems with the proposed method of separation.

CONCLUSIONS

CONCLUSIONS

1. The possibilities of using ethanol as a precipitant in the separation of plant enzymes have been demonstrated in the specific case of lipoxidase.

2. While isolation of the enzyme in a homogeneous state was not achieved, a useful degree of purification has been obtained.

3. The variables involved in the separation have been studied, and the limiting conditions defined.

4. On the basis of the work carried out a simple separation has been devised which will give in four steps a useful concentration of the enzyme.

5. For large-scale development of the proposed separation, special equipment would be required. A suitable design has been prepared.

SUMMARY

SUMMARY

While some progress has been made in defining general conditions of protein solubility, problems of enzyme purification have been solved in the past by methods which are largely empirical. While a range of isolation procedures and combinations of these procedures are at present available, the isolation of any particular system depends largely on the application of trial-and-error methods.

The isolation of lipoxidase has been achieved in only one recorded case, by a complex combination of precipitative and electrophoretic methods. The work reported in this thesis describes the application of the use of ethanol as a precipitant in a purification procedure for lipoxidase.

A study of precipitation conditions is reported, and arising from the data obtained, routes of separation are described on an experimental basis. From these studies and the experience accumulated, a simple procedure for obtaining a useful degree of concentration of the enzyme is described.

The procedure calls for experimental arrangements to permit working at controlled temperatures between

0^{o} and -5^{o} with a sufficient reserve of refrigerative capacity to rapidly dissipitate the heat of solution of ethanol. The bulk of the activity is removed in the fraction between 30-45% ethanol and the subsequent precipitate which is readily obtainable in quantity, may be further concentrated by continuous electrophoresis at pH 6.0.

The conditions, required to prevent undue losses, are shown to be somewhat critical but given these conditions, the procedure evolved is simple.

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APPENDIX I

HOLMAN'S PURIFICATION OF LIPOXIDASE

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HOLMAN'S PURIFICATION OF LIPOXIDASE

Fat-free, low-temperature extracted soya-bean flour (15 kg.) was suspended in 100 litres 0.1 M acetate buffer at pH 4.5. Centrifugation by a basket centrifuge removed the insoluble material and the extract was adjusted to pH 6.7 with ammonia. Five volumes of 20% barium acetate, 10 volumes acetone and 2 volumes of basic lead acetate were added per 100 volumes of extract. The inactive precipitate was removed in a large separator.

The inactive precipitate produced by addition of 25 g. $(NH_4)_2SO_4$ per 100 ml. extract was allowed to settle and the supernatant fluid was decanted. More $(NH_4)_2SO_4$ was added to bring its concentration to 40 g./100 ml. and the precipitate containing the activity was recovered by centrifugation.

The solid was redissolved in a small quantity of water and heated to 63^0 for 5 mins. to coagulate inactive albumins. The precipitate was centrifuged off. The supernatant liquid was fractionated with ammonium sulphate and that which precipitated between 35% and 50% saturation retained. The precipitate was

dialysed against 0.02 M phosphate buffer at pH 5.5, and the undissolved material was discarded.

This was fractionated with 90% ethanol at 0° at pH 5.5 and the initial precipitate deposited below 12% contained the activity. This solution was dissolved again and fractionation with $(NH_4)_2SO_4$ between 50% and 60% saturation yielded an active precipitate. This was dissolved in M/50 phosphate buffer at pH 7.5 and was separated in the large Tiselius apparatus. This was concentrated by dialysing against $(NH_4)_2SO_4$ solution, the concentration of the latter being gradually increased. The lipoxidase crystallised out and was then washed free of amorphous material with slightly less concentrated ammonium sulphate.

APPENDIX XI

LIPOXIDASE ASSAY

APPENDIX II

LIPOXIDASE ASSAY

Reagents

Reaction buffer

M/50 pH 9.0 ammonia/ammonium chloride buffer

10 mls. N ammonia and 30 mls. N ammonium chloride diluted to 2,000 mls.

Alcoholic sodium hydroxide

4 g. of sodium hydroxide are dissolved in 5 ml. of warm distilled water and diluted to 100 ml. with ethanol. The solution is standardised in the usual way.

Substrate solution

1 g. of pure linoleic acid obtained from the Hormel Institute, Minnesota, is saponified overnight with a slight excess of alcoholic N sodium hydroxide solution. This is diluted to 500 ml. with distilled water.

Aqueous sodium hydroxide

A 20% solution in distilled water is used to stop the enzyme reaction.

Aqueous potassium cyanide

A 5% solution in distilled water is used.

Spectrophotometric measurements

The Hilger Uvispek spectrophotometer is used with wavelength at 234 mμ, slit width 51 and 1 cm. cells.

Reaction flasks

The reaction is carried out in 175 ml. conical flasks. These are thoroughly cleaned by soaking overnight in a strong sodium hydroxide solution. Then they are thoroughly rinsed with tap water and then distilled water before drying for use. Between assays they may be rinsed thoroughly with distilled water and dried. Soaps and detergents should never be used for cleaning these, since the presence of such surface active agents may interfere with the assay.

Method

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.	Substrate solution, 1 ml.
3	Aqueous potassium cyanide, 1 ml.	Aqueous potassium cyanide, 1 ml.
4	Enzyme solution, x ml.	Aqueous sodium hydroxide, 1 ml.
5	Aqueous sodium hydroxide, 1 ml.	Enzyme solution, x ml.

The reaction buffer is added to the flasks and is thoroughly shaken up. After the substrate solution and the potassium cyanide have been added the flask is shaken up. The enzyme solution, 0.05 to 0.3 ml., is added from a microburette with a capillary attachment. The flask is rotated during the addition and also during the time the reaction is proceeding. As soon as the enzyme is added, a stop watch is started and after exactly one minute the sodium hydroxide is added to stop the reaction. The optical density of the solution is measured in the spectrophotometer against the blank solution prepared as above.

APPENDIX III

METHODS OF DEVELOPING PROTEIN BANDS ON ELECTROPHORESIS PAPER STRIPS

APPENDIX III

METHODS OF DEVELOPING PROTEIN BANDS ON ELECTROPHORESIS PAPER STRIPS

1. Hydrolysis of the protein spots by trypsin and development of the amino acids produced with ninhydrin.

The paper strip was dried at 105° for 5 mins. and then a strip of filter paper soaked in 0.1% trypsin was placed on top of it. They were then held tightly between glass plates and held at a temperature of 50° for 30 mins. The protein strip was then removed and dried for 1 minute at 105°. After spraying with 0.1% ninhydrin it was dried again at 105° for 5 mins.

2. Hydrolysis of proteins with alkyl sulphates and development of amino acids with ninhydrin.

The paper was dried at 105° for 5 mins. then a filter paper, soaked in a 10% solution of a commercial detergent, "Teepol", was placed on top and the whole placed between two glass plates. This was kept at 60° for 30 mins. and then the test paper was dried for one minute at 105°, sprayed with ninhydrin and dried again for 5 mins. at 105°.

3. Naphthalene black.

Solutions:- (a) Saturated solution of naphthalene black 12.B.200 in methyl alcohol containing 10% acetic acid.

(b) Methyl alcohol containing acetic acid.

(c) Methyl alcohol.

The paper strip after being dried for 30 mins. at 105° was immersed in the dye solution for 10 mins. Then after being washed with solution (b) until the background colour was a pale blue and washing with methyl alcohol, the paper strip was dried in a current of warm air.

4. "Photographic" method.

Solutions:- (a) 0.5 g. silver nitrate in 100 ml. of water and one drop of dilute nitric acid added.

(b) 175 g. sodium carbonate in 1 litre distilled water.

(c) 10 g. hydroquinone in 100 ml. alcohol.

(d) 100 g. sodium thiosulphate in 1 litre distilled water.

The paper was dried as before at 105° for 5 mins. then dipped in solution (a) for 5 mins., then washed in water for 5 mins. It was then dipped in a solution containing 10 ml. of solution (c) to 100 ml. of solution (b) for two and a half minutes. It was washed for half a minute and then dipped in solution (d) for two and a half minutes. After washing for 5 mins. it was dried in a warm current of air.

5. Bromophenol blue.

Solutions:- (a) 1% bromophenol blue in 95% ethyl alcohol saturated with mercuric chloride.

(b) Methyl alcohol containing 1% mercuric chloride.

(c) Methyl alcohol.

The strips were dried for 30 mins. at 105°. They were first immersed for 5 mins. in the bromophenol blue solution and the excess of dye removed by four washings in methyl alcohol containing mercuric chloride. This washing took half an hour to complete. The solution was kept moving over the strips. The excess mercuric chloride was removed by washing the strips in methyl alcohol. The strips were finally dried in a current of warm air.

APPENDIX IV

METHOD OF ELECTROPHORESIS

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METHOD OF ELECTROPHORESIS

Apparatus

Two types of apparatus were used.

1. This is a perspex box, measuring 45 x 12 x 20 cm. with a heavy glass lid. Four partitions run the length of the box at its base dividing it into five compartments. All of these compartments, except the centre contain the buffer solution, the outer pair also contain the carbon electrodes (diameter 6 mm.) which protrude from the ends of the tank through rubber bungs. The outer partition on each side has six holes (diameter 6 mm.) spaced at equal intervals along its length near its free upper edge. Small wicks fill these and dip into the buffer solution on either side connecting the inner and outer compartments electrically. The purpose of this arrangement is to prevent the pH changes at the electrodes from reaching the inner compartments. The slender glass rod which runs down the centre of the box 19.5 cm. above the base (17 cm. above the liquid levels) suspends the filter paper strips. The whole apparatus is mounted on a base with levelling screws to give a level base to prevent siphoning between the compartments by way of the filter paper strips and wicks.

2. This apparatus is a perspex block 11" x 8" x $\frac{1}{8}$" at the sides of which are fixed two perspex supports $\frac{1}{8}$" thick, 2" wide and 21 cm. in height, holding a 6 mm. diameter glass rod 19.5 cm. above the base.

There are four compartments - $10\frac{5}{8}$" x $\frac{3}{4}$" x $\frac{1}{4}$" - connected at equidistant points by wicks, $\frac{1}{8}$" diameter, passing through holes in the partition $\frac{1}{8}$" below the surface of the partition.

The carbon electrodes are 6 mm. in diameter with brass terminals and are held in position by rubber bungs 1.2 cm. in diameter.

The whole is mounted on a base 13" x 10" x $\frac{1}{8}$" held in position by strips of perspex and then this is held on a wooden base 15" x 12" x $\frac{1}{8}$" by wooden strips. The whole is mounted on levelling screws provided with locknuts.

The hood covering the whole is made of $\frac{1}{8}$" perspex and is $12\frac{1}{2}$" high, resting on a rubber ring on a perspex base.

Electrolyte

M/15 phosphate buffer - 84 mls. M/5 potassium dihydrogen phosphate and 16 mls. M/5 disodium hydrogen phosphate, diluted to 300 mls. with distilled water.

Developing solutions

1. 1% Bromophenol blue in 95% ethyl alcohol saturated with mercurie chloride.
2. Methyl alcohol containing 1% mercurie chloride.
3. Methyl alcohol.

Paper

Whatman No.1 for apparatus 1. strips 36 x 5 cm.
" " " " 2. " 37 x 6 cm.

Procedure

The four compartments in the tank are filled with the buffer to the levels of the wicks and strips of Whatman No.1 filter paper are placed in position. When the tank is loaded the lid is replaced and the strips are allowed to become saturated by capillarity. After an hour the strips are straightened out with glass rods so that they are held at their ends to the inner partitions by surface tension, and all liquid levels are adjusted to the same height. Then the liquid to be tested is applied by means of a micro-syringe at the apex of the filter paper strip in a narrow band extending to 5 mm. of the edge on either side. The lid is replaced and the potential applied.

The apparatus is allowed to run the allotted time and the strips are then removed from it and dried for

30 mins. at 105°. They are then developed as follows. They are first immersed for 5 mins. in the bromo-phenol blue solution and the excess of dye is removed by four washings in methyl alcohol solution containing mercuric chloride. This washing takes half an hour to complete and it was found that the solution must be kept moving over the strips. The excess mercuric chloride is removed by washing the strips in methyl alcohol. The strips are finally dried in a current of warm air.

THE COMPARISON OF THE EFFECTS OF CATHODE RAYS ON CAROTENE, LINOLEIC ACID AND BUTTER

by

Mary Davidson Cameron, B.Sc., A.R.T.C., A.R.I.C.

An additional paper to the thesis submitted in accordance with the requirements of the Faculty of Science of the University of Glasgow for the Degree of Doctor of Philosophy.

June, 1956.

INDEX

SUMMARY

SUMMARY

When exposed to high voltage cathode radiation the carotenoid pigments of butter are bleached and the butter becomes rancid. Since it is well known that such pigments are destroyed by a coupled reaction with fatty peroxides catalysed by the unsaturated fat oxidases the possibility of a similar reaction mechanism initiated by the ionising effect of the radiation has been investigated.

Linoleic acid and β-carotene were used as the model system. Although the proportion of octadecanoic acid present in butter fat is 18.9% while there is only 2.1% octadecadienoic acid it was decided to use linoleic acid because at its more reactive unsaturated centres oxidation is first likely to take place in butter.

Carotene was directly affected by radiation, but when irradiated in the presence of linoleic acid, the latter, instead of having an accelerating effect on the bleaching of the carotene, actually protected it to a certain extent from oxidation. There was also found to be a formation of conjugated hydroperoxides on exposure of linoleic acid by itself and in the presence of carotene. The results showed no direct similarity to the lipoxidase reaction.

A different pattern of results was found on irradiation of butter and therefore there appears to be no correlation between the reaction of butter and that of the model system. There are less conjugated hydroperoxides present in butter at the higher dosages due, perhaps, to secondary oxidation taking place to a greater extent than the primary formation of conjugated hydroperoxides. The amount of carotenoids destroyed increased with rising dosage.

The effect of temperature on the reactions caused by irradiation confirmed the direct effect of the rays on both carotene and linoleic acid as with lowering of the temperature they were increasingly stable.

INTRODUCTION

INTRODUCTION

During the past few years considerable interest has been aroused in the possibility of sterilising foodstuffs with either β or γ -radiations obtained from electrostatic generators of the Van der Graaf or linear accelerator types or from radioactive waste products. Amongst many reactions induced in foodstuffs by the ionising effects of these radiations, oxidative deterioration of fat has presented a major problem.

In 1950 Mukerjee[10] reported that ultra-violet light and X-rays induced rancidity in butter fat. This only took place if oxygen was present during the radiation and there was no peroxide formation when irradiated in vacuo. After irradiation, there was an increase in the amount of peroxide formed during storage when the butter had been irradiated in oxygen but no increase in after effects when irradiated in vacuo even if exposed to air afterwards. He also treated various pure acids with U.V. light and found there was a feeble formation of aldehydes with lower fatty acids. The unsaturated acids, linoleic acid and linolenic acid, formed peroxides.

Dunn et al in 1948[3] and again Ede in 1950[4] found that high speed cathode rays produced similar rancidity in butter. The peroxide values increased with the amount of dosage received but the rate of increase did not appear to be linear. The orange-yellow colour of the butter was destroyed during radiation. This was confirmed by Hannan and Boag[5] in 1952 using the electron beam of a Van der Graaf generator at 1.2 MeV. and they found that the amount of peroxides formed varied with the temperature of the radiation. Also, it appeared that the carotenoid destruction was proportional to the dosage and the water content of the system. Later Hannan and Shepherd[8] reported discovering certain secondary effects on storage as Mukherjee had found with X-rays and these varied with the temperature of storage. The lower the temperature of both irradiation and storage the greater was this increase in peroxides. Hannan and Boag[6,7] have shown that there are two reactions going on in the butter, firstly the reaction of the unsaturated acids and secondly a reaction which they have tentatively called the free radicle chain reaction. The after effect was found not to be related to the

physical state of the butter but only due to a chemical effect induced at low temperature. The amount of peroxide formed depends on the amount of oxygen present in the butter, the peroxide value reaching a limiting value at each oxygen level.

This after effect has been reported by Astrach, Sorbye, Brasch and Huber[1] in irradiated fish oils, but they also report an initial destruction of the peroxides present in untreated oil.

In 1952 Mead[9] showed that when linoleic acid was irradiated with X-ray the amount of conjugated, oxidised acids formed varied with the radiation dosage used but that if the amount of radiation was kept constant the concentration of product bears an approximately linear relationship to the concentration of the substrate. From his results he suggested that the reaction was similar to the free radicle theory of autoxidation. However, he reported that trans:trans isomers were formed during radiation but in autoxidation cis:cis isomers have been always reported.

The striking feature observed during the radiation of butter was the bleaching of the yellow carotenoid pigments present. This bleaching which occurred during the radiation continued on storage for several weeks

after irradiation. Since lipoxidase induced rancidity of fats is characterised by a similar bleaching of associated carotenoids it was a matter of some interest to revise experiments with a view to examining similarities and differences in the reaction mechanisms.

EXPERIMENTAL

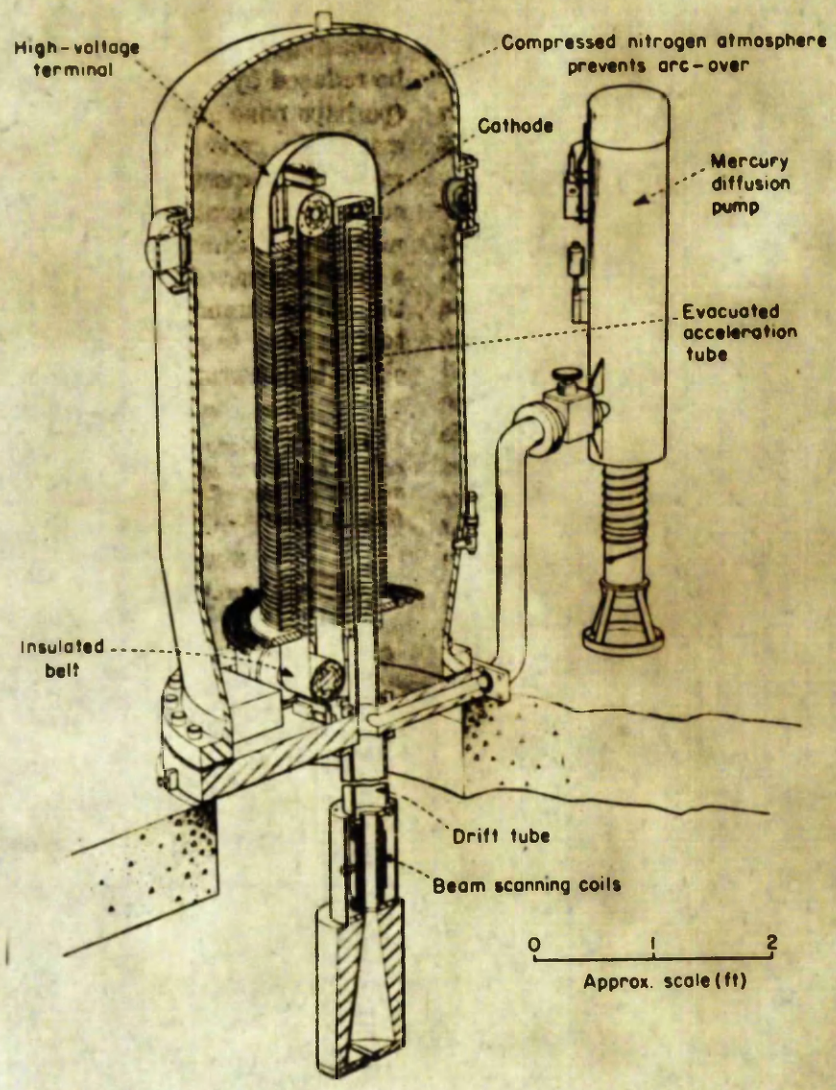

Fig. 1

Van der Graaf Electrostatic Generator

EXPERIMENTAL

The experiments can be divided into two sections, the effect of the radiation on firstly pure linoleic acid and pure β-carotene and secondly on butter.

The source of the high speed cathode rays was a Van Der Graaf generator capable of delivering cathode rays at energies up to 2.8 MeV in a working power range of about 3,000,000 to 1,000,000,000 gram-rep. per minute. The electron beam from this machine has a penetration in water of 2 cm. but it can only penetrate 1 cm. into solid material such as butter.

The Van der Graaf generator is shown in Fig. 1. An electron charge is applied to the fast moving insulated belt and the belt carries the charge to a high voltage terminal, thus creating an electric field down the length of the evacuated acceleration tube. Electrons emitted from a heated cathode are accelerated down the tube by this potential difference between the high voltage terminal and the lower end of the accelerator. This electron beam passes through beam-scanning magnetic or electric fields which control the lateral beam intensity distribution. After passing through a six-inch aluminium window the beam strikes

the material being irradiated. This is passed through on a continuous belt.

The radiation doses were estimated from the time interval of exposure along with beam power calculations[11]. Independent determination of the dose for each sample was made by chemical dosimetry. The oxidation of ferrous solutions by the radiation to ferric was estimated by means of the Beckman model DU spectrophotometer[12]. These solutions were placed in ampoules and irradiated, lying horizontally in the belt, beside the sample.

The roentgen is the fundamental dosage unit for radiation and it is defined as a certain quantity of ionisation, one roentgen being that quantity of radiation which produces in 1 cc. of air ions carrying 1 esu of electricity of either sign. That is one roentgen forms 2.083×10^9 ion pairs/cc. As the energy necessary to produce one ion pair is constant the roentgen is also an energy unit, 83 ergs per gram of air or 93 ergs per gram of tissue. For alpha and beta rays the unit of dosage is roentgen-equivalent-physical (rep) which is defined as that dose which produces energy absorption of 93 ergs per gram of tissue.

1. Linoleic acid and β-carotene

The linoleic acid was examined for the development of conjugated hydroperoxides by the study of the ultra-violet absorption at 234 mμ. The absorptions were measured on a Beckmann Spectrophotometer and the systems used were as described by Blain, Hawthorn and Todd[2].

The following reagents were used:-

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.

Carotene solution

1.5 mg. of carotene (90% β, 10% α) dissolved in 100 ml. of a mixture of acetone-ethanol (75/25V/V).

Aqueous caustic soda

20% solution used to clarify solution.

Alcoholic caustic soda

4 g. of caustic soda pellets dissolved in 5 ml. of warm distilled water and diluted to 100 ml. with ethanol.

Sodium linoleate solution

Linoleic acid from the Hormel Institution

(iodine value 180.87) was saponified overnight with slight excess of normal alcoholic caustic soda solution and then diluted with distilled water so that one ml. of this solution contains 1 mg. of linoleic acid.

The containers used were glass crystallising dishes covered with aluminium foil and the depth of the solution radiated was 1 cm. from one direction only.

Method

(a) Carotene

4 ml. of carotene solution were added to 50 ml. of pH 6.0 phosphate buffer and this solution was placed in a container after thorough mixing. After irradiation of this container 20% caustic soda was added in the amount of 2 ml. per 50 ml. reaction mixture and after mixing the absorption at 465 mμ was immediately found. The blank used contained only buffer and caustic soda in the amounts given above. The control, made up in a similar manner to the sample, was kept at room temperature for the same time as the sample was being irradiated.

(b) Linoleic acid

0.5 ml. of the linoleic acid was diluted with 50 ml. of pH 6.0 phosphate buffer and irradiated. After

irradiation the pH was increased by the addition of caustic soda as in the carotene determination. The solution was tested against a buffer/caustic soda blank at 234 mμ and a control similar to the reaction solution was tested at the same time.

(c) Linoleic acid and carotene

A solution was irradiated containing 0.5 ml. of linoleic acid solution, 4 ml. carotene solution and 50 ml. of phosphate buffer. After irradiation 20% caustic soda was added as in (a) and (b) and the solution was shaken thoroughly. It was tested at 465 mμ against a phosphate buffer/caustic soda blank and then it was diluted 1:4 with pH 6.0 phosphate buffer and the U.V. absorption determined at 234 mμ against a phosphate blank. A control was run with the same solution keeping it for the same length of time as the irradiated sample.

Notes

1. The carotene and linoleic acid should not be mixed until just before irradiating.

2. The linoleic acid must be fresh because of the danger of the bleaching of the carotene by preformed peroxides.

3. The acetone absorbs in the region of 234 mμ which

increases the error of the method and if continued work was being done on this it would be better to improve the system by using another solvent. However, the error is small with careful preparation of blanks.

4. The carotene should be stored in a brown or covered bottle at room temperature and the linoleic acid should be firmly sealed and stored at 0^{o} to 4^{o}.

5. The solution must be tested as soon as possible after irradiation.

2. Butter

The butter was irradiated in circular tins 2 cm. thick and of diameter 7 cm. It was Grade AAA household butter and the whole butter was used. The butter was used as delivered without any attempt to include more water or oxygen as it was thought that equilibrium conditions would have been reached by the butter from the time of packing. It would have been difficult to reproduce unnatural conditions and in the past it has been found that there is disagreement between the results when fat has been separated from the butter and washed with distilled water and saturated with air[5]. All comparative tests were done on the same sample of butter. The cans were

filled with butter, which was smoothed down firmly, and then sealed in air and irradiated on both sides at approximately 0^{o} unless otherwise stated.

The butter was sampled in two ways, to study firstly the amount of conjugated hydroperoxides and, secondly, the amount of carotenoids.

Method.

(a) Conjugation

0.5 g. of fat were saponified with 2.5 ml. of normal alcoholic sodium hydroxide overnight at room temperature and then dissolved in distilled water and made up to 50 ml. 0.5 ml. of this solution were added to 100 ml. phosphate buffer and then 2 ml. of 20% sodium hydroxide solution were added. After thorough shaking it was tested immediately at 230 mμ against a buffer/sodium hydroxide blank. A control butter, unirradiated, was tested with the sample to make the correction for autoxidation.

(b) Carotenoids

5 g. of butter were shaken up with approximately 35 ml. of alcohol/acetone mixture (75/25V/V) and then left overnight at room temperature in a dark cupboard. The volume of the solution was made up to 100 ml. with the alcohol/acetone solution and then the solution was

thoroughly shaken up. After filtering the solution was tested at 465 mμ using an acetone/alcohol blank. A control butter was tested in the same way as the sample.

Note

The fat and the alcoholic sodium hydroxide are heated gently at the beginning of the saponification in order to get thorough mixing. On calculating the results the values of pigment destruction and increase in absorption at 230 mμ were corrected by the control values so as to eliminate the ordinary autoxidation effects taking place at the same time.

Results.

The formation of conjugated hydroperoxides was followed by comparing directly the optical densities at 234 mμ of the solutions. The percentage of carotene destroyed in the solution was found by calculating what fraction the decrease in the optical density was of the optical density at 465 mμ of the original solution.

With butter the optical densities recorded were corrected to 5.0 g. for the carotenoid destruction and 0.5 g. for the formation of conjugated hydroperoxides. The results were then treated as above with the pure chemicals.

Where the controls are reported with the results the latter have not been corrected with respect to the controls.

With the linoleic-carotene systems the different sets of results are not directly comparable. The age of the linoleic acid was different in some cases and it was found that slightly oxidised linoleic acid forms conjugated peroxides more readily than freshly prepared linoleic acid.

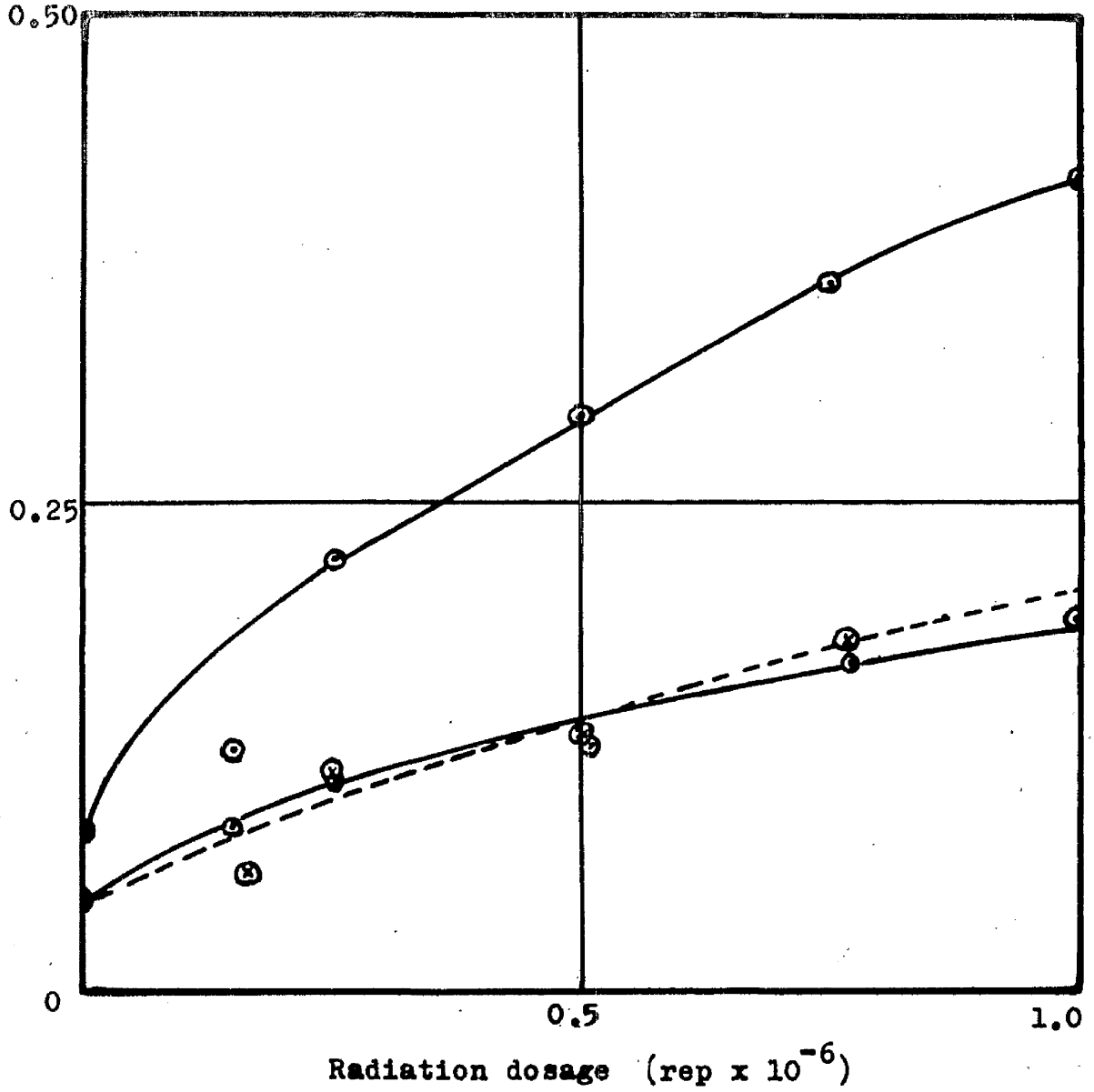

Fig. 2

The relationship between diene conjugation and radiation dosage in phosphate buffer at pH 6.0

Upper curve: 2 mg. per cent linoleate

Lower curves: 1 mg. per cent linoleate

1. Linoleic acid and carotene

(a) Linoleic acid.

In Fig. 2 are recorded the results of two experiments comparing the formation of conjugated hydroperoxides at different levels of cathode radiation and it can be seen that the method is reproduceable. The amount of conjugated hydroperoxides formed on radiation increases with increasing dosage and the relationship is approximately linear with no tailing off at higher dosages. It was also found that increasing the concentration of linoleic acid in the solution increased the conjugation approximately in proportion.

TABLE I

Effect of temperature on irradiation of linoleic acid
(concentration of linoleic acid 1 mg./100 ml.)
(Dosage = 639,000 rep)

Temperature °C	-	40	3	-16	-30
Absorption at 234 mμ	0.042	0.103	0.101	0.075	0.064

Table I shows the effect of temperature of irradiation on the degree of conjugation in the linoleic acid. There is a decrease in conjugation as the

temperature is lowered. This is not in agreement with the results obtained by others[8] on butter which showed an increase in the amount of oxidation as determined by other methods with lowering of temperature of radiation. Hydroperoxide formation associated with diene conjugation would normally lead to subsequent polymerisation or other secondary reactions of the fatty acid chains, which in turn would be associated with loss of absorption at 234 mμ. There are thus two possible explanations of this Table. The lowering of temperature might either result in an overall decrease in oxidation, or alternatively an increase may have taken place which was masked by secondary reaction products.

The second conclusion seems the more probable in view of Hannan's[8] data on butter, and the storage data given in Table II lends support to this view.

TABLE II

Effect of storage on linoleic acid after irradiation
(concentration of linoleic acid 1 mg./100 Ml.)

(Dosage = 639,000 rep)

Temperature of storage °C		40	3	-16	-30
Absorption at 234 mμ on irradiation	Control 0.042	0.103	0.101	0.075	0.064
Absorption at 234 mμ after storage for 4 days		0.113	0.062	0.042	0.018
Absorption of control 234 mμ after 4 days		0.125	0.084	0.042	-

It appears that storage at high temperatures caused increased conjugation while at lower temperatures it is gradually decreasing. When compared with the controls it is seen that there is an increase of conjugation in them in all cases except -16° which remains the same and that in all cases irradiation decreases the amount of conjugation taking place in linoleic acids on storage. The rapid increase in absorption of the controls stored at 40° and 3° is of the pattern to be expected from normal autoxidation under these conditions. Since dual interpretations may be placed on these results, arising from the use of diene

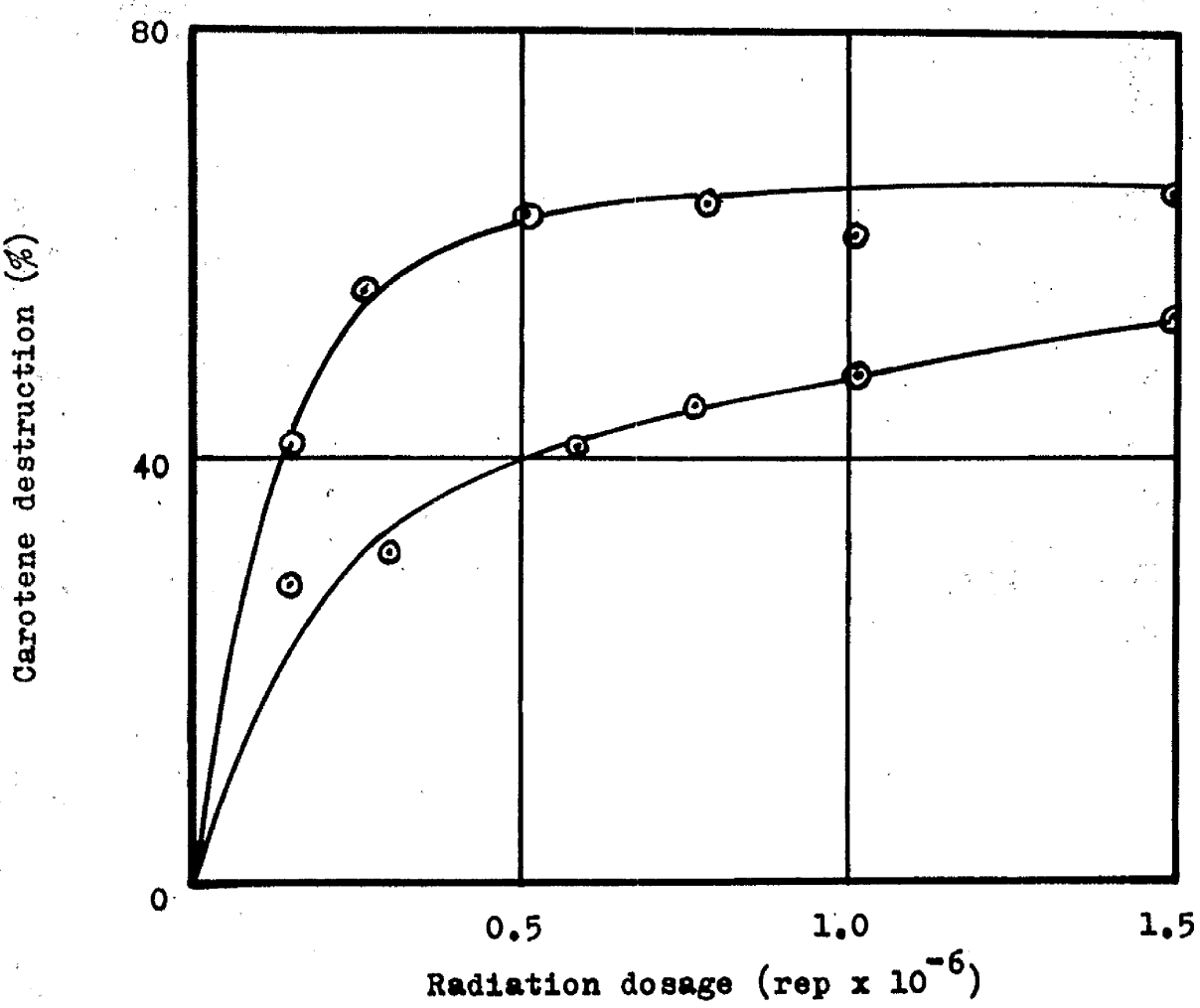

Fig. 3

The relationship between carotene destruction and radiation dosage

Upper curve: 0.12 mg. per cent carotene dispersed in phosphate buffer at pH 6.0

Lower curve: 0.12 mg. per cent carotene in presence of 1 mg. per cent linoleate in phosphate buffer at pH 6.0

conjugation as a criterion of degree of oxidation, the question of the validity of this method arises. This will be dealt with in the discussion.

(b) Carotene.

Fig. 3 shows the effect of irradiation on systems containing β-carotene in the absence of a fatty substrate. The carotene is directly destroyed by the radiation and although there is a tailing off at higher dosages this could be due to lack of availability of oxygen or of carotene.

TABLE III

Effect of temperature on irradiation of carotene

(Dosage = 639,000 rep)

Temperature ^{o}C	40	3	-16	-30
% Destruction	91	56	67	42

As Table III shows freezing decreases greatly the effect of irradiation as would be expected from standard radiation theories, if carotene is directly affected by the ionising rays. It was attempted to directly obtain storage effects, but bleaching was so rapid in this system that the results were inconclusive.

(c) Carotene and linoleic acid together.

The lower curve in Fig. 3 shows that the linoleic acid instead of acting as an activating agent, as would be expected were the lipoxidase analogy correct, actually has a protective action towards the carotene. With higher dosages the amount of carotene destroyed, instead of being constant with increasing dosage as when carotene is irradiated alone, increases gradually. Although treated to the same dosage the amount of carotene destroyed did not reach the maximum amount, namely 65%, obtained on irradiating carotene alone.

TABLE IV

Effect of temperature on radiation and storage of linoleate-carotene mixtures

(Dosage = 639,000 rep)

Temperature ^{o}C	40	3	-16	-30
% Destruction of Carotene				
Zero storage	72	80	57	57
4 days storage	85	94	-	83

There is least destruction of carotene at lower temperatures again confirming the direct action of the

radiations on the carotene instead of through the hydroperoxides as predicted. There is a slightly greater destruction of the carotene at 3^{0} than at 40^{0} which may indicate that there may be some action through the peroxides. Again there is less destruction of the pigments than when carotene is irradiated alone at 40^{0} and at -16^{0} but at 3^{0} and -30^{0} there is more destruction of the carotene in the presence of linoleic acid.

In the storage tests the amount of carotene destroyed during the storage period is greatest at -30^{0} although the total amount of carotene destroyed during irradiation and storage is less than at 3^{0}.

TABLE V

Variation of linoleic acid concentration

(Dosage = 297,000 rep)

Concentration of linoleic acid ($\times 10^{-5}$M)	0	1.78	3.58	7.15	10.71
% Destruction of carotene	57	36	23	50	70

From the above table it is seen that as the amount of linoleic acid is increased in the solution the amount of carotene destroyed firstly decreases but then after a certain minimum of destruction at about

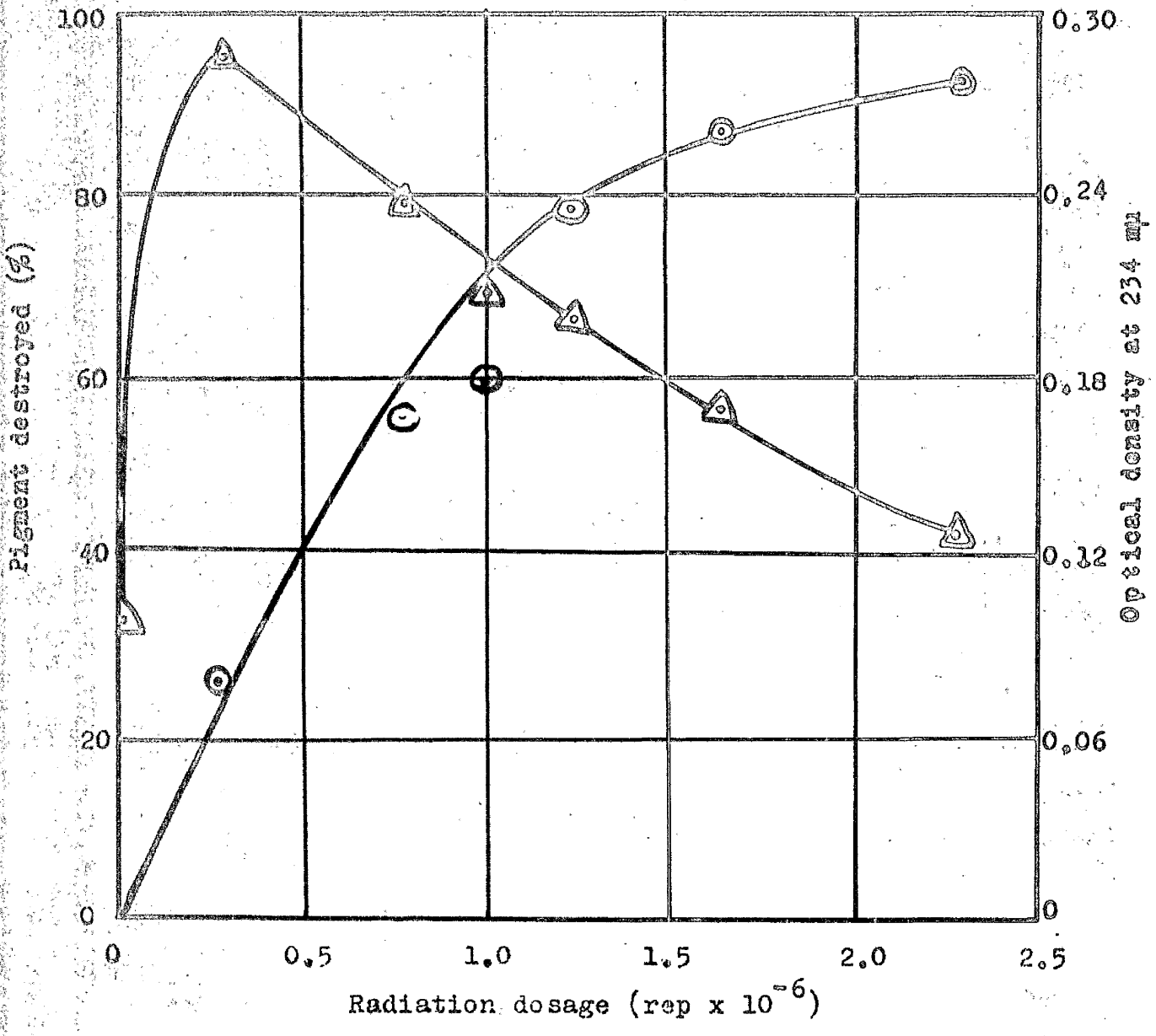

Fig. 4

Effect of cathode rays on butter fat and butter pigments

—△— Conjugation of diene

—⊙— Destruction of pigments

3.58×10^{-5}M linoleic acid the amount of carotene destroyed increases. The carotene destruction thus varies irregularly with the amount of unsaturated acid present in the solution and a possible explanation is suggested in the discussion.

2. Butter

From the results shown in Fig. 4 it is seen that β-rays attack the fat and pigments in butter in a different way from that with the pure chemical system. The conjugated hydroperoxides and the pigment in the butter are both decreasing with increasing dosage but there is no simple relationship between them.

Table VI shows the destruction of pigments under varying conditions of irradiation and storage.

TABLE VI

Effect of storage on butter after irradiation

% Destruction of pigments

Storage Temperature °C	-	40	3	-16	-30
Time of storage (days)	0	3	3	3	3
Irradiated Temperature °C					
40	20	32	36	35	57
3	24	35	52	69	90
-16	-	37	58	61	90
-30	24	40	39	56	67

Contrary to expectation, higher destruction values were observed at lower temperatures.

TABLE VIX

Effect of storage on butter after irradiation

Formation of conjugated hydroperoxides

Storage Temperature °C		-		40		5	
Time of storage (days)		0		5		5	
Irradiated Temperature °C		Cont.	Irrd.	Cont.	Irrd.	Cont.	Irrd.
	40	0.097	0.148	0.327	0.107	0.143	0.202
	5	0.097	0.321	0.327	0.155	0.143	0.190
	-16	0.097	-	0.327	0.201	0.143	0.105
	-30	0.097	0.005	0.327	0.096	0.143	0.229

Storage Temperature °C		-16		-30	
Time of storage (days)		5		5	
Irradiated Temperature °C		Cont.	Irrd.	Cont.	Irrd.
	40	0.215	0.113	0.199	0.177
	5	0.215	0.010	0.199	0.290
	-16	0.215	0.061	0.199	0.224
	-30	0.215	0.103	0.199	0.242

The destruction of pigment increases with decrease of temperature of storage but those with highest destruction are not those stored and irradiated at -30^{0} as would be

thought but those irradiated at -16^{0} and 3^{0} and stored at -30^{0}. The pigments in the controls were all destroyed to a lesser extent than those irradiated.

In most cases there is more conjugation at 3^{0} than at 40^{0} but there is a decrease again to -16^{0} and then a rise at -30^{0}. The highest amount of conjugation occurs at 3^{0} as irradiated temperature and -30^{0} storage temperature, one of the temperature combinations where there is the highest destruction of pigment.

Again there is less formation of conjugated hydroperoxides at 3^{0} and -16^{0} in the irradiated sample compared with the unirradiated sample. Again in this experiment the possibilities of a dual interpretation arises and this will be discussed later.

DISCUSSION

DISCUSSION

It is evident from the experimental data that there is no parallel between the effect of β -radiation and that of lipoxidase on the carotene-linoleate system described. Moreover, the bleaching of butter under the same conditions of radiation appears to follow a somewhat different mechanism to that of the carotene-linoleate system.

Carotene is directly destroyed by β -radiation. The presence of linoleic acid in concentrations of up to 2 mg. per 100 ml. exerts a certain protective influence on the carotene but at higher concentrations this effect decreases. This effect may reflect the dispersion of the system rather than a fundamental characteristic of the radiation effect. At low levels of radiation the carotene destruction curves are similar in the presence and absence of linoleic acid, but at higher levels the rate of carotene destruction is greater in the presence of linoleic acid. The destruction curve for carotenoids in butter is of a similar form to that of the linoleic acid-carotene system and it may be that analogous reactions are taking place.

In the absence of carotene, linoleic acid oxidises under the influence of β -radiation. Using conjugation

as a criterion of oxidation the reaction approximates to a linear function of radiation dosage. Owing to serious interference from the blanks it was not possible to obtain reliable figures for diene conjugation in presence of carotene in the model system. In butter, however, the absorption at 234 mμ decreased with increasing dosage. No explanation can be given for this phenomenon on the available data but the possibilities of polymerisation might be looked at in future studies. The oxidation of butter fat on irradiation has been reported by previous workers,[5, 6,7,8].

Hannan and Boag[6,7] found that the effects of the irradiation of butter varied with the temperature of the material at the time of irradiation. Studies on both the model system and on butter at various temperatures of irradiation revealed a rather complex picture. Lower temperatures decreased irradiation effects on both carotene and linoleic acid when they were irradiated alone. In each other's presence the effect on carotene decreased with decreasing temperature. With butter the greatest destruction of carotenoids took place at -16^{0}.

The effect of storage after irradiation at various temperatures was also complex but the generalisation may

be made that the lower the storage temperature and the lower the irradiation temperature of butter the greater is the loss of pigment.

The data on absorption at 234 mμ with butter shows a decrease in absorption with increasing dosage and it would be of interest to seek further information on this point. At the same time irradiated samples of linoleic acid after a marked increase in conjugation immediately after irradiation showed a subsequent decrease in storage. Both require further explanation.

One explanation of the variation in these results may be that two reactions are taking place on irradiation and storage. Firstly, there is a formation of conjugated hydroperoxides and, secondly, a breakdown or polymerisation of these to further oxidation products. In some cases the second reaction may be taking place to a greater extent than the first and therefore there is apparently less oxidation as measured by absorption at 234 mμ.

It would therefore appear that this method of determining fat oxidation due to radiation might not show the whole oxidation that was taking place as the primary oxidation at high dosages would proceed too quickly to the secondary products and therefore would

not be measured. However, it has indicated the route by which these oxidations take place, and if used in conjunction with other methods of measuring fat oxidation would show more clearly the breakdown of fats by β-radiation.

The system used, with linoleic acid and carotene would seem to be well adapted to the study of the effect on antioxidants in the radiation of fats.

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