LIPID BINDING IN WORKED WHEAT FLOUR DOUGHS

by

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SUMMARY.

A study has been made of factors affecting lipid binding in wheat flour doughs. Simple flour water doughs, wetted by a method which avoided the introduction of mechanical work, were used to follow changes in lipid binding during the initial stages of dough mixing. Varying the moisture content of such doughs showed that there was a "critical" moisture content below which mechanical work did not cause any appreciable increase in lipid binding. Above this moisture content there was a very rapid binding of flour lipids in the early stages of dough mixing, the total amount of lipid bound increasing as the mixing speed was raised. It was concluded that gluten development was the major factor affecting lipid binding.

When fully hydrated doughs containing lipoxygenase-active soya flour were mixed in air this initial rapid increase in bound lipid was followed by a release of bound lipid. The onset of lipid release was shown to occur after the same mixing time irrespective of mixing speed. However the time taken was dependent upon the availability of oxygen in the system.

To examine these effects in greater detail the binding of glyceryl tri (oleate-9,10-^{3}H), glyceryl tri (palmitate-1-^{14}C) and palmitic acid-1-^{14}C, has been studied by the use of liquid scintillation counting. The use of these labelled compounds revealed a difference in the behaviour of the free fatty acids compared with the triglycerides. Whereas triolein and tripalmitin showed similar overall binding patterns to the total flour lipids, palmitic acid gradually became less extractable with the solvents used to determine free lipid. Moreover it was shown that in both air and nitrogen there was a very rapid interchange of triolein between the free and bound lipid.

A new theory of lipid behaviour in dough has been proposed in which it was suggested that the triglycerides were in dynamic equilibrium between the free and bound states. Mixing in nitrogen was thought to shift the equilibrium towards the bound state whilst mixing in air had the opposite effect.

The relevance of these observations to current concepts of the nature of lipid binding in wheat flour doughs has been discussed with particular reference to the effect of lipids on baking performance.

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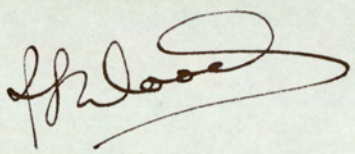
This is to certify that no part of the work described in this thesis was done in collaboration except where specifically mentioned. This work has not been submitted for any other award.



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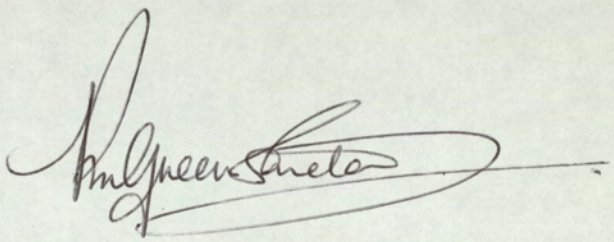


SECTION 1

INTRODUCTION

1.1 INTRODUCTION.

Whilst the art of breadmaking has been practised since the Stone Age, it is only during the past twenty years that there have been any fundamental changes in the methods of bread production. Today in many commercial bakeries the traditional method of breadmaking (see Fance and Wragg[1]) is giving way to new processes which replace three hour fermentation of the dough with a short period of intense mechanical work.[2] Under the influence of this work certain chemical reactions, involving lipids, are initiated which have a profound influence upon the quality of bread produced. It is these reactions, their nature and relative importance, particularly at low levels of mechanical work input, which will be the subject of this thesis.

The literature survey which follows will review what is known of the role lipids play in breadmaking. It will also examine the oxidative reactions in which flour lipids are involved and the influence these have upon the interactions of lipids with other dough constituents. However it is not possible to discuss the role of lipids during dough mixing without first reviewing the physical and chemical constitution of the basic dough ingredient, flour.

1.2 FLOUR COMPOSITION.

(a) Microscopic structure.

Cereal grains are the seeds of certain grasses and as such contain a germ, from which the new plant will develop, and a food store, the endosperm, on which the plant will initially grow. The endosperm consists of a collection of cells which contain starch granules of various sizes

embedded in a protein matrix.[3,4] Several workers have distinguished between the protein adherant to the starch granules, which is probably the remnants of amyloplast membranes,[5] and storage protein, which fills the interstices between the starch granules. Lipid droplets may be seen as inclusions in the protein matrix.[6]

On milling, the endosperm is separated from the germ and fragmented into a range of particle sizes which tend to increase in protein content as they decrease in size.[7] In the flour the fat content of the endosperm[6] is further supplemented by fat squeezed from the germ during the milling process.[8]

(b) Biochemical composition.

(i) Protein.

Chemically, wheat flour is unique amongst cereals in that part of its proteins are capable of forming (on hydration) a visco-elastic mass known as "gluten". When flour is made into a dough with water and then kneaded under a stream of water, the gluten readily separates as a rubbery mass. It is this material which is mainly responsible for the ability of wheat flour to hold stable the gas cells, formed by yeast fermentation, as they expand in the oven.

Gluten has been divided into two main fractions, glutenin and gliadin, on the basis of the latters solubility in 70% ethanol.[9] The introduction of more sophisticated techniques such as gel-electrophoresis has enabled gliadin, the viscous component of gluten, to be further separated. Woychik et al.[10] have found at least eight components in gliadin and subsequent workers have found almost twice as many.[11] However gel-filtration chromatography has shown that all the gliadin

components are approximately the same size.[12] Glutenin, responsible for the elastic properties of gluten, is a very high molecular-weight protein which appears to be composed of smaller protein units crosslinked by disulphide bonds. There has been some discussion whether the component proteins of glutenin are in fact gliadins[13] and Ewart has suggested that glutenins and gliadins may both be related to a single evolutionary precursor.[16]

The soluble proteins of wheat flour, the albumins and globulins, have also been examined by electrophoresis and have shown similarities to the soluble proteins of other cereals.[14] The globulin-like protein purothionin is the most investigated and will be discussed in more detail later. The soluble proteins also include several classes of enzyme such as the proteases and amylases. Lipoxygenase, lipase and ascorbic acid reductase and oxidase, are also present and play important roles in the chemistry of wheat flour.[15]

(ii) Lipids.

When flour is extracted with a non-polar solvent such as petroleum-ether, up to 1% of a yellow oil can be extracted which is termed "free lipid".[17] If the remaining flour is then extracted with a more polar solvent, such as water-saturated butanol,[18] a further 0.5% of fat, termed "bound lipid", is obtained. These fats are very complex mixtures of lipids, which, as a result of the advances in analytical techniques made during the last twenty years, are now becoming well characterized. The components have been separated by thin-layer chromatography[19,20,21] and silicic acid column chromatography, identified by infra-red, ultra-violet, nuclear magnetic resonance and mass spectrometry and their fatty acid composition determined by gas-liquid chromatography.[21]

The flour lipids can be further divided into two broad categories, neutral and polar[22,23] on the basis of their chemical structure. In practice these two groups are conveniently separated by differential adsorption on silicic acid (from chloroform solution).[24] The non-adsorped neutral lipids consist mainly of triglycerides,[25,26] diglycerides, sterol esters,[27,28,29,30,31] fatty acids[26,32,33] and traces of carotenoids,[34,35] hydrocarbons,[36] and monoglycerides. The polar lipids which may be washed from the silicic acid by methanol, are composed of almost equal amounts of phospholipids and glycolipids,[27,37] the most abundant being lecithin,[27] lysolecithin,[38,39] and digalactosyl diglyceride.[27,40,41,42,43,44] MacMurray and Morrison[27] have recently published a complete analysis of flour oil with fatty acid analyses of many of the lipid classes.

(iii) Starch.

Whilst starch comprises over 80% of flour and is essential for the formation of bread structure it is generally accepted that the rheological properties of dough are contributed almost exclusively by the gluten. During mixing the starch granules become thoroughly embedded in the protein matrix and it has been suggested that the starch and gluten are held together by strong electrostatic forces.[45] Small amounts of lysolecithin are present in the starch granules[39] but whilst lipids are known to affect the starch pasting properties and its subsequent retrogradation,[46,47,48,49,50] little has been reported on starch-lipid interactions in dough. The undamaged starch granules can be regarded therefore as an inert filler until the onset of gelation. However the damaged starch granules are a source of fermentable sugar through amylase hydrolysis and can also absorb up to 30% of their own weight of water. The water-soluble polysaccharides known as pentosans contribute only 2-3% of the flour weight but can be responsible for absorbing one quarter of the dough water. They can there-

-fore compete for available water with the dough proteins. The function of starch in dough has been reviewed by Medcalf and Gilles.[51]

1.3 LIPIDS IN BREADMAKING.

(a) Function of flour lipids.

Early research into the role of lipids in baking was often contradictory and confusing. Differences in grists, milling techniques, formulations, mixers and mixing times led to results that were often conflicting and difficult to correlate. One of the major difficulties in this work was that of removing all of the flour lipids without adversely affecting the breadmaking potential[52,53,54,55,56,57] of the extracted flour.

Most experiments concerning lipid functionality have been performed with petroleum-ether extracted flours which still contained a proportion of natural flour lipids thus making interpretation difficult. Some workers found that fat-extraction improved loaf volume and quality whilst others felt it had a detrimental effect. These studies have been the subject of reviews by Cookson and Coppock,[52] Fisher,[29] Daniels,[58] Mecham and Pence,[59] and Pomeranz.[60]

A possible cause of some of this confusion and contradiction may lie in differences in the flour proteins, which have been shown by Pomeranz et al.[61] to influence the behaviour of fat-extracted flours on baking. Petroleum-ether extraction of both strong and weak flours improved loaf volumes whilst reconstitution of the extracted flours restored all the original properties to both flours. However the addition of shortening to the fat-extracted soft flours was beneficial to loaf volume but was detrimental when added to the fat-extracted strong flours.

The same workers showed that reconstitution of fat-extracted strong flours with the oil from a soft flour restored the original properties typical of the strong flour. This was a further demonstration that the beneficial effect of flour lipids was dependent upon the flour proteins. Other workers have shown that whilst there were quantitative differences both physically[62] and chemically[63,64,65,66] associated with variety, season and environment, these could not be equated with baking performance.[67,68]

(b) Function of flour lipid classes.

Daftary et al.[68] have reconstituted fat-extracted strong flours with various flour oil fractions. The beneficial effect of fat extraction was shown to be due to removal of free non-polar lipids. Reconstitution with free or total polar lipids improved loaf volume but the addition of shortening as well as a flour fraction always gave an improvement over the flour fraction alone. Free polar lipids appeared to be better improvers than bound polar lipids.[56,69]

Further fractionation into chemical classes showed that the main improving compounds present in the polar lipids were the glycolipids.[68] Their usage and occurrence have recently been reviewed by Pomeranz and Finney.[40] However, the phospholipids also had an improving effect and in both cases the addition of shortening brought further improvement.

(c) Function of added shortenings.

The well known but by no means essential improving action of added fats (shortening fats) in the conventional three-hour fermentation became more intriguing when modern high-speed methods of dough development

were introduced.[70] These processes required the inclusion in the dough formula of a shortening fat which would be 10% solid at the proof temperature.[71,72,73] Without a fat of this specification the loaf volume was poor and there was no "oven spring".

This phenomenon was investigated by Elton and Fisher[74,75] who baked loaves containing mixtures of groundnut oils and long chain hydrocarbons and came to the conclusion that the improving effect was directly related to chain length. Evidence that a physical rather than a chemical effect was involved has been provided by Pomeranz et al.[76] They baked loaves containing mineral oils and waxes and found that waxes with a melting point around 50°C had a deleterious effect whilst those with a melting point between 61 and 85°C had an improving effect on loaf volume and crumb texture.

Many theories have been put forward to explain the action of flour oils and shortenings. Baker and Mize[77] suggested that added semi-solid fats blended with the flour oil and raised its melting point and viscosity. The hard fat was said to be able to 'plug' holes in the gas cell walls, thus aiding gas retention and resulting in larger loaves.[72]

1.4 LIPID OXIDATION REACTIONS.

The role of lipids in the oxidative reactions occuring during dough-mixing is now becoming fairly well understood after many years of intensive research. Initially, studies were directed towards elucidating the part played by lipids in the ageing of flour. The enzymic formation of free fatty acids from flour lipid[78,79] was shown to be related to flour ageing by Kozmin[80] who rejuvenated an aged flour by replacing its lipids with those from a fresh flour. The addition of oleic acid to a dough produced an

effect similar to ageing,[81,82,83] and extreme ageing was simulated by Sullivan who added oxidized fatty acids to a dough.[84] These phenomena appeared to be related to interactions between oxidized lipids and dough proteins[82] giving rise to alteration of the dough rheology, an effect which will be dealt with later.

The incorporation of soya flour, a rich source of lipoxygenase, into doughs as a bleaching agent has formed the basis of several patents[85,86] and the bleaching of pigments in model systems has been shown to occur through a coupled reaction involving lipid oxidation and atmospheric oxygen.[86,87,88,89,90] However oxygen also influences dough rheology[91,92,93] and is incorporated as an improver into the dough during the initial batter stage of the Rank and Hay[86] process. This improving effect was also shown to involve the oxidation of lipid by lipoxygenase,[89,94] although Morrison has suggested that all the lipid losses during mixing could not be accounted for by lipoxygenase action and that a β-oxidation mechanism might be involved.[95,96]

Oxygen uptake during dough mixing has been shown to be related to flour age[97] and particularly to linoleic acid content and the presence in flour of a lipoxygenase specific to fatty acids.[98,99] The addition of soya flour substantially shortened the time for doughs to reach maximum strength in the batter process[89] and Daniels[100] has suggested that the presence of a triglyceride lipoxygenase in soya flour[99] contributed to its effectiveness as a bread improver.

Smith et al.[101] noted that oxygen uptake was not prevented by fat-extraction and that it was related to the loss of thiol groups.[102] The bound lipids were thought to be the oxidative substrate by Koch[103] and Glass[104] and this view was supported by the results of Cunningham et al.[105]

They noted a link between lipids and the improver potassium bromate, which was thought to act at disulphide bonds. There was less loss of potassium bromate in doughs prepared from fat-extracted flour than in doughs from the original flour.

Bloksma[106] suggested that the flour lipids might act to protect the protein thiol groups from oxidation whilst Hawthorn and Todd[87] felt that the improvement brought about by fat-extraction was due to molecular oxygen independent of fat oxidation.

Whilst the involvement of sulphydryl groups in oxidative reactions is now well known, the products of these reactions are not known. Some authors have felt that oxidation of thiols to disulphides[107,108] or even further to sulphonic acid[100] was solely responsible for the improving action whilst others felt that oxidation of thiols facilitated sulphydryl-disulphide interchange.[109,110] As a result of several studies[109,111,112] it would appear that during the initial stages of doughmaking there is a rapid loss of sulphydryl groups. This initial loss is followed in nitrogen by an increase but, in the presence of oxygen or oxidizing agents, by a further decrease in sulphydryl groups. Mecham and Knapp[109] noted that fat-extraction eliminated the initial loss of thiols in nitrogen. Tsen and Hlynka[113] showed that lipid peroxides were formed in dough as a result of lipoxygenase activity and that when sulphydryl blocking compounds were incorporated into the dough the rate of peroxide formation increased. They postulated that the polyunsaturated lipid-lipoxygenase system in flour competes for available oxygen with the thiol groups and the lipid peroxides formed could also oxidize the thiols. This theory was supported by rheological studies with fat-extracted flours.[114] Further work by Tsen and Hlynka[115] showed that lipid peroxides when incorporated into a dough

increased the rate of thiol oxidation and exerted an improving effect. Bloksma[116] confirmed Tsen and Hlynka's results but suggested that the oxidation of thiols by lipid peroxides was a fast reaction relative to the direct oxidation by molecular oxygen.

The linoleate-lipoxygenase-sulphydryl interaction was confirmed by Dahle and Sullivan[117] but they felt that the role of the peroxide lipid was a minor one. The reactivity of lipid-lipoxygenase to glutathione was found to be low although other workers have found a rapid oxidation of glutathione in the presence of lipid-lipoxygenase and 2-octanol.[118]

Recent studies[119] have demonstrated the effectiveness of lipid peroxides in oxidizing thiols and have provided evidence that thiols can reduce hydroperoxy acids to hydroxy acids. Furthermore a factor associated with gluten is involved in the alternative reduction route of hydroperoxy acids to hydroepoxy acids on hydrolysis.[120]

Daniels et al. have shown that the increase in lipid binding normally found when doughs were mixed in nitrogen[121] or a vacuum,[122] was reversed in air and that this was related to the presence of lipoxygenase. Further studies[100] confirmed that during initial stages of the lipid-lipoxygenase reaction there was bleaching of the flour pigments which was followed by a rapid release of bound lipids. No lipid peroxides were found in the bound lipid or in free lipid during the period when bound lipid was being released. They made the hypothesis that the free lipid-lipoxygenase system oxidized the sites of lipid binding by a mechanism similar to that involved in pigment bleaching. Furthermore they suggested that this oxidation led to a drastic change in the electrostatic equilibrium of the protein network, causing disruption of the lipid-protein complex.

1.5 LIPID-PROTEIN INTERACTIONS.

(a) Lipid binding.

The formation of a complex between flour lipids and other dough constituents, (most probably protein), is generally referred to as lipid binding. The importance of the lipids involved, particularly the polar lipids, on the rheology of the resultant dough was realised by many early workers.[82,123,124] However it was Olcott and Mecham[125] who first recognized the importance of water content in the formation of this complex. They showed that as the water content of a high protein flour was increased the amount of free lipid (extractable with a non-polar solvent) fell, and after the introduction of mechanical work only 6% could be extracted.

These findings were subsequently confirmed by Pomeranz *et al.*[126] and Davies.[127] In a simple flour-water system phospholipids were preferentially bound, but the addition of shortening[128] or a modification of the polar-nonpolar[126,127] lipid ratio considerably altered the pattern of binding. The lipid binding potential of the flour protein was quite considerable as Olcott and Mecham[125] showed; up to three times the normal fat content could become bound.

The observations of Daniels *et al.*[121] that lipid binding was less in air than in nitrogen has already been mentioned, the effect being due to a lipoxygenase-coupled oxidation of the gluten protein.[100] This explanation also accounted for the increase in lipid binding noted when fat-extracted flours with added shortening were mixed in air, since the shortening provided no substrate for lipoxygenase activity.[23]

Lipid binding is probably influenced to some extent by the physical properties of the added fat.[126,129] Daniels *et al.*[23] found no evidence

of preferential binding of unsaturated triglycerides although the highly unsaturated polar lipids were preferentially bound. This point has been overlooked by other workers who found a connection between unsaturation and lipid binding.[72,130]

Technologically these observations are most important in the light of the requirements of modern high-speed mixing processes for a proportion of hard fat in the dough recipe.[72,73,131] Baldwin et al.[72] have reported differences in the amount of lipid bound in doughs prepared by conventional and continuous process. Daniels et al.[22] have confirmed this and shown lipid binding to be dependent upon the total work performed upon the dough, the rate of work input, and, as already explained, the mixer atmosphere.[121]

Olcott and Mecham[125] observed that after dough formation most of the lipids were associated with the glutenin fraction, but Ponte et al.[132,133] have since pointed out that this may be an artifact associated with the relocation of the lipids during the alcoholic extraction of the gliadin. A gluten fraction which was insoluble in dilute aqueous formic acid but was made soluble by oxidation was shown by Meredith to contain 88% of the gluten lipids.[134]

(b) Nature of the lipid-protein bond.

The nature of lipid binding is still somewhat speculative and several kinds of binding have been postulated. Hosenay et al.[135] suggested that glycolipids may be bound to the glutenin by hydrophobic bonds and to the gliadin by hydrophilic bonds thus forming a link between the gluten proteins. The addition of salt to a dough has been shown to reduce lipid binding,[126,127,128] but since it is not clear as to whether the polar or

the neutral fraction is affected opinions differ as to whether salt-like linkages are involved. Pomeranz et al.[126] and Davies[127] found that neutral lipid was released by the addition of salt whilst Wooton[136] and Mecham and Weinstein[128] found polar lipids released.

Wooton[136] demonstrated that solvents of high dielectric constant, such as formamide or formic acid, gave a dough like structure with flour in which there was evidence of lipid binding. He suggested that hydrogen bonding and electrostatic bonding are involved, whilst Schulerud[137] has suggested that lipid double bonds may be involved in the formation of the lipoprotein complex.

The problem of lipid-protein interactions in flour-water systems has been reviewed by Fullington[138] who, on the basis of his observations, has suggested that mixed chelates of protein and phospholipid on a divalent cation might be involved. This sort of intermolecular chelate has also been noticed in the envelopes of bacteria.[139]

Evidence for hydrophobic bonding can also be produced from other fields. Hydrocarbons such as butane and pentane[140,141,142] and also long chain fatty acids[143] have been observed to bind to bovine serum albumins and β-lactoglobulins. This is particularly interesting in the light of the remarkable improving properties of hexane and heptane.[144,145,146,147] When added to doughs containing shortening these hydrocarbons cause a considerable increase in loaf volume and quality. The requirement for added shortening and the increase in lipid binding noted, particularly of phospholipids, led Ponte et al. to suggest that the addition of hexane favours protein aggregation by increasing the hydrophobicity of the gluten proteins.[148]

1.6 GROSS STRUCTURE OF THE LIPOPROTEIN COMPLEX.

Such considerations of the lipid-protein link prompts speculation as to the physical structure of lipoprotein in gluten. Most theories are based on analogies with theories of cell membrane structure. Thus Coppock[149] speculated that gluten might prove to be an immature myelin. X-ray investigations by Traub[150] showed that endosperm sections of wheat grains had unique spacings at 47Å (4.7nm) and that these spacings were due to phospholipids. D.G.H.Daniels[151] proved that the spacings depend upon the presence of lecithin and the extracted phospholipids produced the same pattern when wetted. Similar X-ray experiments led Grosskreutz[152] to suggest that the gluten protein existed as platelets separated by well oriented lipid bimolecular leaflets which acted as slip planes. Extraction of the lipid was thought not to affect the basic platelets but to impair their ability to withstand large plastic deformation.

This bimolecular leaflet idea is analagous to the Davson-Danielli model of biological membranes in which a double layer of lipid molecules held together by hydrophobic bonds, is attached to a protein layer by electrostatic bonds.[152] Modifications to this concept, such as were introduced by Vandenheuvals[154] particulate concept or Lucy and Glauverts[155] idea that the membrane lipids are in globular micelles rather than layers, have generally been attempts to explain the porosity of physiological membranes.

Other workers, such as Benson,[156] considered the alternative arrangement most feasible, the membrane being envisaged as a layer of protein with certain hydrophobic amino-acid sequences which compliment hydrocarbon chains of the lipids. This results in a two dimensional lipoprotein aggregate which possesses the strongly anionic charged groups of the phospholipids

on its surface, and restores functionality to the protein. It is however generally recognized that such theories must be regarded as speculative since the obtaining of X-ray diffraction patterns and electron micrographs on which these theories are based involves drastic preparative procedures. Thus the possibility of artifact formation cannot be excluded.

This problem of isolating pure lipoprotein without disrupting or considerably modifying the molecule has been a major stumbling block to research in this field. Therefore the isolation by Balls and Hale of a lipoprotein, named lipopurothionin,[157] from petroleum-ether extracts of flour and the crystallization of the protein[158] moiety prompted considerable research. The high content of arginine and lysine[159,160] and the relatively low content of glutamic acid,[159] plus the presence of lecithin[161] in the complex pointed towards ionic binding. The high content of cysteine[157,159] has also prompted interest[162] because of the involvement of this amino acid in gluten development. However the lipoprotein had no bread improving effects.

1.7 OBJECTIVES.

From the previous sections it can be seen that the oxidative improving action of flour lipids on dough proteins is probably very much interlinked with the formation of the lipid-protein complex during the initial stages of doughmaking. Therefore the first objective of this thesis will be to gain more insight into the factors influencing the formation of this complex. The observation of Davies *et al.*[163] that lipid binding in unworked doughs increases with moisture content will be investigated further by studying the effect of work at a series of moisture levels.

A further objective is to verify that there is an initial increase in bound lipid at the onset of aerobic mixing. That this probably occurs can be deduced from a comparison of the results of Davies et al.[127,163] with Daniels et al.[169] The former found that at 47% moisture the amount of lipid bound was 46% and that this rose rapidly to nearly 90% when salted doughs were mixed to 0.4 H.P.min./lb.[163] Similarly Daniels et al.[169] have found that while work free wetting of a commercial dough gave 30% bound lipid this rose to about 45% at 0.1 H.P.min./lb. in air. Thus, even in air, when lipid release takes place there must have been an increase in bound lipid when mechanical work was introduced to the system.

From these investigations it is hoped to learn more about the factors limiting and controlling lipid binding and to gain a better understanding of the nature and sites of the lipid-protein bonds. Such a fuller understanding of the mechanism of these processes may eventually lead to technological advances particularly in the field of bread staling and improver action.

SECTION 2

EXPERIMENTAL

2.1 MATERIALS.

(a) Flours.

The flour samples (A, B and C) used in this work were unbleached, untreated bread flours milled from a commercial grist to 74% extraction. They were obtained from the mill as four separate mill streams, taken from a point before the inclusion of additives at the mill. The four streams were mixed in the correct proportions in a ribbon mixer for two hours. The flour was then canned (4 x 40lb.) and stored at 4°C until required.

The grists and analyses of the flours are given in Tables 2.1 and 2.2.

TABLE 2.1

Grists of flours used in these experiments (as percentages).

	Flour A	Flour B	Flour C
English		17.5	10
W. Australian 1969/70		10	10
Russian		25	20
No. 2 Manitobas	25	32.5	
No. 3 Manitobas	25		
N. Springs		15	
N.S.W. Australian 1970	5		
N.S.W. Australian 1969	25		
No. 2 U.S. H.Winter	20		15
No. 1 Canadian Red Springs			45

TABLE 2.2

Analyses of flours used in the experiments.

	Flour A	Flour B	Flour C
Moisture %	14.2	13.3	13.5
Protein (N x 5.7)%	11.7	12.3	12.1
Oil (Soxhlet) %	1.0	1.05	1.1
Total Ash %	0.5	0.45	0.5
Water Absorption (as rec'd.) galls./sk	16.35	17.40	18.1

(b) Other dough ingredients.

The soya flour used was a commercial full-fat enzyme-active soya flour. The lipoxygenase activity as determined by the method of Surrey[164] was 1420 units/g. Distilled water was used throughout. Where stated (Section 4.4) dissolved air was removed from the distilled water by boiling. The water was then saturated with either oxygen or nitrogen by bubbling the appropriate gas through the water.

(c) Chemicals.

Reagent grade chemicals were used unless stated otherwise.

All 40-60°C petroleum-ether was freshly distilled in glass apparatus before use and only the fraction distilling below 50°C was taken.

Methanol, chloroform and toluene were A.R. grade.

The radiochemicals were obtained from the Radiochemical Centre, Amersham, (triolein and palmitic acid) and from I.C.N. Tracerlab, (tripalmitin). The specific activities were as follows: glycerol tri(oleate-9,10-^{3}H), 500 mCi/mmole ($2.14 \times 10^{13} s^{-1} kg^{-1}$); glycerol tri (palmitate-1-^{14}C), 14.3 mCi/mmole ($6.74 \times 10^{11} s^{-1} kg^{-1}$); palmitic acid-1-$^{14}C$, 55 mCi/mmole ($7.94 \times 10^{12} s^{-1} kg^{-1}$).

2.2 METHODS.

(a) Work-free wetting.

Flour was wetted to the required moisture content without the

introduction of mechanical work by the method of Davies et al.[163] The method enables the ingredients to be blended together by slurrying in liquid nitrogen. This avoids the formation of a dough and hence the introduction of mechanical work.

A beaker (5l) was half filled with liquid nitrogen and the top covered with a silk screen (5XX). The flour and soya, if included, were gradually added to the liquid nitrogen by brushing through the silk screen. The required amount of ice was prepared by adding water, dropwise, into a steel mortar and pestle containing liquid nitrogen. When the nitrogen had evaporated the ice pellets formed were ground to a fine powder. This ice powder was then tipped into the liquid nitrogen-flour slurry and the whole stirred.

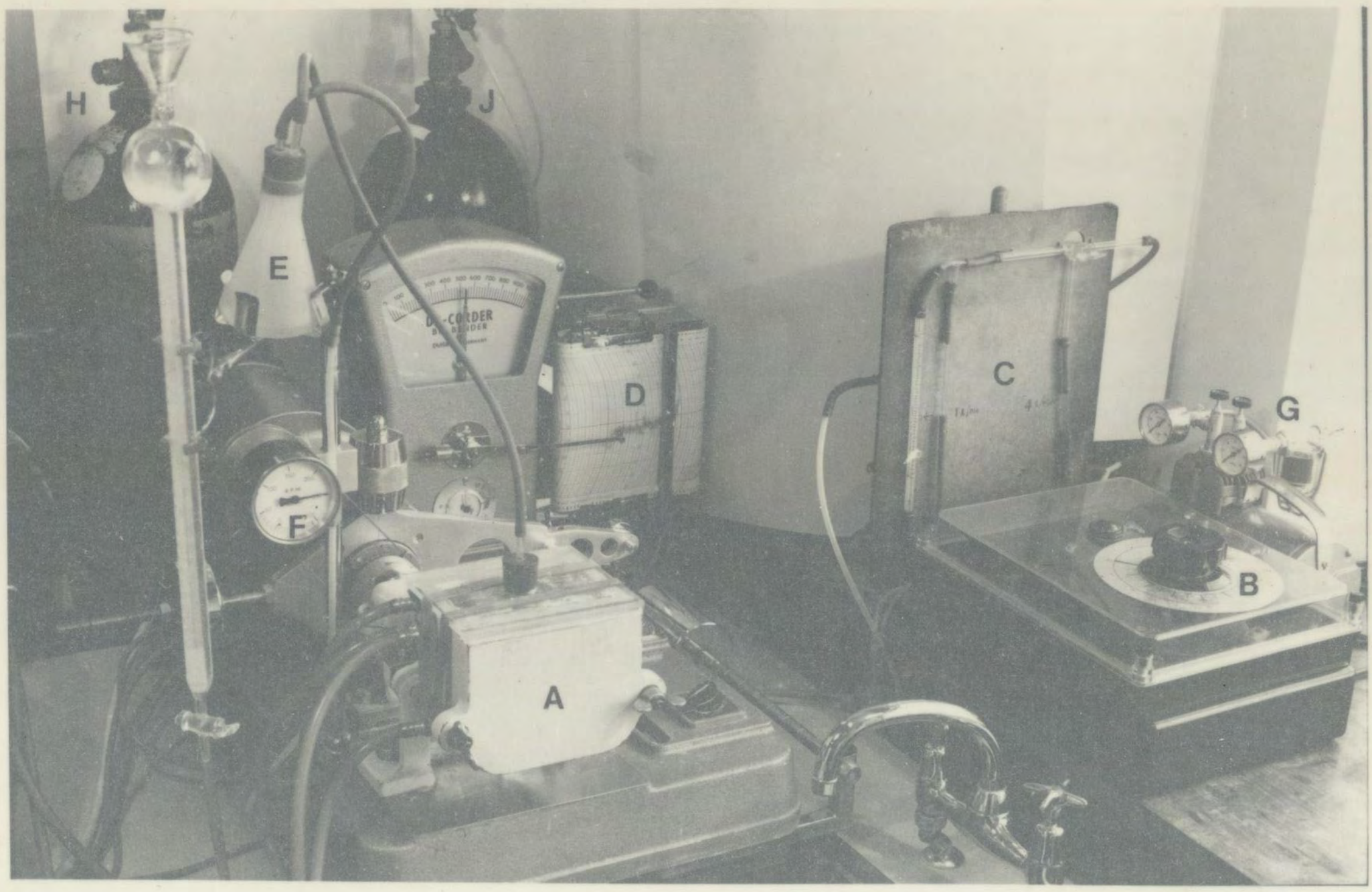
The beaker was covered with aluminium foil to prevent the loss of fine flour and ice powder as the nitrogen boiled off. The prepared slurry was placed in a deep freeze at -40°C. and the liquid nitrogen was allowed to evaporate off overnight. The ice-flour powder was vigourously stirred with a cooled spatula before weighing out into cooled containers.

Normally 2000g of flour-ice mixture was prepared which was sufficient to mix six doughs.

(b) Dough mixing.

Doughs were formed from the frozen flour-ice mixture with or without the introduction of mechanical work. In the case of "work-free" doughs the frozen ice-flour powder was placed on a tray, covered with aluminium foil and allowed to reach room temperature. This effectively avoided any loss of moisture once the ice had thawed, and prevented

PLATE 2.1 Illustration of the apparatus used to mix doughs in these experiments



A. Farinograph bowl

B. Speed control

C. Gas metering system

D. Torque recorder

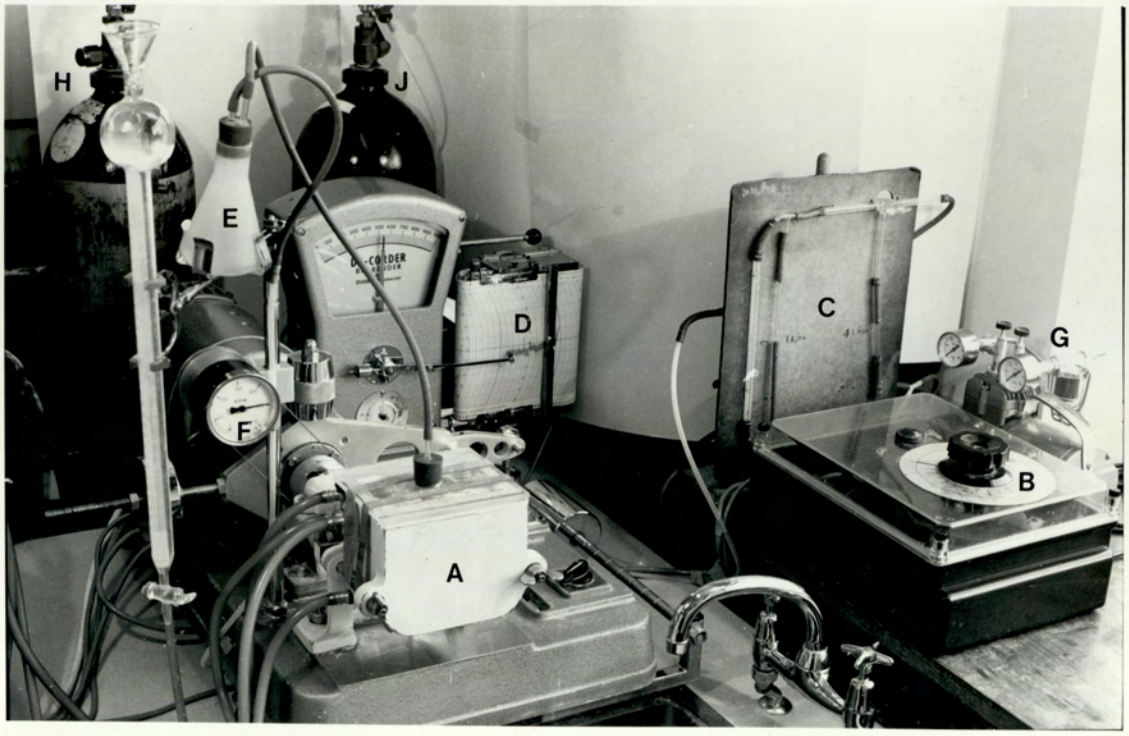
E. Flask containing ingredient water

F. Tachometer

G. Air pump

H. Nitrogen

J. Oxygen


H
J
E
D
C
G
F
B
A

"skinning" of the dough.

Where the introduction of mechanical work was required the flour-ice mixture (300g) was placed in a Farinograph bowl with a capacity of 500g (see Plate 2.1). The bowl (A) was clad in stainless steel and attached to a Brabender Do-corder modified to allow continious variation (B) of the mixing speed (F). It was found that if more than 300g of flour-ice mixture was mixed at low speeds a layer of dough rode above the blades and was not mixed homogenously. The frozen mixture was allowed to thaw for $1\frac{1}{2}$ hours in the Farinograph bowl held at $30 \pm 1^{\circ}C$.

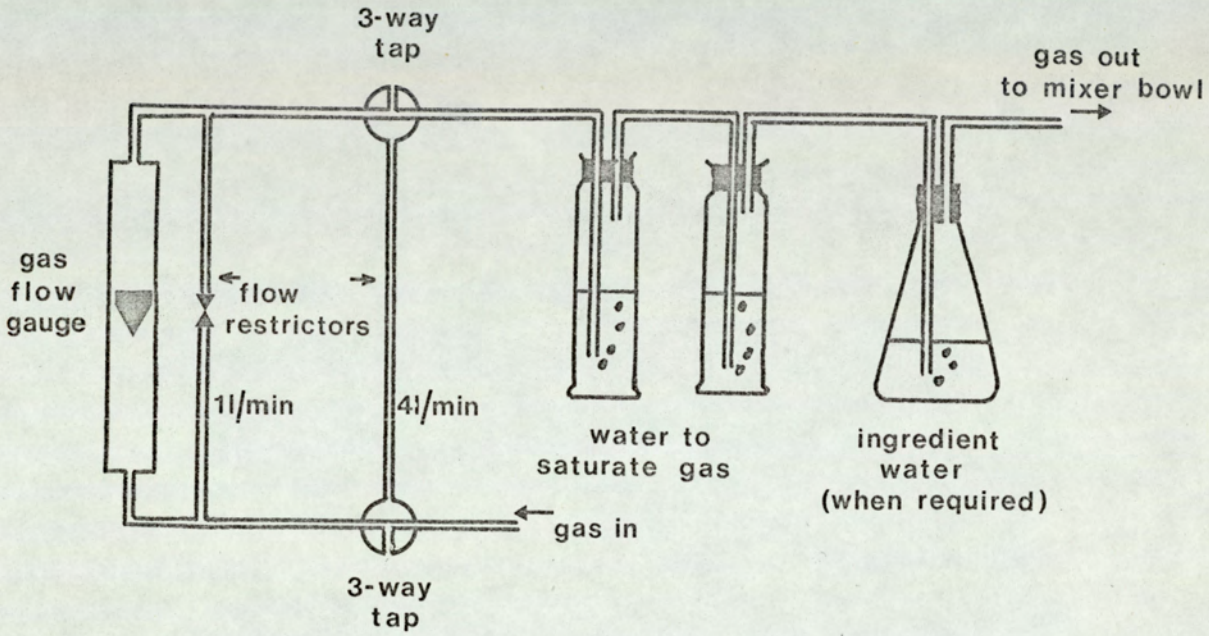
Control of the mixer atmosphere was achieved by means of the system (C) illustrated in Fig.2.1. If doughs were to be mixed in nitrogen the bowl was flushed with oxygen-free nitrogen as the powder was introduced. The atmosphere during mixing was either oxygen-free nitrogen, oxygen or air. All the gases were metered in at 1l/min. and saturated with water vapour (see Fig.2.1).

Where strict exclusion of oxygen was necessary (Section 4.4) the weighed powder was reslurried in liquid nitrogen by carefully pouring the liquid nitrogen onto the frozen mixture in its container. The slurry was transferred to the mixer bowl and the nitrogen allowed to evaporate off, thus keeping an atmosphere of nitrogen above and within the wet flour.

The doughs were mixed at either constant speed (Section 3 and 4) or constant work rate (Section 5). In the former case the total work input was calculated from the following basic equation:-

$$\text{Power per unit dough mass} = \frac{n\,T}{m} \quad W\,kg^{-1} \qquad \ldots\ldots\ldots\ldots\ldots\ldots (1)$$

FIG.2.1 Diagram of the system used to control and humidify the gas flow to the mixing chamber.



Where n = angular velocity of mixer shaft in rad s^{-1}

T = torque in N m

m = dough mass in kg

It was desired to work in the traditional bakery work rate units of H.P.min./lb./min. The Do-corder chart (D) was calibrated in m kg so inserting these units the equation becomes:

Work per unit dough mass = $6.2423 \times 10^{-4} \frac{N.T.\tau}{m}$ H.P.min./lb./min.....(2)

Where N = mixer speed in r.p.m.

T = time in minutes

τ = torque in kg

and m = dough weight in kg

A convenient conversion of these units to S.I. units is:-

1 H.P.min./lb. = 98.71kJ kg^{-1}

or 1kJ/kg = 0.01013 H.P.min./lb.

In practise the product of T and τ was obtained by measuring the area under the torque curve (see Fig.2.2) by counting squares.

Where doughs were required to be mixed at a constant work rate (Section 5) the work-free wetting procedure was dispensed with and the mixing procedure of Daniels et al.[22] was followed. The dry ingredients were blended at 15 r.p.m. in the mixer bowl for two minutes whilst the gas stream was bubbled through a flask (E) containing the required amount of water. At the end of the blending period the flask was inverted allowing the gas pressure to drive the solution into the mixing bowl. The wet ingredients were then premixed for a further minute at 15 r.p.m. There after the dough was mixed to the required work input at a rate of 0.2

H.P.min./lb./min. This was attained by keeping the product of N and τ equal to a constant obtained from equation 2.

In practise a calibrated scale fixed to the torque recorder (see Fig.2.2) enabled the mixing speed to be adjusted as required. The calibration data are given in Table 2.3.

TABLE 2.3

Calibration figures to mix 500g of dough (wet weight) at 0.2 H.P.min./lb./min. in the Brabender Do-corder.

Torque m kg	Mixing speed r.p.m.	Torque m kg	Mixing speed r.p.m.
550	291	1000	160
600	268	1050	152
650	246	1100	145
700	229	1150	139
750	213	1200	133
800	200	1300	123
850	188		
900	178		
950	168		

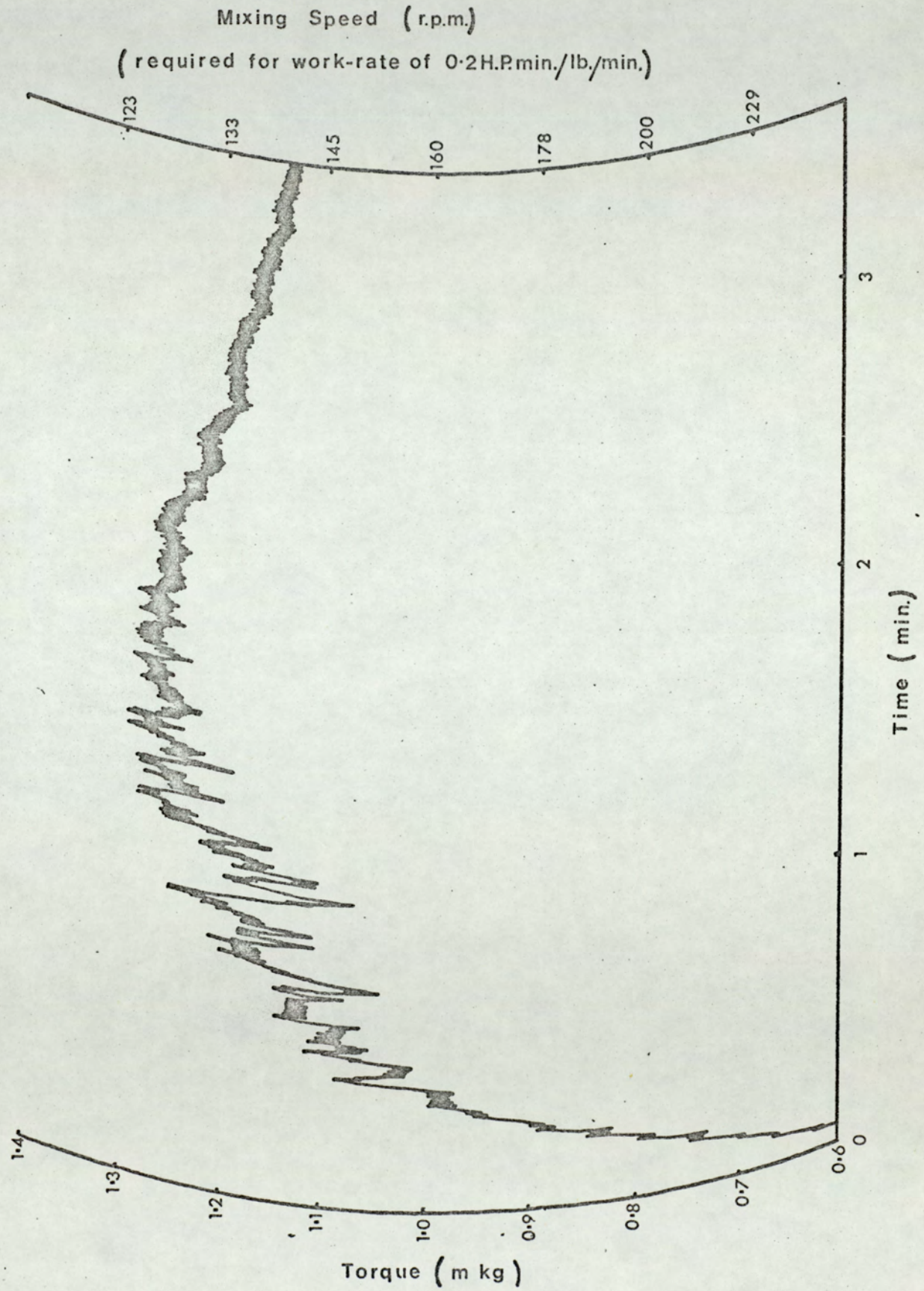
(c) Free and bound lipid extraction.

(i) Freeze-drying and grinding.

Dough prepared by mechanical mixing was cut into small pieces and quickly frozen (30 minutes) in a blast freezer (supplied by Frigidaire). Work-free doughs were frozen on the trays in which they had thawed out. The frozen dough or dough pieces were quickly transferred to a freeze-drier (Edwards High Vacuum Ltd., Model 30PI/484) and dried overnight. The final moisture content aimed at was 4.0%.

The dried dough pieces were ground in a end-runner mill (Pascall

FIG.2.2 Typical torque recording of a dough mixed at 0.2H.P./min./lb. in the Brabender Do-corder, illustrating the adjustments to mixing speed required to maintain a constant work rate.



Model O) and the ground dough sieved to pass a 5XX silk screen.

(ii) Free lipid determinations.

The proportions of "free" and "bound" lipid were determined by the procedures adopted by Daniels et al.[22]

Free lipid was defined as that removed by a 7hr Soxhlet extraction with 40-60°C petroleum-ether. Duplicate determinations were made on about 30g of the dough powder. After extraction the solvent was removed from the lipid on a rotary film evaporator (Buchi) and the lipid transferred to a weighed vial. The extracts were dried to constant weight at 70°C. Determinations were corrected to a dough dry weight basis.

This procedure was modified slightly when radioactive doughs were being handled, to minimize the hazard to personnel from the dust produced by grinding in an end-runner mill. The freeze-dried dough pieces (20g) were lightly ground by hand in a mortar and pestle until about 1cm. diameter. They were then transferred with petroleum-ether to a tall-form beaker and disintegrated with a homogeniser (Silverson Machines Ltd., Laboratory Model). The petroleum-ether slurry was carefully transferred through a filter funnel into an extraction thimble (118 x 33mm.) placed in a Soxhlet extractor. After all the slurry had been washed into the thimble it was plugged with cotton wool and extracted for 7hr. The extract was dried to constant weight in a scintillation vial. No correction was made in these determinations for moisture content since determinations on the freeze-dried dough pieces was highly inaccurate and moreover they absorbed atmospheric moisture during grinding.

A comparison of the two methods of grinding, using the same dough

pieces, showed that there was no significant difference (P = 0.10) between them.

Statistical analysis of all the results of free lipid determinations by the method of analysis of variance showed that the standard error of the mean duplicate determinations was ± 0.01.

(iii) Bound lipid.

Bound lipid was defined as that removed from the petroleum-ether extracted dough powder by the solvent system of Tsen, Levi and Hlynka.[165]

The extracted dough powder was transferred to the chilled stainless steel container of a blender (M.S.E. Atomix) containing a mixture of chloroform (25ml), methanol (50ml), and water (20ml). The sample was mixed for two minutes, more chloroform (25ml) was added and the sample mixed for a further half a minute. Finally 25ml of water was added and mixed in for half a minute. All mixing was at the half speed setting of the blender. The homogenate was transferred to a centrifuge bottle (250ml) and cooled to below 8°C in a salt-ice bath. It was found that this aided the separation of the phases.

The homogenates were centrifuged at 2000 r.p.m. (1100xg) for 15 minutes. Three layers were produced, a lower chloroform layer containing the extracted lipids separated from the upper aqueous methanol layer by a solid plug of hydrated dough. The upper methanolic solution was carefully poured away and an aliquot of the chloroform solution removed with a pipette. This solution was filtered through cotton wool and the solvent removed on a rotary film evaporator. The extract was transferred to a weighed vial and dried to constant weight in an oven at 70°C. The total amount of

lipid in 50ml of chloroform was calculated from the weight of extract in the aliquot.

Statistical analysis of the results of bound lipid determinations by the method of analysis of variance showed that the standard error of the mean of the duplicate determinations was $\pm$ 0.03.

The same procedure was used for radioactive samples except the extract was transferred to a weighed scintillation vial.

(d) Moisture determinations.

Moisture contents were determined by the vacuum oven method of the American Association of Cereal Chemists.[166]

(e) Bound water.

Bound water (Section 3.3) was determined by the method of Davies and Webb[167] on doughs of 29.8% moisture content.

A Differential Thermal Analyser (Dupont 900) fitted with a Differential Scanning Calorimeter (D.S.C.) cell was used. It was found difficult to obtain a representative sample of dough because of the small sample size required by this equipment. Furthermore there was considerable loss of water from the sample during handling and weighing. To overcome these problems the mixed dough was cut into approximately half-inch square pieces and frozen in liquid nitrogen. The frozen dough pieces were ground for one minute at maximum speed in the cooled stainless steel container of a blender. Samples (15mg) were taken from the powder produced and

accurately weighed (± 0.01mg) into the D.S.C. aluminium sample pots using a torsion balance. The cell was cooled to -60°C with the Dupont cooling attachment and then heated at 10°C/min. to +20°C. The area under the ice-water exothermic peak at 0°C was measured with a planimeter, the mean of six determinations being taken on different samples.

Calibrating the instrument against distilled water a figure of 1.65mg water/in.^{2} was obtained at a heating rate of 10°C/min. and recorder sensitivities of 1°C/in. (ΔT axis) and 20°C/in. (T axis).

(f) Radioactive studies.

(i) Handling.

In evaluating the amount of radiotracer to be incorporated into each dough consideration was given to a number of factors. These included the size of the dough sample to be analysed, the likely counting efficiency of the isotope, and the counting rate required for good statistical significance in reasonable counting time. From such considerations it was decided to incorporate 0.5μCi of ^{3}H and 0.1μCi of ^{14}C into each dough. In the case of ^{3}H-triolein and ^{14}C-palmitic acid this was equivalent to about 0.05mg and 0.03mg respectively. Because of the difficulties of accurately handling such quantities, the radiotracers were coated onto an inert "extender" of glass beads.

The radiochemicals were coated onto the glass beads (Sigma Chemical Co., Type 1. 75-105nm) by evaporation from a petroleum-ether solution in a rotary film evaporator. Initial experiments showed that as much as 5% of the added activity became bound to the beads if they were used as received. For this reason the glass beads were deactivated by the following procedure. The beads (100g) were boiled in 1% Decon 75 for

15 minutes then washed with distilled water (4 x 100ml) and dried under vacuum. A slurry of the beads in 1% dichlorodimethylsilane in chloroform was dried on a rotary film evaporator, washed with chloroform (4 x 100ml) and finally air-dried. Binding of the radiochemicals to the glass beads was reduced to 0.5% by this procedure.

The "radioactive bound lipid" used in Section 5.4 was prepared by mixing a dough containing flour B (50g), water (28g), 25μCi of glycerol tri(oleate-9,10-^{3}H) and 5μCi of palmitic acid-1-^{14}C, to 3.0 H.P.min./lb. at 0.3 H.P.min./lb./min. in nitrogen. The dough was freeze-dried and ground in an homogeniser as described in Section 2.2 c (ii). The free lipid was removed by an 18hr petroleum-ether extraction in a Soxhlet extractor. The amount of free radioactivity remaining was determined by a further 7hr petroleum-ether extraction on the dough powder (2g), which removed 1460 dpm/g of ^{3}H and 860 dpm/g of ^{14}C.

The radiochemicals, either as freeze-dried dough powder or on glass beads were added either to the dry ingredients (Sections 5.2, 5.4, 5.5) or to the dough after a period of mixing had taken place (Section 5.3). In the latter case the mixing was stopped and the mixing chamber lid slid aside sufficiently to allow the glass beads to be added. Since the gas flow was not stopped this procedure avoided the incorporation of air into anaerobic dough mixes.

(ii) Counting.

The extracted lipids (Section 2.2 c) were dissolved in a scintillant mixture (15ml) composed of 2,5-diphenyl-oxazole (P.P.O.) (5g/l) and 1,4-di-(2(5-phenyl-oxazolyl)) benzene (P.O.P.O.P.) (o.3g/l) in toluene.

An International Chemical and Nuclear Corporation Corumatic 25 liquid scintillation counter was used to count the samples. The channel settings were as follows:-

	^{3}H	^{14}C (Counting Channel)	^{14}C (Ratio Channel)
Gain	64	8 x 1.5	8 x 1.5
Threshold	25	70	70
Window	140	35.5	60

These settings gave counts of approximately 10^{4} in 20min. for ^{14}C and 5 x 10^{4} in 10min. for ^{3}H which ensured a standard deviation of less than 1%. All counts were corrected for background.

Quench correction was determined by the method of sample channels ratio.[168] The calibration curves were prepared by using glycerol tri (oleate-9,10,^{3}H) and palmitic acid-1-^{14}C with free flour oil as the quenching agent. The absolute disintegrations per minute were determined by comparison with sealed standards obtained from I.C.N. Tracerlab.

SECTION 3

THE EFFECT OF MOISTURE ON LIPID BINDING AT LOW WORK LEVELS

3.1 INTRODUCTION.

The origins of the work described in this thesis lie, in part, in the findings of Davies *et al.*[163] who investigated lipid binding occurring as a consequence of the wetting of flour. They reported that this binding effect, originally investigated by Olcott and Mecham,[125] started to occur when the moisture content of flour was raised above 20% Thereafter the bound lipid increased from 30% of the total lipid, to 45.5% at 50% moisture content. When mechanical work was performed on this work-free system the amount of bound lipid rose even further.[125]

The binding of lipids during the mixing of commercial doughs containing shortening, soya flour and salt was studied in detail by Daniels *et al.*[121] They demonstrated that the amount of lipid bound was dependent upon the rate of work input, the amount of work performed on the dough and the atmosphere in the mixing chamber. At the lowest work level investigated 0.1 H.P.min./lb,, 45% of the total lipid was bound in both air and nitrogen. Continued mixing in nitrogen (at the highest work rate of 0.3 H.P.min./lb./min.) caused a steady increase to 75% bound lipid at about 4.0 H.P.min./lb. The slower mixing rate of 0.1 H.P.min./lb./min. brought about a slower increase in binding to only 65% bound lipid at the same work level. A complete reversal of this lipid behaviour was observed in doughs mixed in air when the bound lipid decreased as the doughs were mixed to increasing work levels.

Comparing the investigations of Davies *et al.*[163] and those of Daniels *et al.*[121] raises two questions. Firstly is there a rapid initial increase in lipid binding when mechanical work is started either on wetted flour or on a commercial dough formulation. At the commercial water content of 45% used by Daniels *et al.*,[121] Davies *et al.*[163] found 0.7%

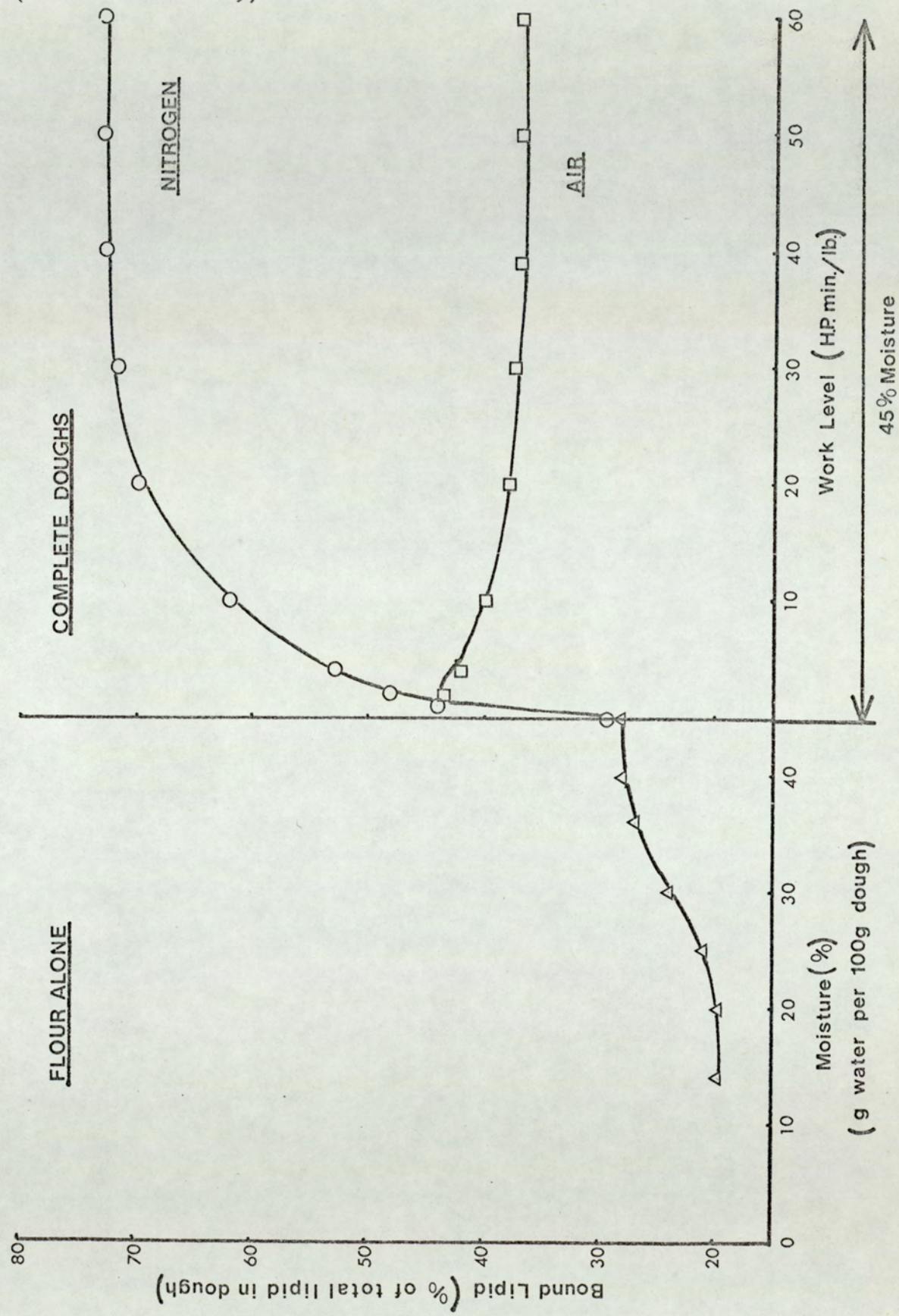
bound lipid (flour dry weight basis) compared with the 1.1% lipid (dough dry weight basis) bound at 0.1 H.P.min./lb. noted by Daniels et al. There was of course considerably more lipid in the latter system due to the added shortening. It seems unlikely that this would be bound during work free wetting because Davies et al.[127] found no evidence of extra lipid binding when flours with augmented levels of flour oil were wetted to 50% moisture. Daniels et al.[169] wetted their commercial dough formulation to 45% water without introducing mechanical work and found only 30% lipid binding. On the basis of this result they superimposed the results of their experiments on those of Davies et al.[163] and produced the graph reproduced here in Fig.3.1.

The implication of this result is that even in air it would appear that there is indeed a rapid initial binding of lipid at the onset of mixing. In fact, this increase must have occured so rapidly that the oxidative processes leading to a release of lipid in air did not have time to take place.

The second question raised is whether or not lipid binding is linked to the availability of water within a dough system. As the water content of flour is increased in the absence of work the lipid binding increases up to 50% moisture. The influence of mixing is to increase the homogeneity of the system bringing about a more even distribution of the water. The increase in lipid binding during both work-free wetting and mechanical mixing could therefore be related to the same effect of water availability. This being so, mechanical work may cause an increase in lipid binding in work-free doughs of quite low moisture content.

The following experiments were intended to answer these questions by observing the effect that very low levels of work had upon flour wetted

FIG. 3.1 Composite diagram of the changes in lipid distribution that take place when flour and ingredients are wetted and mixed into a dough. (from reference 169)



to various moisture contents. It was hoped to determine whether mechanical work caused a rapid increase in lipid binding and if so, whether this was related to water distribution within the dough.

3.2 INITIAL INVESTIGATIONS ON MIXING DOUGHS TO LOW WORK LEVELS.

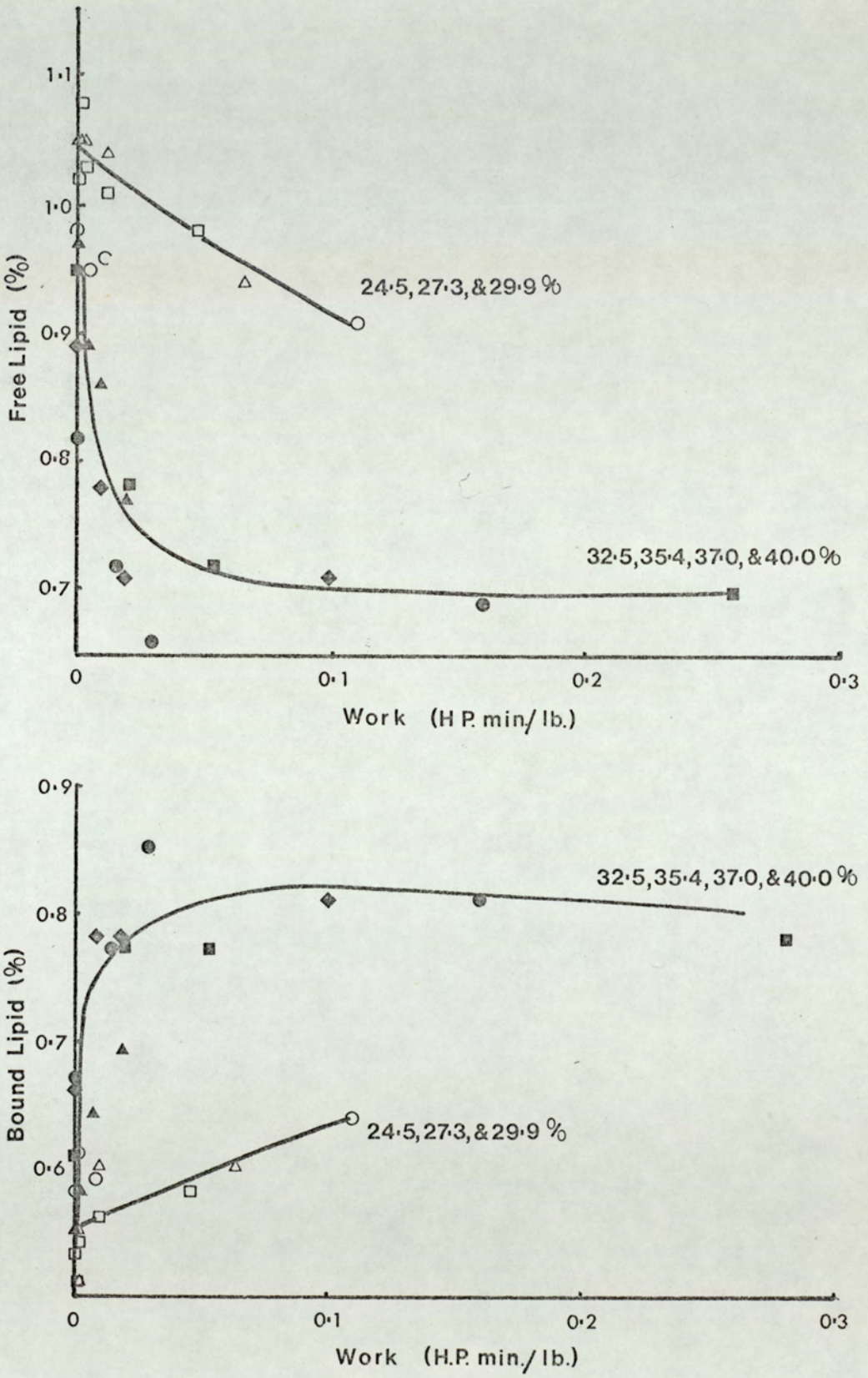
Flour A was wetted to various moisture contents by the liquid nitrogen slurry technique of Davies et al.[163] The wetted flour was then mixed for 0.5, 1.0 and 5.0 minutes at the lowest speed attainable on the Do-corder (15 r.p.m.). All the doughs were mixed under nitrogen to avoid the reduction in lipid binding during mixing in air described by Daniels et al.

It will be seen from Fig.3.2 that there was a very rapid initial increase in bound lipid at the onset of mixing in doughs containing more than 32.5% moisture. This increase occurred so rapidly that the free lipid fell from 1.0% to 0.8% at 0.01 H.P.min./lb. and to 0.7% by 0.1 H.P.min./lb. Thereafter further work caused no further lipid binding.

This result would appear to confirm that the onset of mechanical mixing causes a sudden increase in the level of bound lipid, an effect which will be discussed and investigated in more detail later (Section 4). This finding also confirmed that mechanical work induces lipid binding at moisture levels as low as 32.5%. The most surprising feature of these results, however, was the sudden onset of work-induced lipid binding between 30 and 32.5% moisture. Below 30% water there was a slight steady fall in free lipid with increasing work content but the rapid initial increase in bound lipid, which occurred at moisture levels above 32.5% did not take place. The large difference in lipid binding obtained above and below 30-33% moisture suggested that there may in fact be a "critical" moisture content below which work induced lipid binding is unable to occur.

FIG.3.2 Variation of lipid distribution with mechanical mixing at various moisture contents (N_2 atmosphere)

Symbols: △ 24.5%; □ 27.3%; ○ 29.9%; ▲ 32.5%; ■ 35.4%; ● 37.0%; ◆ 40.0%.



To confirm whether this observation was correct and if so to determine the "critical" moisture content more accurately, the moisture range 25-33% was studied in greater detail.

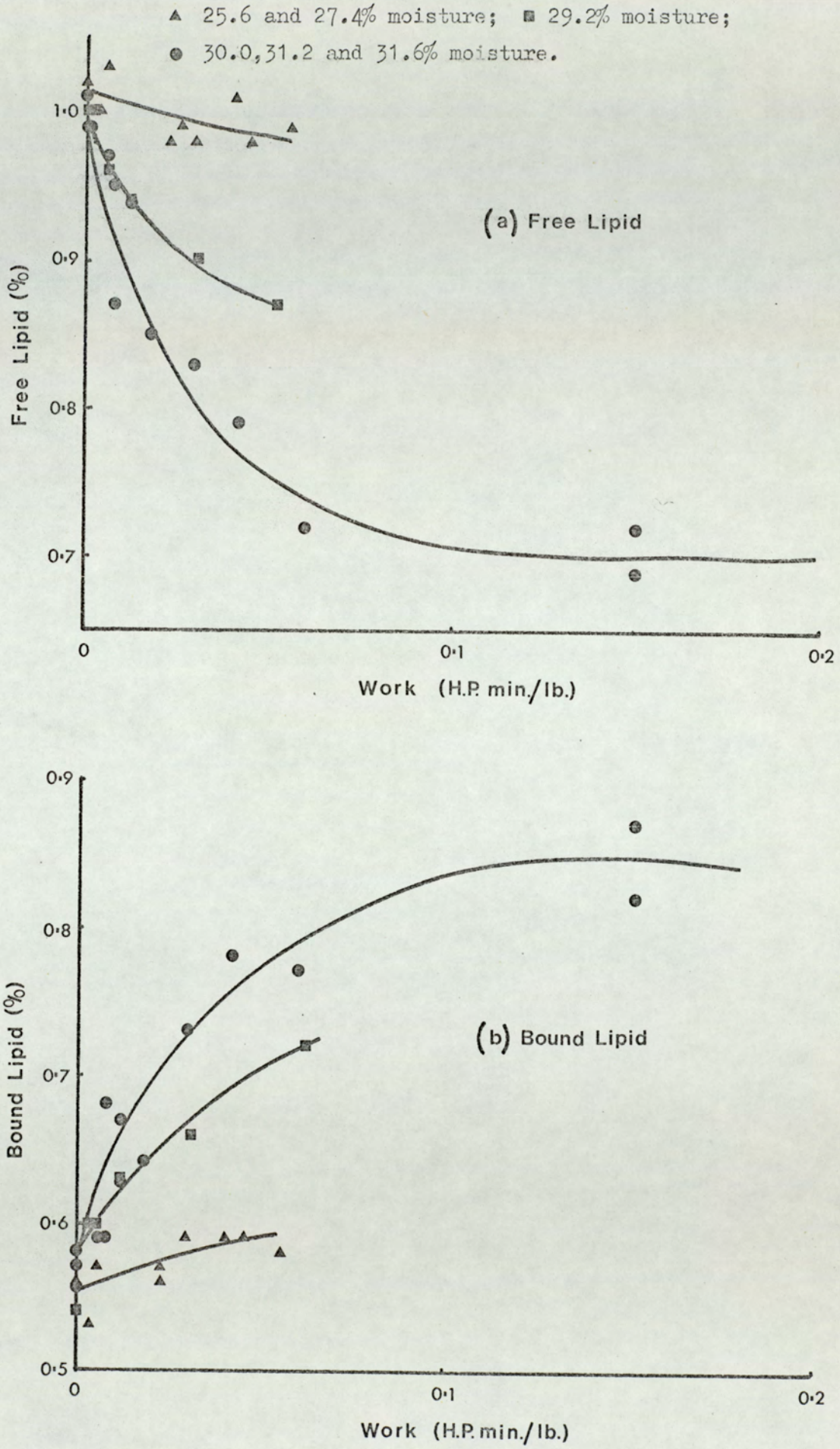
3.3 THE "CRITICAL" MOISTURE CONTENT FOR WORK INDUCED LIPID BINDING.

Figs. 3.3a and 3.3b show the influence of mixing in nitrogen on the lipid distribution of flour (A) wetted to various moisture contents in the range 25.6 to 31.6%. All the doughs were mixed at 15 r.p.m. and since work is derived from the product of time and torque, the time of mixing was adjusted to allow for variations in torque between the doughs. The curves for 30.0, 31.2, and 31.6% moisture, and for 25.6 and 27.4% moisture coincided and for the sake of clarity have been superimposed.

Up to 27.4% moisture, work had little effect on the level of free lipid. However at 30% moisture, and above, work caused an immediate rapid increase in the amount of bound lipid up to a work input of 0.1 H.P.min./lb. This rapid initial increase confirmed the previous findings but occured at a slightly lower moisture content than found previously.

The moisture content of 29.2% would appear to be close to the "critical" moisture content discussed above. Mechanical work at this moisture content did not produce such a rapid increase in bound lipid as at 30% moisture and the amount of lipid bound was not as great. The fact that the lipid binding curve falls midway between those for 27.4% and 30.0% moisture suggests that the mechanism by which water enables work induced lipid binding to occur is not an "on-off" mechanism but one which occurs progressively over a narrow moisture range.

FIG.3.3 Variation of lipid distribution with mechanical mixing at various moisture contents (N_2 atmosphere)



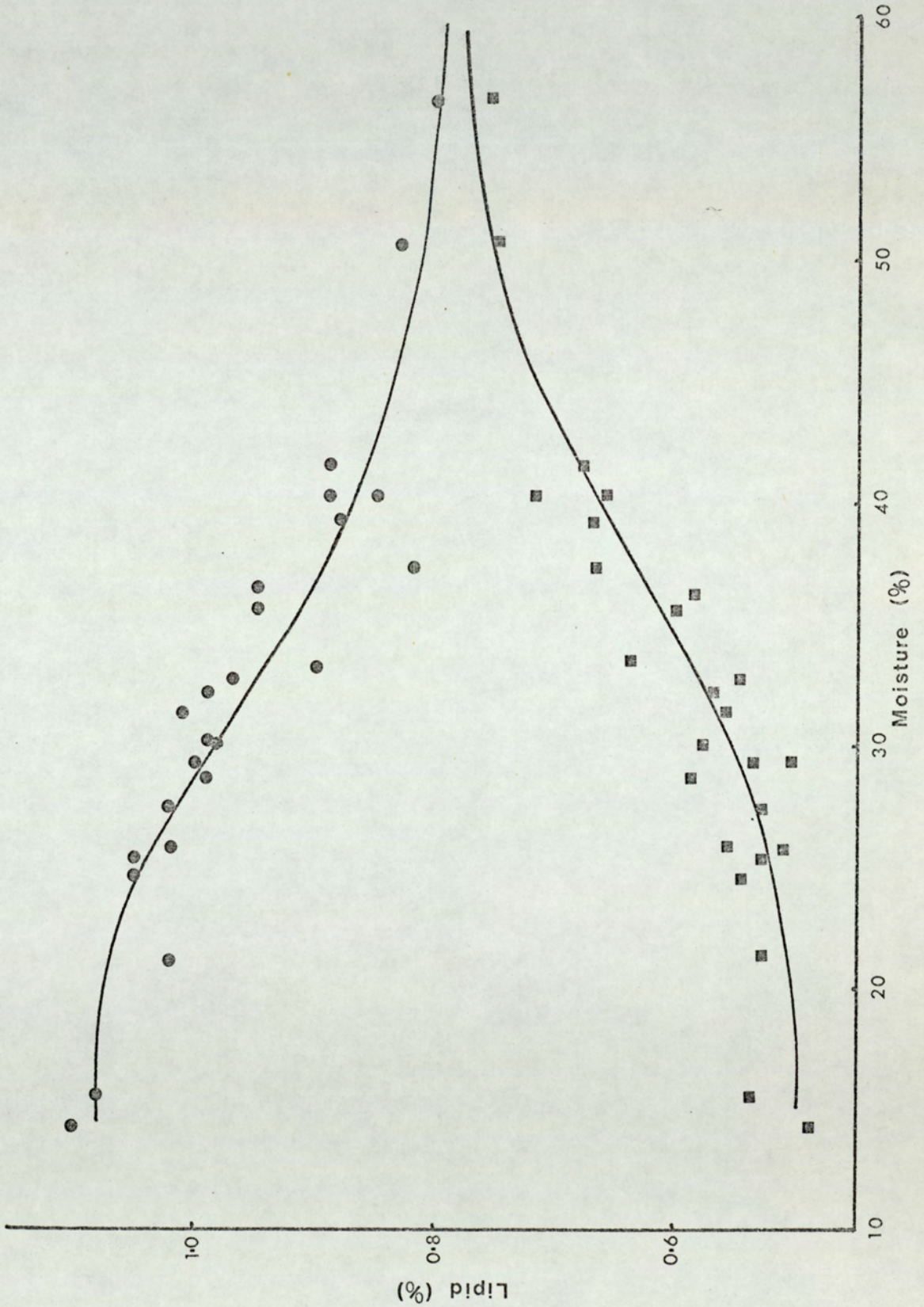
The findings of Davies et al.[163] have been confirmed in Fig.3.4. Flour (A) was wetted to various moisture contents by the liquid nitrogen slurry method and the lipid distribution determined. The onset of lipid binding occured at about 20% moisture and continued up to about 50% moisture. There was no evidence of a critical moisture content around 29%.

It would appear at first sight that the lipid binding induced by work-free wetting is caused by a different mechanism than that inducing lipid binding when a dough is worked. Lipid binding in the work-free system begins at about 20% moisture, well below the "critical" moisture content for work induced lipid binding. If the same mechanism was causing lipid binding in both systems then little binding would occur below 29.2% water in the work free system. However it is suggested that the same mechanism produces lipid binding in the worked and work-free systems and that the apparent discrepancy in the results may be due to variations inherent in the experimental method, particularly the size of the ice crystals in the liquid nitrogen slurrying technique.

If the onset of lipid binding occured at a "critical" moisture content then, given perfect distribution of the water, below that moisture content no lipid would be bound. If however the ice particles were sufficiently large so that when they melted the local moisture content was above the "critical" level some lipid binding would occur. As the total moisture content of the mixture was increased, more and more local regions would be raised above the critical moisture level with consequent lipid binding in those areas. The overall effect would be an apparent steady increase in lipid binding over a range of moisture contents either side of the critical level. The effect of work on such a system would be to increase the homogeneity and cause an increase in the level of bound

FIG.3.4 Variation of lipid distribution with moisture content in unworked doughs.

Symbols: ● Free lipid; ■ Bound lipid;



lipid. If this were the mechanism by which mixing caused lipid binding then one might expect that at higher overall moisture contents the rate of increase of bound lipid at the onset of mixing would be faster. If there was more moisture in the system it would require less mixing for all the system to be raised above the critical moisture content. The coincidence of the curves above 30% moisture in Figs.3.2a and 3.2b and above 33% moisture in Fig.3.1 does not support this proposition and some mechanism, other than water dispersal, must be involved so far as work-induced lipid binding is concerned.

3.4 THE EFFECT OF WORK ON THE BOUND WATER CONTENT OF DOUGH.

As a result of rheological studies on worked doughs, Webb et al.[170] suggested that work produces an increase in the amount of free water in the dough system. If this increase occurred during the initial stages of mixing it might be related to the phenomenon of lipid binding.

TABLE 3.1

Effect of work on freezable water content of doughs containing 29.8% total water.

Time of mixing at 15 r.p.m. min.	% Freezable water	Least significant difference at P = 0.05			
		min. 1	2	5	10
0	5.56	0.554	0.598	0.609	0.570
1	5.23	-	0.542	0.562	0.518
2	5.64	-	-	0.686	0.653
5	5.38	-	-	-	0.669
10	5.58	-	-	-	-

In an attempt to ascertain if this were the case, a series of doughs containing 29.8% moisture were mixed to various work levels. The free

water in the doughs was determined by a D.S.C. method, and the results are presented in Table 3.1. It can be seen that there was no significant difference between any of the doughs and it would appear that at these low work levels mixing does not alter the proportions of free and bound water. The discrepancy between this result and the findings of Webb et al.[170] may be due to the much higher work levels they investigated.

On the basis that there was no significant difference between the doughs, statistical evaluation of the results in Table 3.1 gives a free water content of 5.3 ± 0.08%. This corresponds to a bound water content of 24.5% which agrees with the 24.8% found by Davies and Webb[164] in worked doughs. Other workers have found 22.5% bound water in wet flour using different methods (Toledo, Steinberg and Nelson;[171] Vail and Bailey;[172] Lee;[173]). As noted by these workers all water added to the flour enters the bound state until the water requirement is satisfied. Thereafter further additions of water remain in the free state. It appears that work in the range studied is likewise without effect on the level of bound water in dough.

3.5 THE EFFECT OF MOISTURE CONTENT ON RESISTANCE TO MIXING.

It was observed throughout these experiments that the flour-water mixtures began to show evidence of gluten formation at about 30% moisture. Below this moisture content the doughs were never more than lumpy powders whilst above 30% moisture a solid dough mass was formed. Davies et al.[163] have illustrated this effect in a photograph showing cylinders of wetted flour which began to shrink above 28-30% moisture content.

The coincidence of this moisture content with the onset of gluten

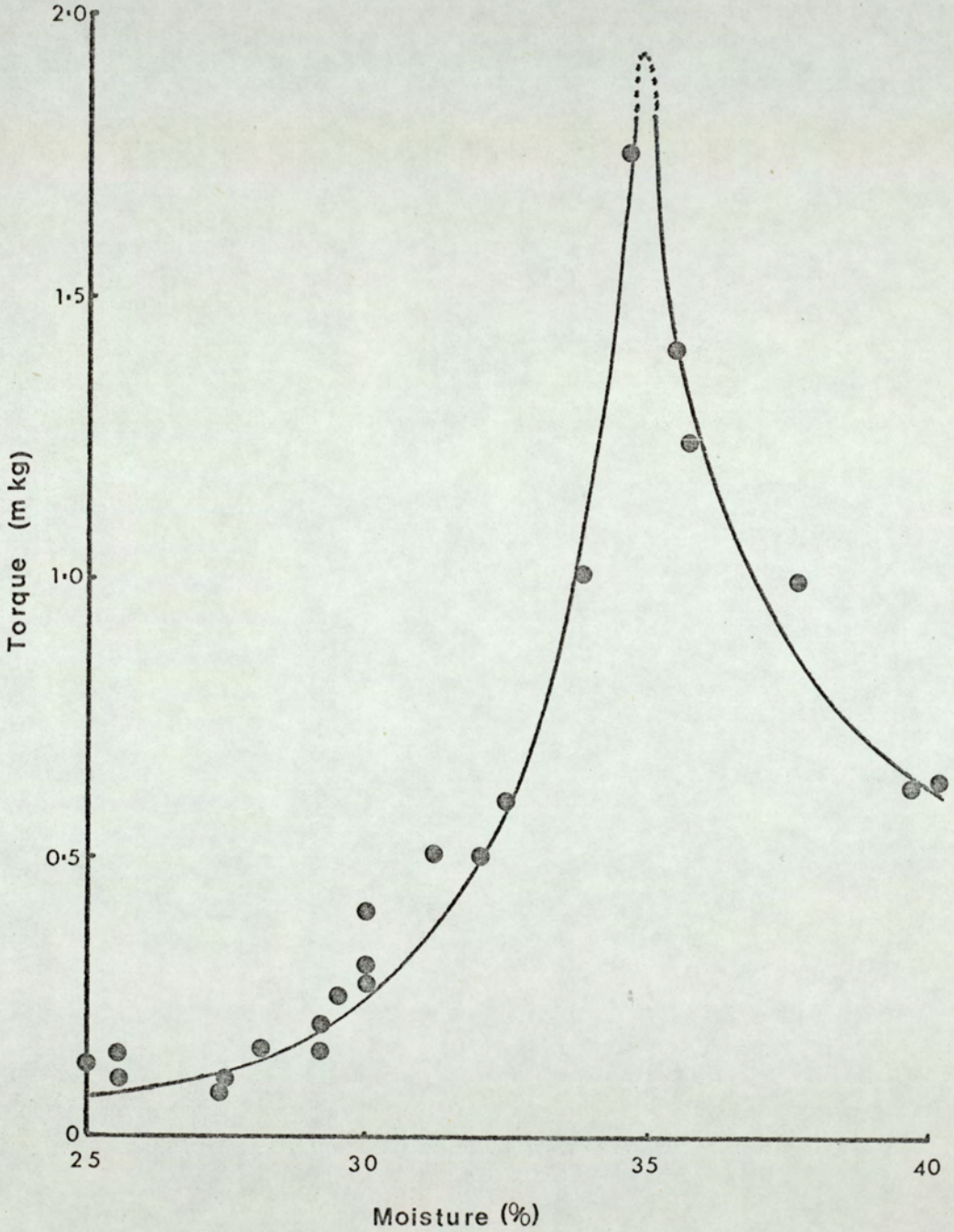
development is further demonstrated in Fig.3.5 in which the resistance to mixing of the flour-water mixtures after 0.5 min. at 15 r.p.m. is plotted against the moisture content. The Do-corder torque measurements were consistently low up to 28% moisture. Thereafter they increased very rapidly to a maximum at 35%, indicating the increasing stiffness of the doughs as the protein chains began to interact even at the low work input used (15 r.p.m.). The decrease in torque above 35% moisture can probably be attributed to the lubricating action of water.[170] Thus it can be seen that the onset of gluten formation as measured by both dough cylinder shrinkage and an increase in Do-corder torque coincides with the "critical" moisture content for work induced lipid binding.

It is concluded from the evidence presented in this section that it is gluten formation and development which is the prime requirement for lipid binding to occur during flour hydration and dough mixing rather than the redistribution of available water. During gluten hydration it would appear that conformational changes in the wheat proteins, under the influence of water cause, or require, the binding of flour lipids to the gluten matrix. Mixing the dough accelerates the rate of gluten development and it is this which gives rise to the observed increase in lipid binding.

3.6 REWETTING DEHYDRATED WORKED DOUGHS.

If indeed it is the development of gluten which causes the binding of lipids then it might be expected that, after gluten development, the addition of more water to the dough would cause no further increase in lipid binding. That is, above 29% moisture the increase in lipid binding with increasing moisture content, noted by Davies *et al.*[163] in the absence of mixing and confirmed in Fig.3.3, would not take place if further water were added after mixing.

FIG. 3.5 Effect of moisture content on resistance to mixing of doughs after 0.5 min. mixing time at 15 r.p.m. in the Brabender Do-corder.



To test this hypothesis, a series of doughs at moisture contents of 25.6, 30.0, 35.2 and 60% were mixed to various work levels. The doughs were freeze-dried, ground and rewetted by the liquid nitrogen technique as closely as possible to 60% moisture without further mixing. Table 3.2 shows that at 25.6% moisture little or no binding of lipid took place during mixing but that when the dough was rewetted to 60% moisture a considerable amount of lipid became bound. The 30% moisture doughs appear similar to those containing 29.2% moisture in Fig.3.2; mechanical work caused a slight increase in bound lipid. Rewetting these doughs caused a further increase in bound lipid. At 35.2 and 60.0% moisture, work caused a large increase in lipid binding as was to be expected, but as predicted by the hypothesis rewetting caused no extra lipid binding in these doughs.

From Fig. 3.4 it might be anticipated that increasing the moisture content from 35 to 60% would cause an increase in bound lipid. The fact that this did not occur after mixing would indicate that the mechanical work must have brought about those conformational changes in the dough protein responsible for lipid binding. It would appear that such changes take place spontaneously under the influence of water in the absence of work.

3.7 DISCUSSION.

The results of these experiments have confirmed the observations of previous workers[125,163] in showing that work free wetting of flour (Fig.3.3) produces binding of flour lipids. This increase in binding takes place over the region 20-50% moisture. The experiments have also demonstrated that mechanical work only caused a significant amount of lipid binding above a critical level of about 29% water. Higher water

TABLE 3.2

Effect of rewetting on the lipid distribution in freeze-dried dough powders obtained from doughs of various moisture contents and mixing times at 15 r.p.m. in the Brabender Do-corder. Nitrogen atmosphere.

Original Dough						Freeze-dried Dough after rewetting			
Moisture Content %	Mixing Time min.	Work Level H.P. min./lb.	Free Lipid %	Bound Lipid %	Total Lipid %	Moisture Content %	Free Lipid %	Bound Lipid %	Total Lipid %
25.6	0	0	1.10	0.53	1.63	60.2	0.75	0.79	1.54
	1	0.003	1.03	0.53	1.56	57.5	0.70	0.77	1.47
	5	0.011	0.99	0.58	1.57	60.1	0.63	0.78	1.41
	10	0.022	1.03	0.56	1.59	62.5	0.71	0.74	1.45
	20	0.050	1.00	0.59	1.59	62.7	0.89	0.67	1.56
30.0	0	0	0.99	0.58	1.57	58.2	0.80	0.76	1.56
	0.5	0.005	0.90	0.64	1.54	60.5	0.77	0.76	1.53
	1	0.008	0.94	0.60	1.54	61.7	0.71	0.76	1.47
	2	0.014	0.91	0.66	1.57	61.0	0.69	0.74	1.43
	5	0.047	0.88	0.69	1.57	65.0	0.78	0.67	1.45
35.2	0	0	0.88	0.65	1.53	59.0	0.77	0.68	1.45
	0.25	0.011	0.73	0.77	1.50	60.6	0.72	0.73	1.45
	0.5	0.023	0.70	0.82	1.52	59.6	0.68	0.76	1.44
	1	0.053	0.65	0.87	1.52	61.2	0.68	-	-
	2	0.112	0.63	0.87	1.50	61.2	0.68	0.72	1.40
60.0	0	0	0.83	0.72	1.55	59.2	0.68	0.74	1.42
	1	0.006	0.70	0.84	1.54	60.7	0.64	0.84	1.48
	2	0.012	0.64	0.84	1.48	61.7	0.57	0.81	1.38
	5	0.031	0.56	0.86	1.42	61.2	0.61	0.86	1.47
	10	0.062	0.48	0.77	1.25	61.1	0.64	0.97	-

contents in a mechanically mixed dough do not alter the amount of lipid bound or the rate at which it becomes bound.

That the onset of lipid binding coincides with the development of gluten structure has been shown by dough stiffness measurements (Fig.3.5) and by the shrinkage of cylinders of wetted flour.[163] Further evidence is afforded by the work of Wooton[136] on mixing flour with various solvents. Doughs were formed with water, formic acid and formamide and in each of these cases there was considerable binding of lipids. None of the other solvents tested formed a dough and correspondingly little lipid was bound. Again it would appear to be the formation of a dough structure which is essential to promote lipid binding.

Measurements of the bound water content of flour and dough have shown no changes in the binding of water during the initial stages of mixing. However the critical moisture content for work-induced lipid binding to occur, coincides with the presence of about 5% free water in the system. Hydrophobic interactions require the presence of free water and it is of interest to speculate on their involvement with lipid binding. There is increasing evidence now, that hydrophobic bonds are involved in lipid-protein interactions[174,176] and a number of workers have implicated such bonds in the gluten network.[175,176]

In the particular system under investigation here the appearance of free water would lead to the formation of hydrophobic areas within the protein. The extent to which this occurred spontaneously, in the absence of work, would depend upon the mobility conferred to the system by excess water. When work was introduced the protein chains would be rearranged and would be able to form more hydrophobic areas. Lipid in the system would naturally migrate to these areas.

Such speculation is somewhat tenuous but from the evidence presented here it would seem very likely that hydrophobic interactions are involved with the phenomenon of lipid binding.

SECTION 4

THE EFFECT OF ATMOSPHERE ON LIPID BINDING AT LOW WORK LEVELS

4.1 INTRODUCTION.

The experiments described in the previous section were concerned solely with the initial changes in lipid binding which take place at the onset of mixing. The experiments in this section were intended to investigate if this initial lipid binding would be modified by the mixing speed or by the atmosphere in the mixing chamber.

The effect of atmosphere on lipid binding was first demonstrated by Daniels et al.[121] They showed that in commercial doughs, containing enzyme-active soya flour and mixed in air, the bound lipid fell from 45% (of the total lipid) at 0.1 H.P.min./lb. to 35% at 3.0 H.P.min./lb. and above. This was a complete reversal of the lipid behaviour during mixing under nitrogen when more lipid became bound as the work level was increased. Similar findings have since been reported by Frazier et al.[178] working with simple flour-salt-water doughs containing full-fat enzyme-active soya flour. Soya flour is a rich source of triglyceride-active lipoxygenase (E.C.1.13.1.13.) and it has been shown that it is the action of this enzyme which causes the reduction in bound lipid in air mixed doughs. Daniels et al.[100] have postulated that the release of bound lipid is brought about by a coupled oxidation of the lipid binding sites in the dough protein by a linoleate-lipoxygenase system, in an analagous manner to the coupled oxidation of pigments by the same system.

In the absence of soya flour Frazier et al.[178] showed that in air there was little change in the level of bound lipid across the work scale. The fact that less lipid was bound than in nitrogen was probably due to the presence in flour of a lipoxygenase specific to unesterified fatty acids.

It was demonstrated in the previous section that the introduction of mechanical work to a wetted flour caused an immediate and rapid increase in the level of bound lipid in the dough. While this increase was observed in nitrogen from the results in Fig.3.3 it seemed likely that a similar increase would occur in air, and by inference must have occured in the doughs examined by Daniels et al.[121] The subsequent lipid release must have caused an inflexion in the lipid binding curve (see Fig.3.1) and at a work level below 0.1 H.P.min./lb. (the lowest work level studied by Daniels et al.). In the absence of soya flour such an inflexion may not occur or may be difficult to detect and soya flour was therefore incorporated into the dough formula for the experiments described in this section.

4.2 THE EFFECT OF AIR AND SOYA FLOUR ON THE "CRITICAL" MOISTURE CONTENT FOR WORK-INDUCED LIPID BINDING.

It was demonstrated in the previous section that the rapid initial increase in lipid binding occuring with the introduction of mechanical work only took place at or above a critical level of about 29% water in the dough. The inclusion of soya flour required that the effect of this additional ingredient in the experimental system should be checked. The results already obtained (Section 3) showed that it was unnecessary to repeat the investigation in detail. Lipid binding in the presence of soya flour was therefore determined in 23, 25, 27, 29 and 31% moisture doughs mixed to 0.05 H.P,min./lb. in both air and nitrogen. This choice was based on the results of Fig.3.3 which shows that at this work level lipid binding is relatively stable and that the difference in the bound lipid of doughs above and below the critical moisture content is at a maximum. All doughs were mixed at 15 r.p.m. as before. Soya flour was

added to the liquid nitrogen slurry as 0.3% of the dough weight. Since analysis of soya shows 20% free lipid this increased the free lipid in the unmixed doughs by 0.09% at 25% moisture and by 0.085% at 31% moisture. Flour B was used throughout.

The results are presented in Table 4.1 and are compared with results predicted from Fig.3.3.

TABLE 4.1

Influence of air and soya flour on the distribution of lipid at various moisture levels.

Formula	Moisture Content %	Mixing Time min.	Work Level H.P. min./lb.	Free Lipid Found %	Free Lipid Predicted %	Bound Lipid Found %	Bound Lipid Predicted %
Flour + water+ soya flour. Mixed in air.	23.2	20	0.037	1.05	1.08	0.56	0.56
	24.8	20	0.044	1.04	1.07	0.59	0.57
	27.7	12	0.045	1.00	1.07	0.60	0.57
	29.1	5	0.070	0.92	0.89	0.70	0.70
	30.7	2.5	0.066	0.85	0.76	0.77	0.77
Flour + water + soya flour. Mixed in nitrogen.	23.5	20	0.031	1.12	1.08	0.50	0.56
	24.9	20	0.062	1.09	1.07	0.55	0.55
	26.6	15	0.042	1.01	1.07	0.55	0.55
	28.8	7	0.044	0.95	0.90	0.60	0.68
	30.9	3	0.065	0.85	0.76	0.74	0.76

Overall the free lipid determinations are somewhat higher due to the presence of the soya flour lipid and suggested that there might be a slight lowering of the critical moisture level. This was not reflected in the bound lipid results which were in good agreement with those predicted from Fig.3.3. It was concluded that the addition of soya flour did not influence to any great extent the "critical" moisture requirement for lipid binding. This observation supports the earlier

postulate that gluten development is responsible for the initial increase in lipid binding following the introduction of mechanical work to moistened flour.

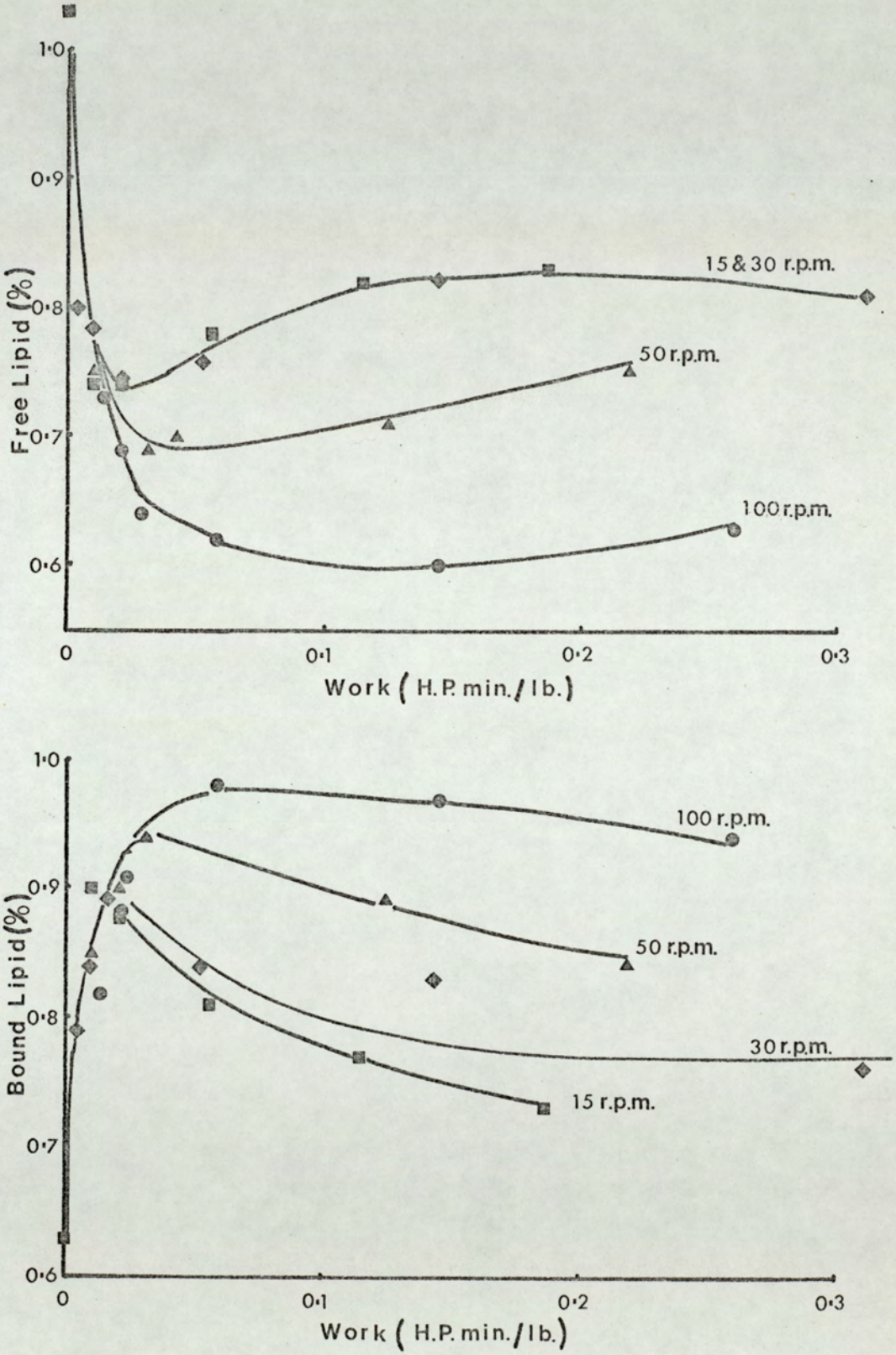
4.3 THE INFLUENCE OF AIR ON LIPID BINDING AT LOW WORK LEVELS.

Having established that soya had little effect on the pattern of lipid binding versus moisture in air or nitrogen mixed doughs, the overall influence of air and soya flour at one moisture level only was studied in more detail. Flour-ice mixtures were prepared as previously, containing 0.44% soya flour, flour B and 45% water. These water and soya flour contents were chosen to be comparable with the work of Daniels *et al.*[121] and Frazier *et al.*[178] to allow practical mixing times (see Fig.3.5) and to ensure that work-free lipid binding would be at a maximum (see Fig.3.4). To observe if the rate of work input modified the lipid binding pattern doughs were mixed over a range of mixer speeds at 15, 30, 50 and 100 r.p.m. During mixing, water saturated air was blown through the mixing bowl at 1l/min.

The results of these experiments are presented in Fig.4.1. The rapid initial increase in lipid binding already observed at lower moisture levels (Figs.3.1 and 3.2) was repeated in all the doughs of this series of experiments. A reversal of the initial binding of lipid quickly occured when these soya-containing doughs were mixed further in air. Moreover it was found that the work level at which the onset of lipid release occured was dependent upon the mixing speed. Thus at 15 and 30 r.p,m. the inflexion in the lipid binding curves occured at 0.02 H.P.min./lb; at 50 r.p.m. the inflexion occured at 0.025 H.P.min./lb. and at 100 r.p.m. there was evidence of an inflexion at around 0.1 H.P,min./lb. Thus increasing the mixing speed tended both to delay lipid release in terms

FIG.4.1 The effect of mixing speed on lipid binding in doughs containing soya flour and mixed in air

Symbols: ■ 15r.p.m.; ◆ 30r.p.m.; ▲ 50r.p.m.; ● 100r.p.m.



of work level and also to increase the amount of lipid bound during mixing.

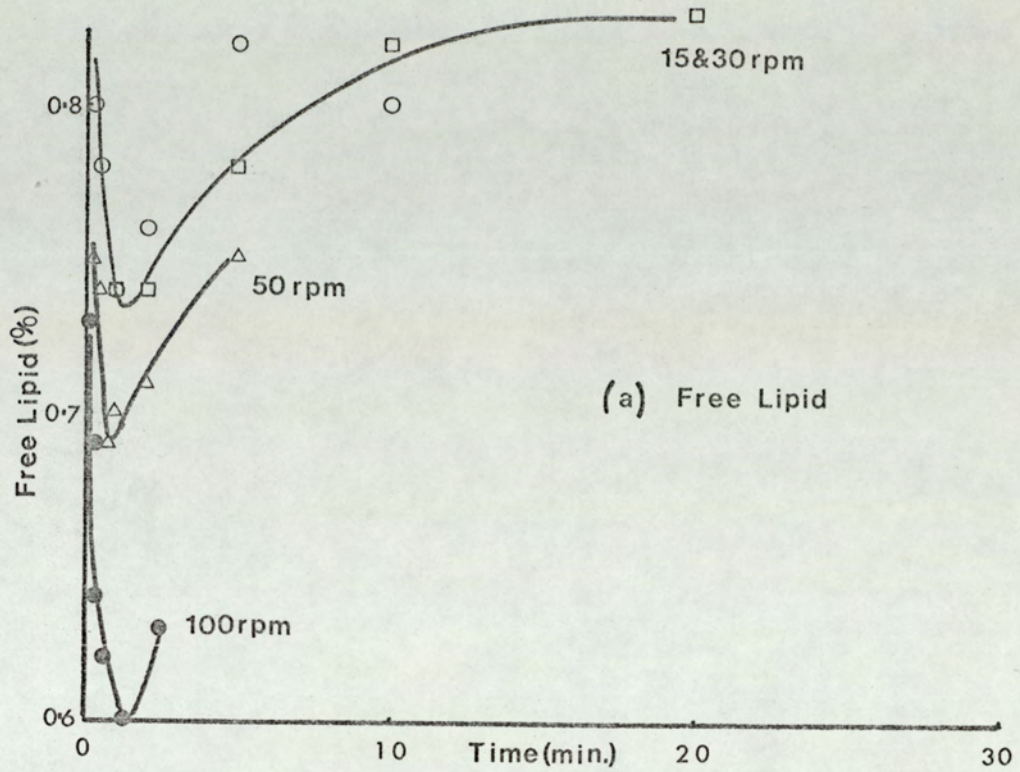
These results show that when soya-containing doughs were mixed in air the initial binding of lipids was followed by lipid release. This was particularly clearly demonstrated at the slower mixing speeds. However these results raise the question of why the release of bound lipids should be greater at low mixing speeds. While this seemed to indicate that lipid release was an inverse function of the mixing speed it was realised that the changes taking place in the dough could also be controlled by a time dependent factor. It was observed that to reach a given work level at 15 r.p.m. took ten times as long as at 100 r.p.m. When the results of Fig.4.1 were replotted on a time scale basis the curves shown in Fig.4.2 were obtained.

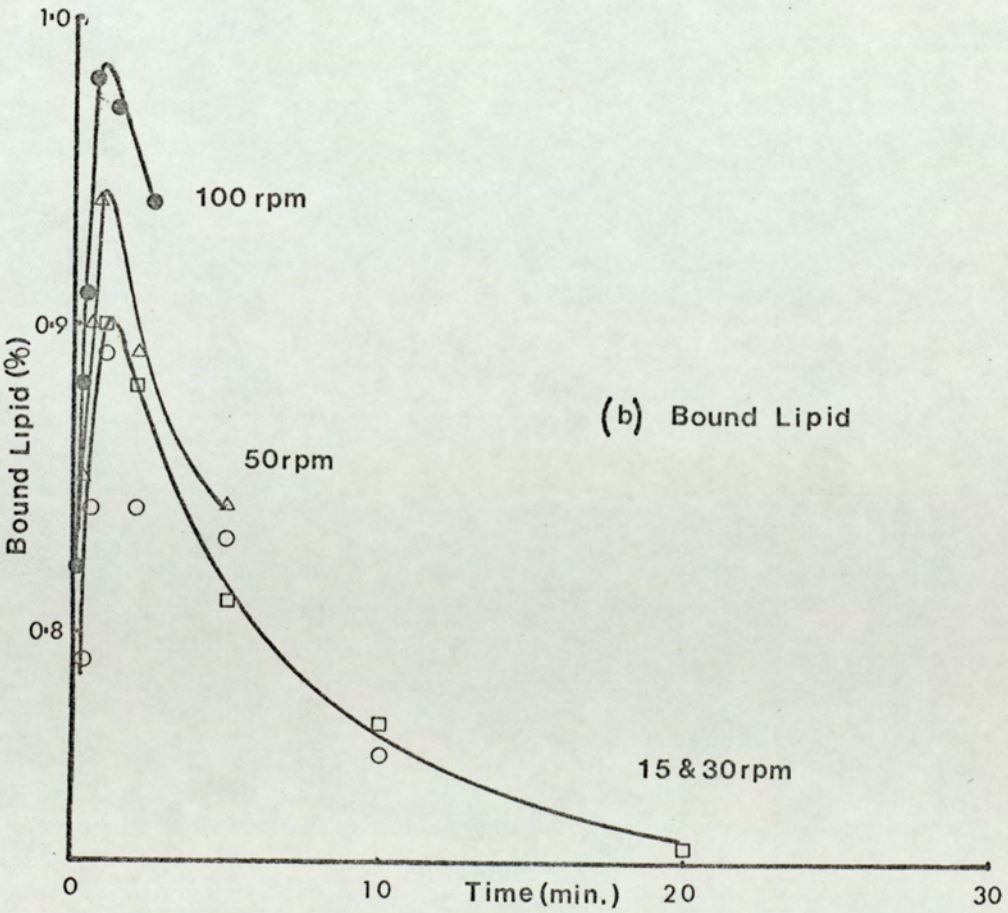
These curves demonstrate quite conclusively that the onset of lipid release in air mixed doughs is a time dependent effect rather than a function of work level or mixer speed. At all mixing speeds the release of lipids began after about one minute. The one minute delay before the release of lipid occurs, may be related to the time required for sufficient air to be incorporated into the dough to activate the enzyme system and induce coupled oxidation of the lipid binding sites.

While the onset of lipid release is thus time dependent, nevertheless it will be observed that mixing speed still influences the amount of lipid binding. Obviously the binding of lipids in air is a complex system influenced by several parameters.

FIG.4.2 The influence of time and mixing speed on free (a) and bound (b) lipid distributions in doughs containing soya flour and mixed in air.

Symbols: □ 15r.p.m.; ○ 30r.p.m.; △ 50r.p.m.; ● 100r.p.m.





4.4 A FURTHER COMPARISON OF THE INFLUENCE OF OXYGEN AND NITROGEN ON LIPID BINDING AT LOW WORK LEVELS.

To further clarify our understanding of the factors controlling lipid binding in air, the experiments of the previous section were repeated using oxygen instead of air and examining all four mixing speeds in nitrogen.

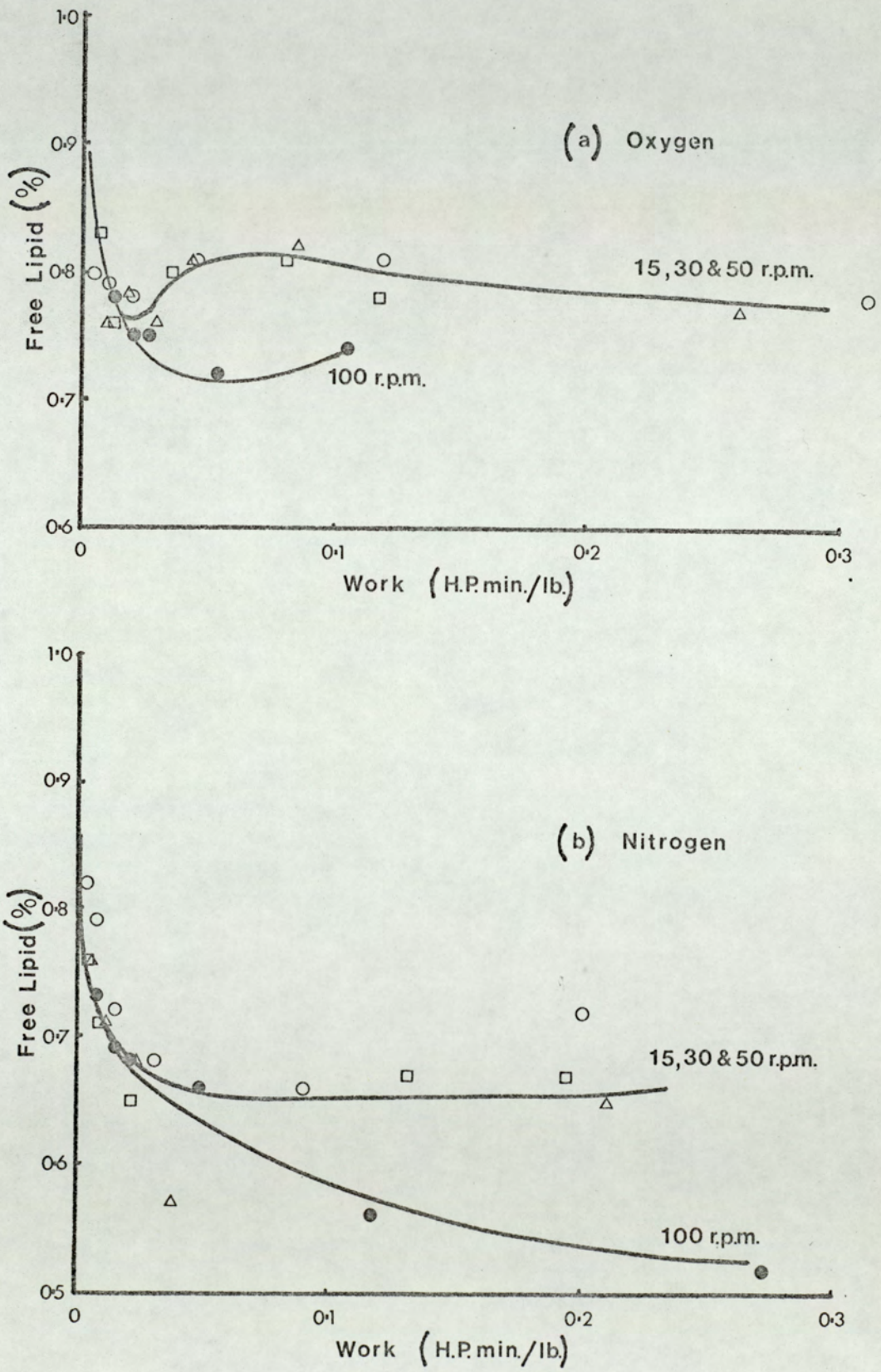
The water which was to be used in the investigations was saturated with the appropriate gas before being ground under liquid nitrogen. As a precaution against air being trapped in the frozen powder intended for an anaerobic dough, the weighed powder was reslurried in liquid nitrogen and poured into the mixing bowl as a slurry. The chamber was then sealed until the flushing nitrogen was admitted.

Only free lipid determinations were made on the freeze dried doughs and these results are presented in Figs.4.3a (oxygen) and 4.3b(nitrogen). Doughs mixed at 15 and 30 r.p.m. in oxygen showed the same binding pattern as previously observed for air mixed doughs. The 50 r.p.m. dough was indistinguishable from them, the inflexion having occured slightly earlier at 0.02 H.P.min./lb. and the subsequent lipid release taking place at lower work levels. The dough mixed at the highest speed of 100 r.p.m. showed much less lipid binding than previously (Fig.4.1) and the inflexion had been shifted to a lower work level. Overall the pattern of more lipid binding at higher speeds was repeated in these oxygen doughs.

At all mixing speeds in nitrogen more lipid was bound than at the corresponding work levels in oxygen. There was little difference between the doughs mixed at 15, 30 and 50 r.p.m. but again more lipid was bound at 100 r.p.m. (than at the slower speeds).

FIG.4.3 The influence of atmosphere on lipid binding in doughs containing soya flour at various mixing speeds.

Symbols: □ 15 r.p.m.; ○ 30 r.p.m.; △ 50 r.p.m.; ● 100 r.p.m.



It was concluded from these observations that in the early stages of mixing the mechanism causing the release of lipids is limited by the availability of oxygen. By mixing in oxygen and using oxygen saturated water the release of lipid was shifted to a lower work level, particularly at the higher mixing speeds. At all speeds the amount of lipid bound was also somewhat reduced. The increased availability of oxygen within the system did not shift the inflexion in the binding curves below 0.02 H.P.min./lb. and it may be that a certain amount of binding must take place before lipid release can begin. Faster mixing speeds would enable this binding to take place in a shorter time and therefore, providing oxygen is not limiting, lipid release would begin earlier.

4.5 DISCUSSION.

The experiments described in this section have demonstrated for the first time that in air-mixed doughs containing soya flour a rapid increase in bound lipid at the onset of mixing is quickly followed by a release of lipid during further dough development.

It is further concluded that the onset of lipid release is independent of the mixing speed but is influenced by the availability of oxygen in the system.

A closer examination of the published results of Daniels *et al.*[121] reveals that whilst they drew no distinction between the three work rates examined, the lower work rates consistently gave slightly lower levels of lipid binding in air. This is in agreement with the results in Figs.4.1 4.2 and 4.3 obtained at lower work levels. It follows therefore that work rate influences lipid binding in air both at the low work levels studied here and at the much higher work levels reported earlier.[121] The general

trend is for higher work rates to cause more lipid binding irrespective of the dough atmosphere even though in air the initial binding may be rapidly overtaken by lipid release during further mixing.

Mixing speed is, however, not the only factor influencing lipid release as is shown by comparing Fig.4.1 with Fig.4.3 Increasing the availability of oxygen in the system caused an apparent decrease in the amount of lipid bound and at higher mixing speeds lipid release occured at a lower work level. As stated previously this may be because lipid must have been actively bound before it may be subsequently released and since higher mixing speeds promote more lipid binding,[121] further mixing in oxygen leads to less bound lipid overall.

Two competing systems seem therefore to govern the binding of lipids in air. Firstly there is the influence of mechanical work which, even in the presence of air and enzyme tends to promote lipid binding. Secondly there is the lipoxygenase system which favours the release of lipids, providing air is present. Results published by Daniels et al.[100,129] suggest that the action of the lipoxygenase system may in fact inhibit or block the binding of lipids as well as promoting their release. When doughs, mixed in air, were exposed to a nitrogen atmosphere there was no increase in lipid binding on further mixing. They concluded that this was due to oxidation of lipid binding sites.

An alternative suggestion arising from the results of this section is that the apparent inhibition might be due to sufficient oxygen having been incorporated into the dough to sustain the lipoxygenase system, even during further mixing in nitrogen. Bloksma has shown that gas occlusion after ten minutes mixing at 66.5 r.p.m. might be as high as 10%.[116] Other results by Daniels et al.[100] have shown that lipid binding is very sensitive

to small amounts of oxygen in the mixing chamber, in line with the possibility that oxygen retention rather than site oxidation could explain the results observed on changing the atmosphere from air to nitrogen.

A possible explanation of the results may be that in air the two systems are operative simultaneously. Lipid binding would be balanced during dough mixing by lipid release caused by the action of the lipoxygenase system. Thus a dynamic equilibrium would be attained when the rate of binding equalled the rate of lipid release; overall the effect would be no further change in the apparent levels of free and bound lipid.

Such a system would explain why the reduction in lipid binding compared with that in nitrogen doughs is only small when either lipoxygenase, linoleate or oxygen are limiting. When doughs are mixed in atmospheres containing progressively less oxygen the lipid binding versus work curves move progressively closer to the nitrogen curve.[100] Similarly when no soya flour is added to the system a lipid binding curve midway between the air and the nitrogen curve is obtained,[178] probably due to the small amounts of free fatty acid specific lipoxygenase in the flour.[98,99] Finally if the flour is fat-extracted and the natural lipid replaced with linoleate-free shortening considerable lipid binding takes place irrespective of atmosphere.[23]

Work to test this hypothesis will be described in the next section in which radiotracer techniques have been used to advantage in following the detailed movement of dough lipids during dough development.

SECTION 5

A CLOSER STUDY OF LIPID BINDING USING RADIOACTIVE LIPID TRACERS

5.1 INTRODUCTION.

(i) Objectives.

It was suggested in the previous section that the changes in lipid binding observed when doughs were mixed in air might be the net effect of two competing systems. The effects of the lipoxygenase system would lead to the release of bound lipid, whilst mechanical work would promote further lipid binding. Thus if both systems were operative in an air-mixed dough the overall observed effect would be dependent on the ratio of the rates of lipid binding and release. When the two rates were equal (and of course opposite) there would be no further change in the level of bound lipid.

Testing such a hypothesis by classical techniques presents many problems. The difference in the fatty acid analyses of the free and bound flour lipids is not great[22,23] and any distributional changes during mixing may be complicated by oxidative losses. Furthermore the initial binding which takes place at the start of mixing would mask any further binding during lipoxygenase promoted release. The incorporation of an unnatural lipid marker, such as margaric acid is also unsatisfactory since the large additions which would be required to be detected by g.l.c. would inevitably cause modifications to the dough system.

(ii) Radiotracer experiments.

The proposed system of lipid binding in air might best be tested by the incorporation of radiotracers into the doughs under investigation. The use of radiotracers would have three advantages in this work. Firstly the physical amount of the radiotracer required would be so small that the dough system under investigation would not be modified by their

inclusion *per se*. Secondly a specific lipid of interest could be used to give a more precise insight into the mechanisms of lipid binding and finally the behaviour of two different lipids could be compared simultaneously by labelling each with a different isotope e.g. ^{3}H and ^{14}C.

Of the various lipid classes present in flour, the triglycerides and free fatty acids were chosen for radioactive tracer studies. Ideally a polar lipid, such as a lecithin, would have been included also, but it was not found possible to obtain this class of lipid in labelled form. In choosing which individual triglyceride and fatty acid to use it was decided to keep as close as possible to the composition of the natural flour lipids. As the flour triglycerides are predominantly unsaturated oils it was decided to use glycerol tri (oleate-9,10-^{3}H). This particular triglyceride had the advantage of being oxidatively stable. Palmitic acid-1-^{14}C was chosen because it is the most abundant of the oxidatively stable fatty acids present in flour lipid.

(iii) Dough mixing.

The inclusion of radioactive materials in the doughs required some modification of the experimental procedures used in the previous section (cf. Section 2). Furthermore the liquid nitrogen slurrying technique for work-free wetting could not be readily modified to avoid the hazard of radioactive dust dispersal and therefore it was not possible to include work-free wetting in the design of these experiments.

Radioactive doughs were therefore prepared using the premix procedure of Daniels *et al*.[22,23] This involved blending the dry ingredients in the mixer bowl for two minutes before adding thewater and then premixing at

15 r.p.m. for one minute before dough development.

A study of Fig.4.1 showed that the onset of lipid release occurred at higher work levels as the work rate was increased. Since the object of these experiments was to study changes taking place before, during and after this occurence it was decided to use a high rate of work input during the mixing of radioactive doughs. As in previous work on mechanically developed doughs, work rate was held constant at 0.2 H.P.min./lb./min. (19.74 kJ kg^{-1}).[100,121,178]

5.2 CONTROL DOUGHS IN AIR AND NITROGEN.

(i) Introduction.

It was realised that these modifications to the mixing procedure might lead to changes in the lipid binding pattern observed in earlier sections. Therefore to establish the basic binding patterns in this modified system a series of doughs were mixed in air and nitrogen. The same dough formulation as used in Section 4 was maintained and the radiotracers (on an inert support of glass beads) were added to the dry ingredients (cf. Section 2). The free and bound lipid distributions are presented in Figs.5.1 and 5.2 together with the distribution of the radioactive tracers ^{3}H-triolein and ^{14}C-palmitic acid.

In studying these graphs it should be remembered that the relative positioning of the axis scales for percentage lipid and disintegrations per minute are purely arbitrary and therefore coincidence of curves does not imply equivalence.

(ii) Total lipid.

Comparing Fig.5.1 with Fig.4.1 it is seen that the changes in the mixing procedure caused some modification in the binding pattern. As expected the release of bound lipid in air-mixed doughs now occurred at a much higher work level than observed previously. Furthermore the level of free lipid at the inflexion fell to 0.37% compared with 0.6% obtained when mixing at a constant 100 r.p.m. This was in agreement with the earlier observation that higher mixing speeds (see Fig.22) shift the inflexion to higher work levels (Fig.4.1) and also promote more lipid binding.[121] The increase in the time taken to reach the inflexion, from one minute to two and a half minutes, is consistent with the omission of the work-free wetting procedure and with the consequent delay in hydration of the dough. While Daniels et al.[121] noted no inflexion in lipid binding above 0.1 H.P.min./lb. in air, differences in dough formulation may have prevented observation of this effect in their earlier work.

The higher mixing speeds also promoted more binding of lipids in nitrogen (Fig.5.2) although the overall increase in bound lipid with work level was maintained.

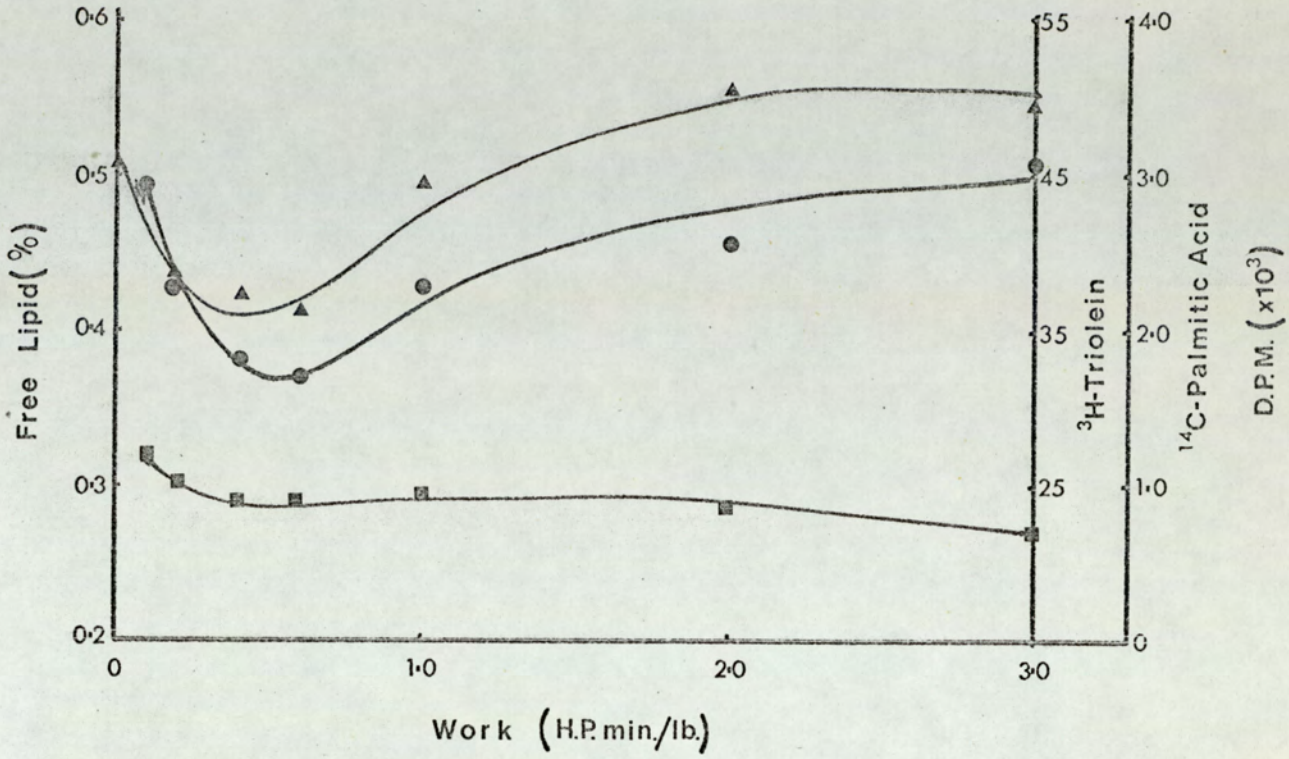
(iii) Triolein and palmitic acid.

The incorporation of radioactive ^{3}H-triolein to the dough revealed that this lipid behaved very similarly to the total flour lipids in its binding pattern in both air and nitrogen.

However the introduction of radioactive ^{14}C-palmitic acid showed that this fatty acid behaved differently from the triglyceride, ^{3}H-triolein, and the natural flour lipids. In air, ^{14}C-palmitic acid was initially bound

FIG. 5.1 The binding of ^{3}H-triolein, ^{14}C-palmitic acid and flour lipids in doughs containing soya flour and mixed at 0.2 H.P.min./lb./min. in air. Radiotracers added to the dry ingredients.

Symbols: ● flour lipids; ▲ ^{3}H-triolein; ■ ^{14}C-palmitic acid



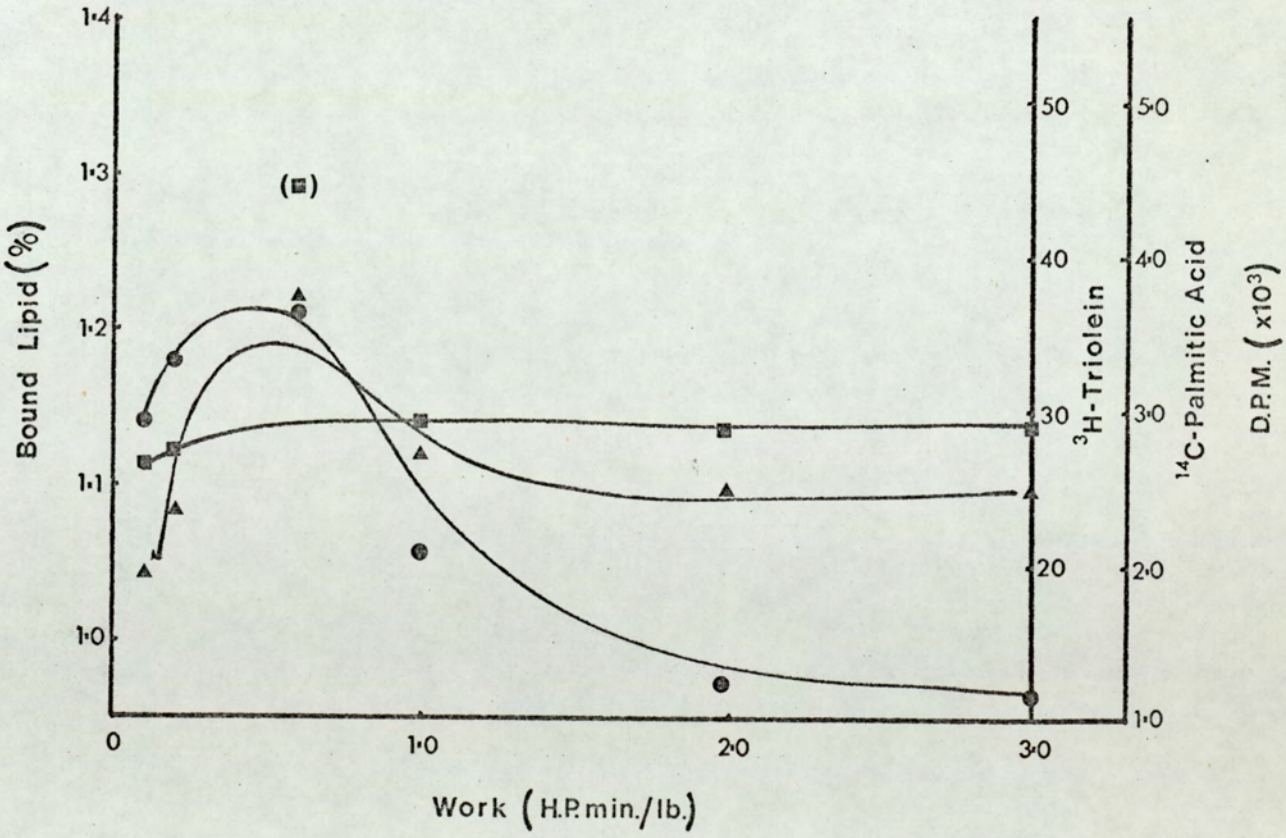
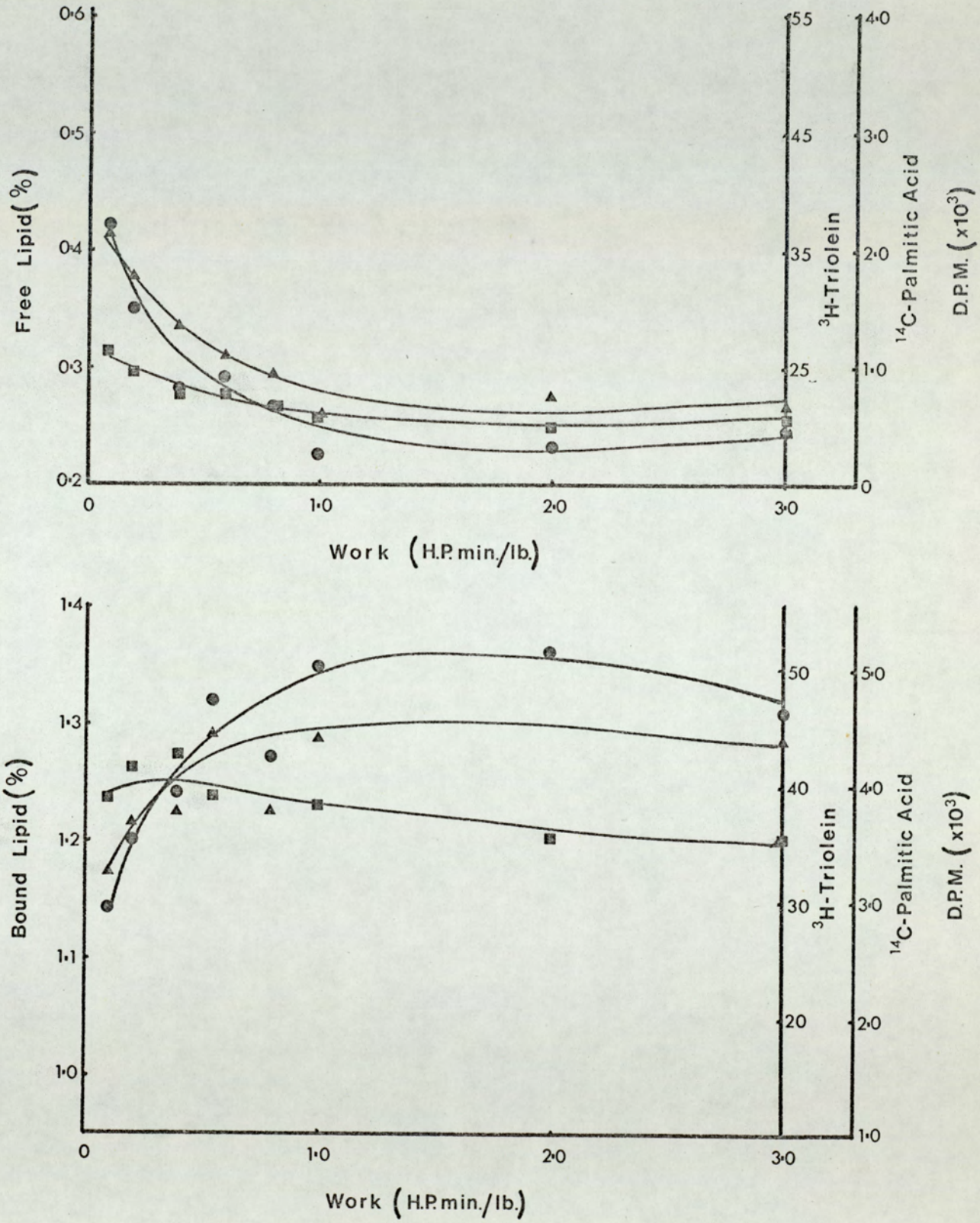


FIG. 5.2 The binding of ^{3}H-triolein, ^{14}C-palmitic acid and flour lipids in doughs containing soya flour and mixed at 0.2 H.P.min./lb./min. in nitrogen. Radiotracers added to the dry ingredients.

Symbols: ● flour lipids; ▲ ^{3}H-triolein; ■ ^{14}C-palmitic acid



as were the other lipids. However unlike ^{3}H-triolein and the dough lipids, ^{14}C-palmitic acid was not subsequently released. Instead the amount in both the free and bound states remained constant. The change of atmosphere to nitrogen produced no marked change in the binding of this fatty acid and the amount in either state remained constant. There was however a slight fall in the total extractable ^{14}C-palmitic acid. The significance of this difference in behaviour between a fatty acid and a triglyceride will be discussed later.

5.3 FURTHER STUDIES ON THE BINDING OF LIPIDS DURING MIXING IN AIR.

(i) Introduction.

Having established the behaviour of these lipid tracers in the control doughs, further experiments were designed to establish whether both lipid binding and lipid release systems were operative during prolonged aerobic mixing. If this were the case then it was predicted that no matter when the radiotracers were added to the dough during mixing some radioactivity would become bound. Thus, even during the active release of lipids, which follows the inflexion in the aerobic binding curve, binding of radiotracers should occur. On the other hand if radioactivity added after the inflexion was not bound then this would imply that the action of the lipoxygenase system blocks further binding of lipids.

(ii) Experimental design.

Doughs were mixed to 0.1, 0.4 and 1.0 H.P.min./lb. in air before the radioactive lipids were added. Mixing was then continued for various periods of time whilst maintaining the same atmosphere. Since the addition of the radiotracers necessitated a slight pause in the mixing, a further series of doughs were mixed in nitrogen as controls. From this series of

of doughs it was possible to establish whether the pause had caused any modification of the lipid binding.

(iii) Control nitrogen-mixed doughs.

Comparison of Fig.5.3 with Fig.5.2 shows that there was nothing in the overall lipid binding of this series of doughs to indicate that the slight pause in the mixing at 0.2 H.P.min./lb. caused any modification to the previously observed binding pattern in nitrogen.

Surprisingly the radioactive ^{3}H-triolein and ^{14}C-palmitic acid were fully incorporated into the bound lipid within one minute (0.2 H.P.min./lb.) from the addition. In fact this binding was so rapid that as much radioactivity became bound after this time, as was bound after 2 minutes (0.4 H.P.min./lb.) when the radiotracers were added to the dry ingredients. Once this rapid initial binding of the radiotracers had taken place the rate of removal from the free lipid changed to a rate similar to that of the rest of the dough lipids.

(iv) Air-mixed doughs.

By adding the radiotracers after one minute mixing (Fig.5.4) their inclusion whilst the dough lipids were still being bound was assured (cf Fig.5.1). It was found that the radiotracers were incorporated into the bound lipid within thirty seconds of addition as was the case in the nitrogen mixed doughs and this rapid initial rate of binding subsequently changed to a slower rate typical of the total lipids. Considering ^{3}H-triolein, as further mixing took the system past the point of inflexion the binding pattern changed to one of lipid release paralleling the overall pattern of release of the dough lipids. Conversely ^{14}C-palmitic acid

FIG. 5.3 The distribution of ^{3}H-triolein and ^{14}C-palmitic acid in doughs containing soya flour and mixed at 0.2 H.P.min./lb./min. in nitrogen. Radiotracers added after 1 minutes mixing, (at arrow).

Symbols: ● flour lipids; ▲ ^{3}H-triolein; ■ ^{14}C-palmitic acid

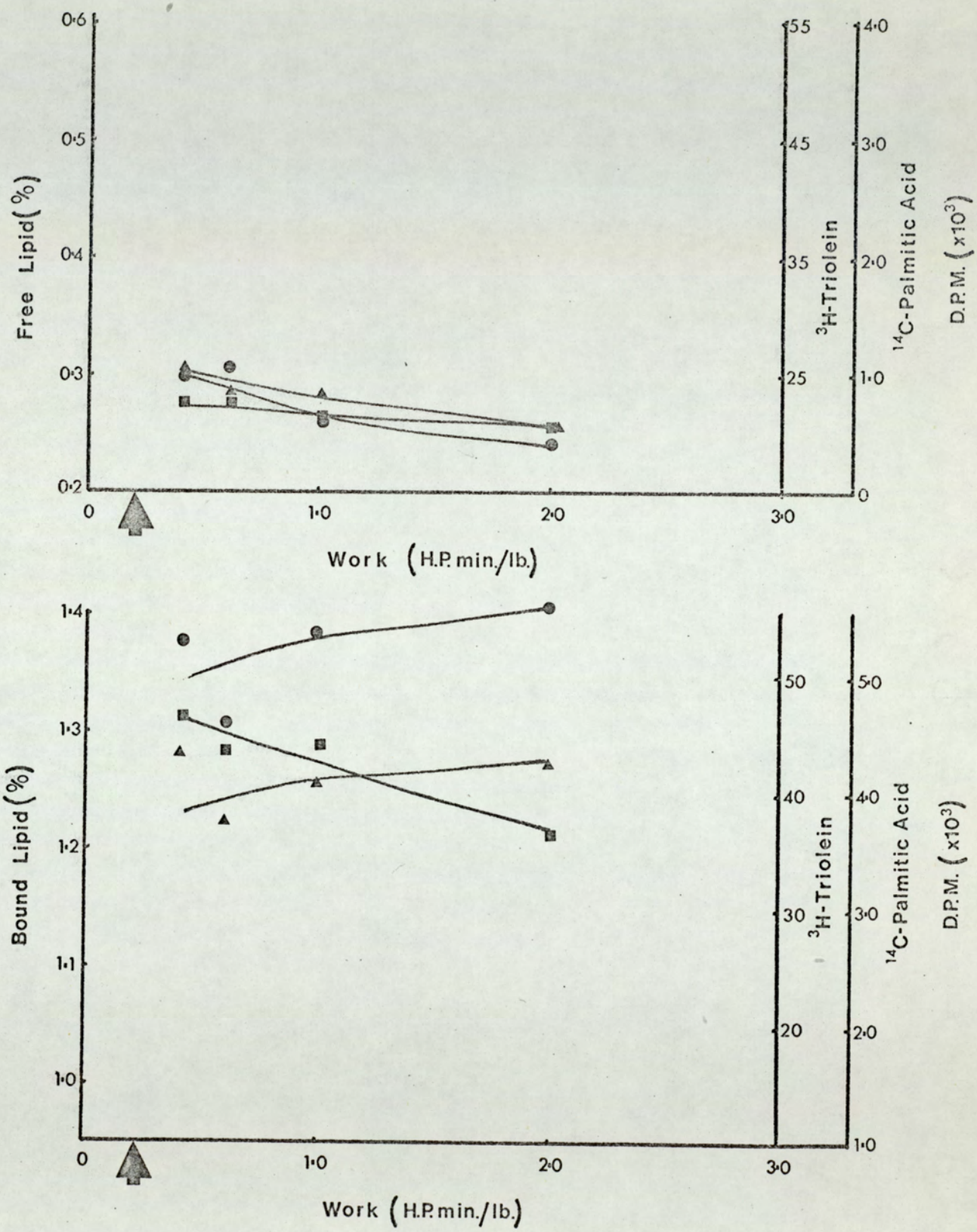
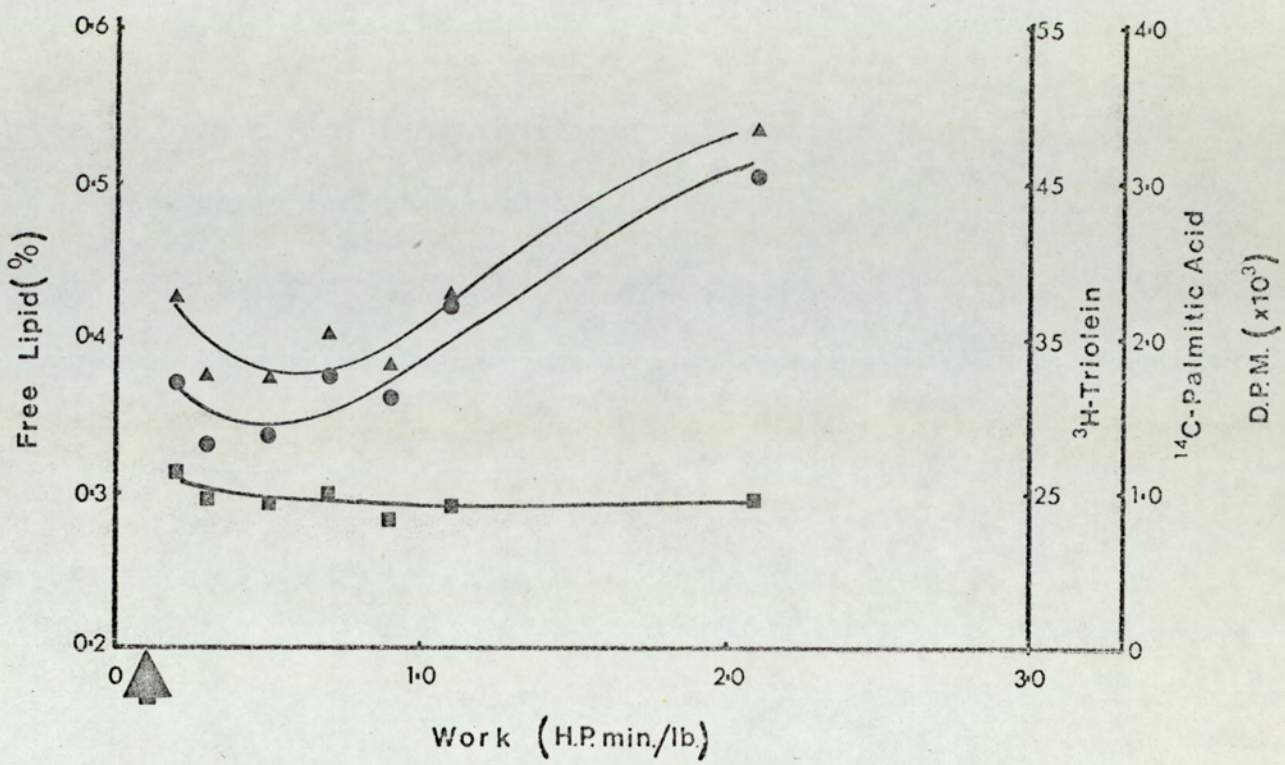
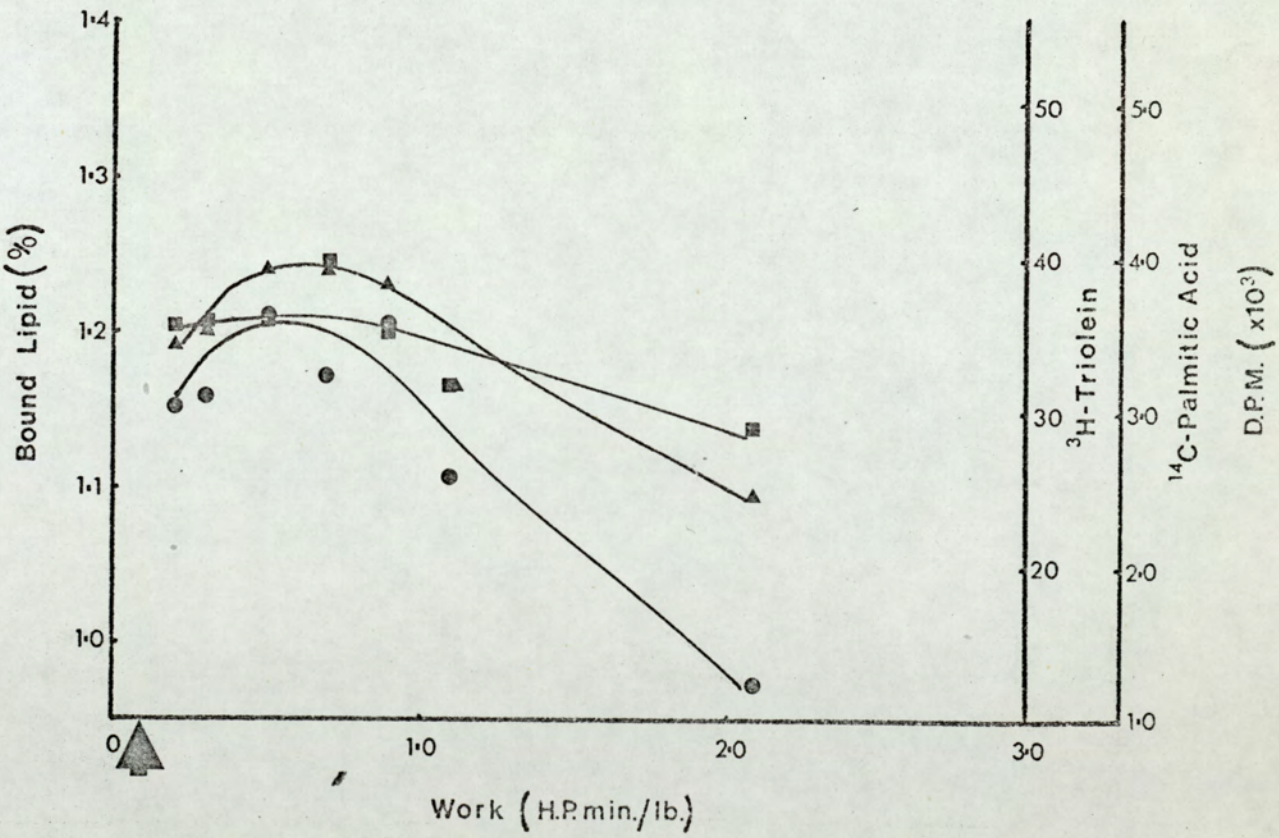


FIG.5.4 The distribution of ^{3}H-triolein and ^{14}C-palmitic acid in doughs containing soya flour and mixed at 0.2 H.P.min./lb./min. in air. Radiotracers added after ½ minute mixing, (point of addition shown by arrow).

Symbols: ● flour lipids; ▲ ^{3}H-triolein; ■ ^{14}C-palmitic acid





remained at a constant level in the free lipid whilst apparently gradually disappearing from the bound lipid.

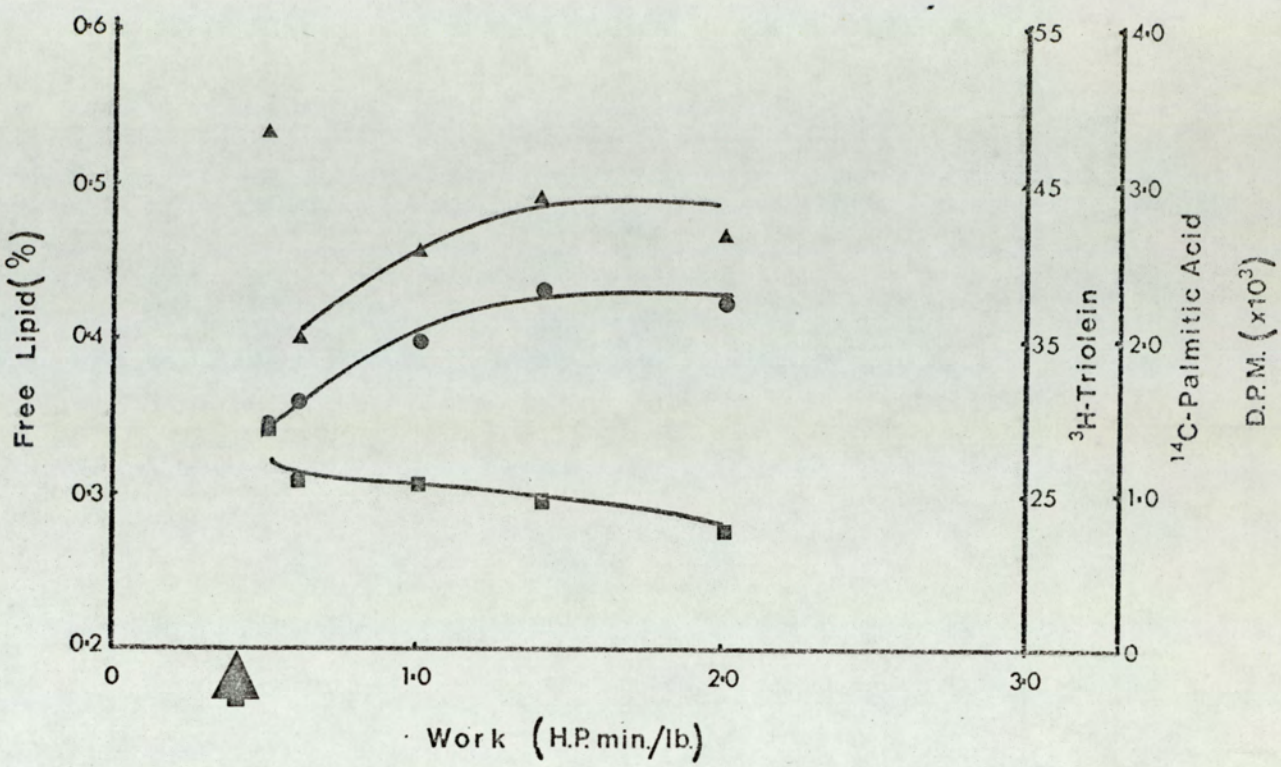
In the second air-mixed experiment the radiotracers were added at a work level close to the inflexion in the aerobic mixing curves (Fig.5.5 cf. Fig.5.1). As had been observed in all this series of experiments the radiotracers were again rapidly bound. In this case however the ^{3}H-triolein immediately began to be released again after one minutes mixing. Again the fatty acid tracer behaved differently from the triglyceride after the initial rapid binding. Instead of the ^{14}C-palmitic acid being released it continued to be bound although fairly slowly. Unfortunately in this experiment, variation in the bound lipid counting results made it difficult to be certain if there was any overall loss of ^{14}C-palmitic acid as observed in Fig.5.4.

In the last of this series of experiments (Fig.5.6) the radiotracers were added after five minutes mixing in air (1.0 H.P.min./lb.) when the amount of free lipid was increasing due to the release of bound lipid through the lipoxygenase system (cf. Fig.5.1). In spite of the continuous release of bound lipid after the addition of the tracers, the results of liquid scintillation counting showed unequivocably that the radiotracers were being actively bound immediately after their introduction to the system.

Whilst the radiotracers subsequently followed the previously observed binding patterns (cf. Fig.5.4 and Fig.5.5) with the ^{3}H-triolein being released and the ^{14}C-palmitic acid preferentially bound there was an apparent inexplicable increase in total ^{14}C-palmitic acid.

FIG.5.5 The distribution of ^{3}H-triolein and ^{14}C-palmitic acid in doughs containing soya flour and mixed at 0.2 H.P.min./lb./min. in air. Radiotracers added after 2 minutes mixing, (point of addition shown by arrow).

Symbols: ● flour lipids; ▲ ^{3}H-triolein; ■ ^{14}C-palmitic acid



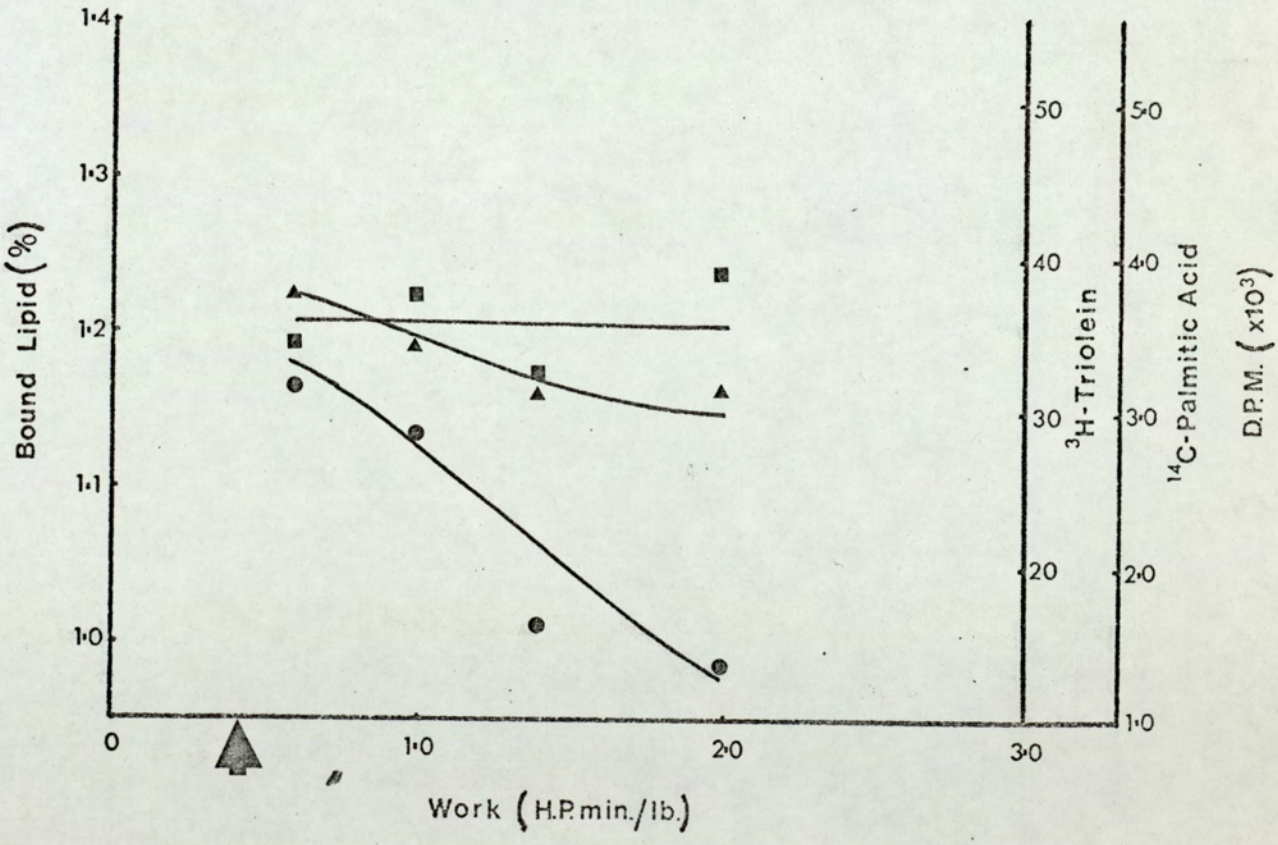
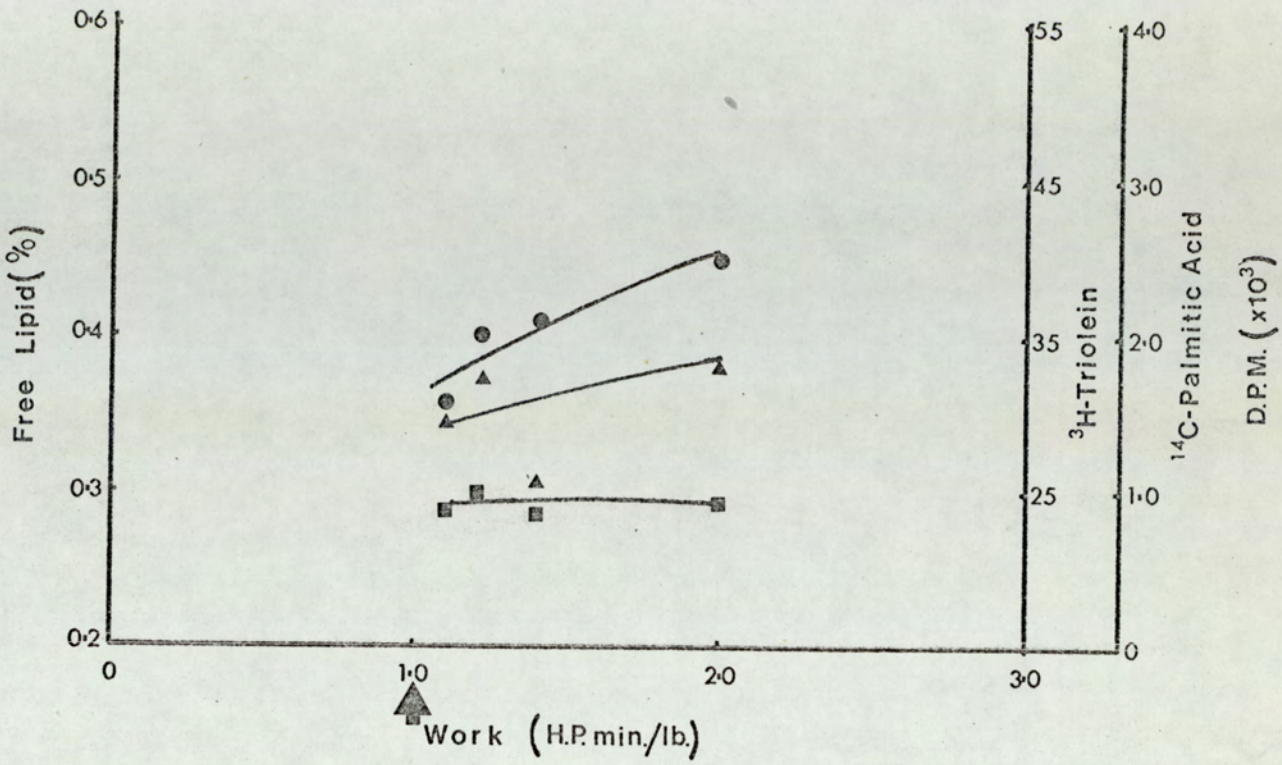
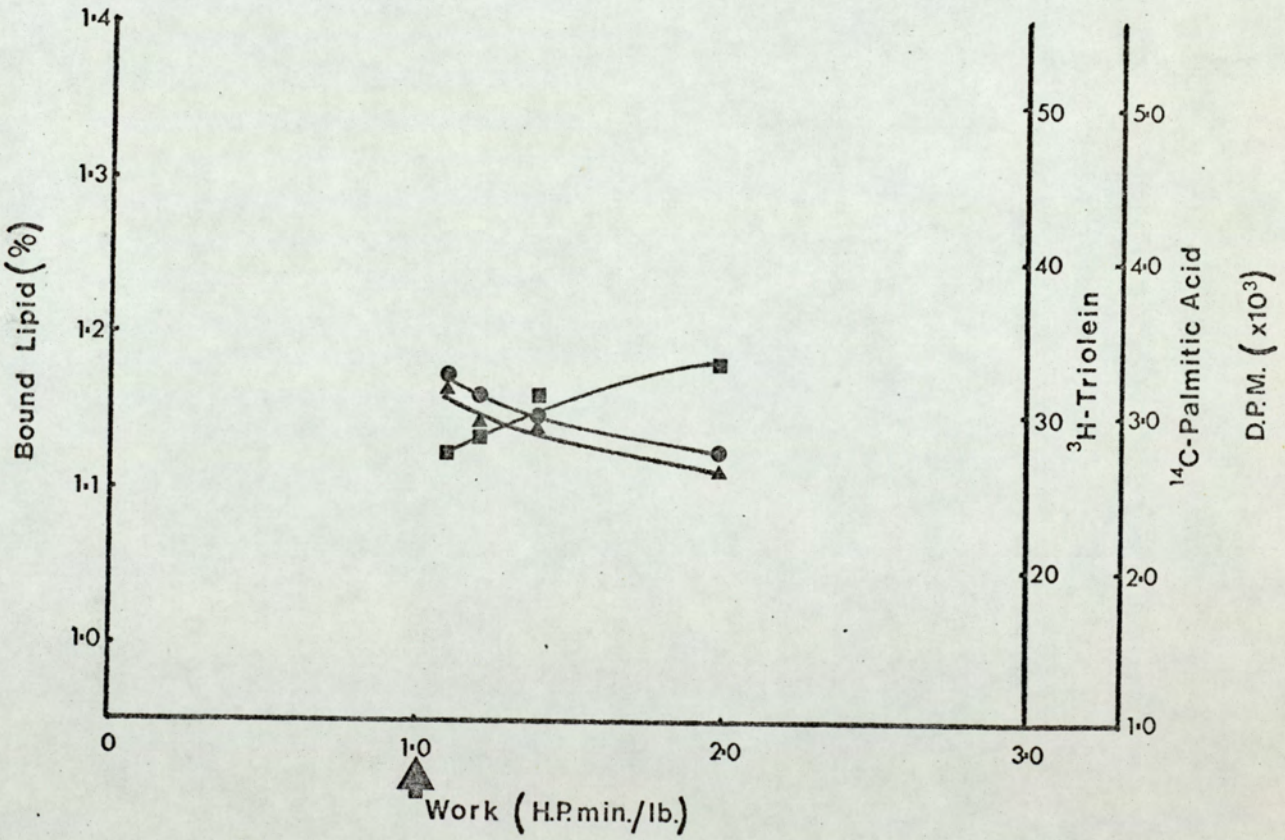


FIG. 5.6 The distribution of ^{3}H-triolein and ^{14}C-palmitic acid in doughs containing soya flour and mixed at 0.2 H.P.min./lb./min. in air. Radiotracers added after five minutes mixing, (point of addition shown by arrow).

Symbols: ● flour lipids; ▲ ^{3}H-triolein; ■ ^{14}C-palmitic acid





(v) Conclusions.

These results have demonstrated for the first time that lipid binding continues to take place throughout aerobic mixing concurrently with the release of bound lipid. They have also shown that lipid binding in wheat flour doughs is a more complex phenomenon than had previously been recognized. It was expected that the radiotracers would be incorporated into the bound lipid at no more than the rate at which lipids are bound during mixing in nitrogen. Lipid release was previously seen as occurring only as a result of the effects of the lipoxygenase system.

In fact the experiments showed that the radiotracers were very rapidly bound following addition to the partly developed dough to the same extent that they would have been bound had they been added to the ingredients. Having reached this level of binding the subsequent binding or release of ^{3}H-triolein occured at the same rate as the total dough lipids. These results indicated therefore that during mixing there was a very rapid interchange of lipids, or at least triglycerides, between the free and bound state. The slow change in the overall level of lipid binding compared with the very rapid initial binding of the radiotracers suggests that the level of free and bound lipid must represent an equilibrium state. This in turn suggests that the effect of prolonged mixing in nitrogen is to shift the equilibrium towards more bound lipid whilst in air the lipoxygenase system shifts the equilibrium the opposite way.

5.4 RELEASE OF BOUND LIPIDS DURING MIXING IN NITROGEN.

(i) Introduction.

If the above explanation was correct then it follows that radioactive lipid would be displaced from the bound lipid even during mixing in nitrogen. The problem was, of course, that using the above system such a change would be impossible to detect because of the radioactivity already present in the

free lipid. This difficulty was overcome by preparing "radioactive bound lipid".

(ii) Experimental method.

A dough containing ^{3}H-triolein and ^{14}C-palmitic acid was mixed in nitrogen to promote a high level of lipid binding. It was then freeze-dried, ground and the free lipid extracted with petroleum-ether. As a result the dough powder contained radioactivity in the bound form only. This powder was then incorporated into a series of anaerobic doughs as a part of the dry ingredients.

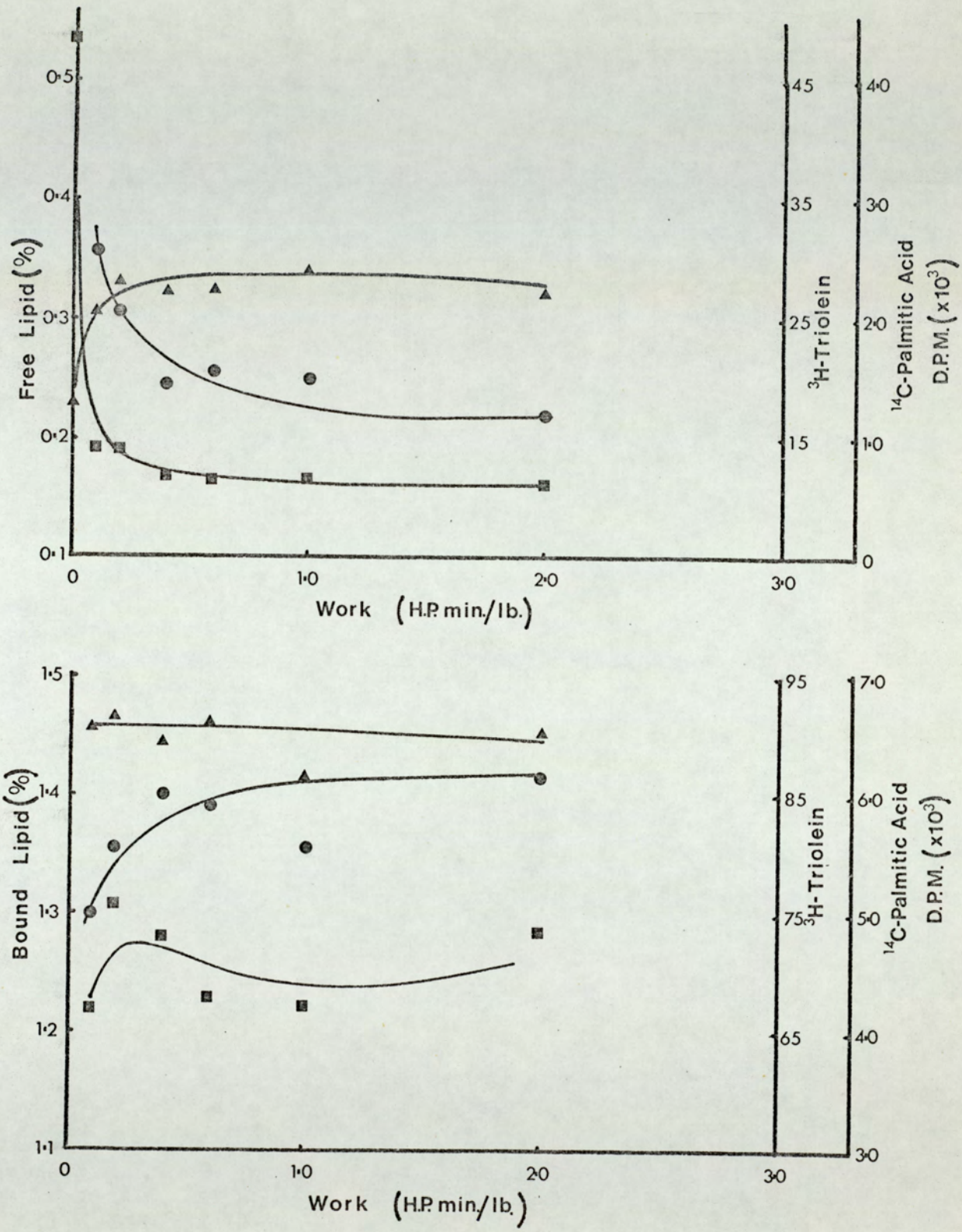
(iii) Results.

It was recognized that, unlike previous experiments using glass beads as a support, the freeze-dried dough powder was not inert and may have been affected by the freeze-drying and fat-extraction. Therefore a sample of the fat-extracted freeze-dried dough powder (18g) was rewetted by the liquid nitrogen slurry technique and after freeze-drying the radioactivity in the free lipid was determined. The equivalent points are marked on the axes in Fig.5.7 which shows also the results of the dough mixings.

In this experiment the level of ^{3}H-triolein in the free lipid clearly increased against the trend of overall lipid binding. Since earlier results had indicated the rapid binding of radioactivity the actual amount of ^{3}H-triolein released overall must have been greater than indicated from the graph. While this observed release of ^{3}H-triolein may have been an extension of the release observed on work-free wetting due to hydration effects, it seems more likely that the effect demonstrates the attainment of the proposed dynamic equilibrium against the flow of free lipid into the

FIG. 5.7 The distribution of ^{3}H-triolein and ^{14}C-palmitic acid in doughs containing soya flour and mixed at 0.2 H.P.min./lb./min. in nitrogen. Radiotracers added to the dry ingredients as "bound lipid" in fat-extracted freeze-dried dough powder.

Symbols: ● flour lipids; ▲ ^{3}H-triolein; ■ ^{14}C-palmitic acid



bound state. (cf. Table 5.1 Section 5.6)

(iv) Conclusions.

A possible cause underlying these effects could be the spontaneous changes in the gluten proteins during rehydration leading to a release of bound lipid due to the equilibrium having been artificially disturbed by fat-extraction.

The rapid rebinding during mixing of ^{14}C-palmitic acid released on rehydration is interesting for two reasons. Firstly it confirms that the released lipids were subsequently rebound and emphasizes that assuming some rebinding of released ^{3}H-triolein, the actual amount of ^{3}H-triolein released overall must have been greater than observed. Secondly it suggests that the ^{14}C-palmitic acid may be bound by two different mechanisms. Part of the ^{14}C-palmitic acid may be bound in a similar way to the ^{3}H-triolein and involved in rapid interchange between free and bound states and part may be more strongly bound. This will be discussed later.

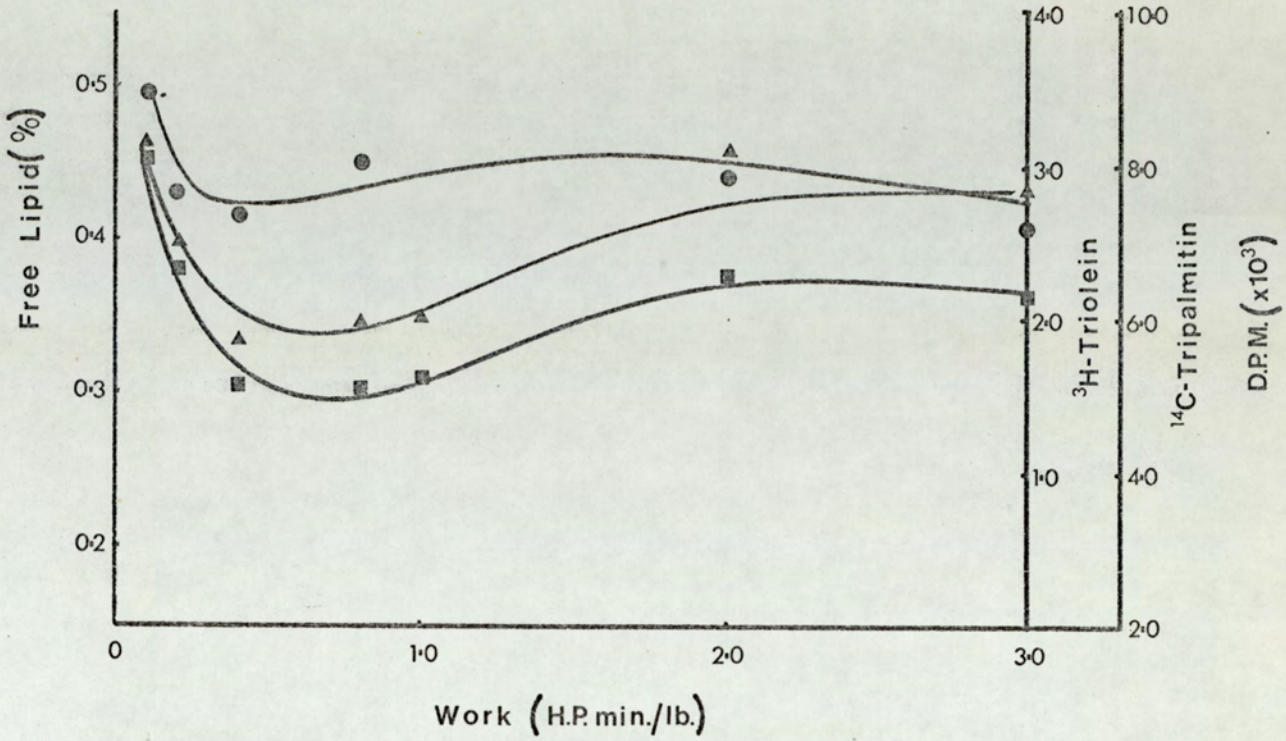
5.5 A COMPARISON OF THE BINDING OF TRIOLEIN WITH TRIPALMITIN.

(i) Introduction.

The evidence presented above for a dynamic equilibrium of dough triglycerides has been based mainly upon results obtained using one triglyceride only, namely ^{3}H-triolein. The assumption that ^{3}H-triolein, being an oil, was typical of the flour triglycerides in its binding pattern was tested by further comparison with another fully saturated triglyceride. ^{14}C-Tripalmitin was chosen as a solid triglyceride and also because of its importance as a bakery additive typical of the hard fat essential to many modern breadmaking processes.

FIG.5.8 The binding of ^{3}H-triolein and ^{14}C-tripalmitin and flour lipid in doughs containing soya flour and mixed at 0.2 H.P.min./lb./min. in air. Radiotracers added to the dry ingredients.

Symbols: ● flour lipids; ▲ ^{3}H-triolein; ■ ^{14}C-tripalmitin



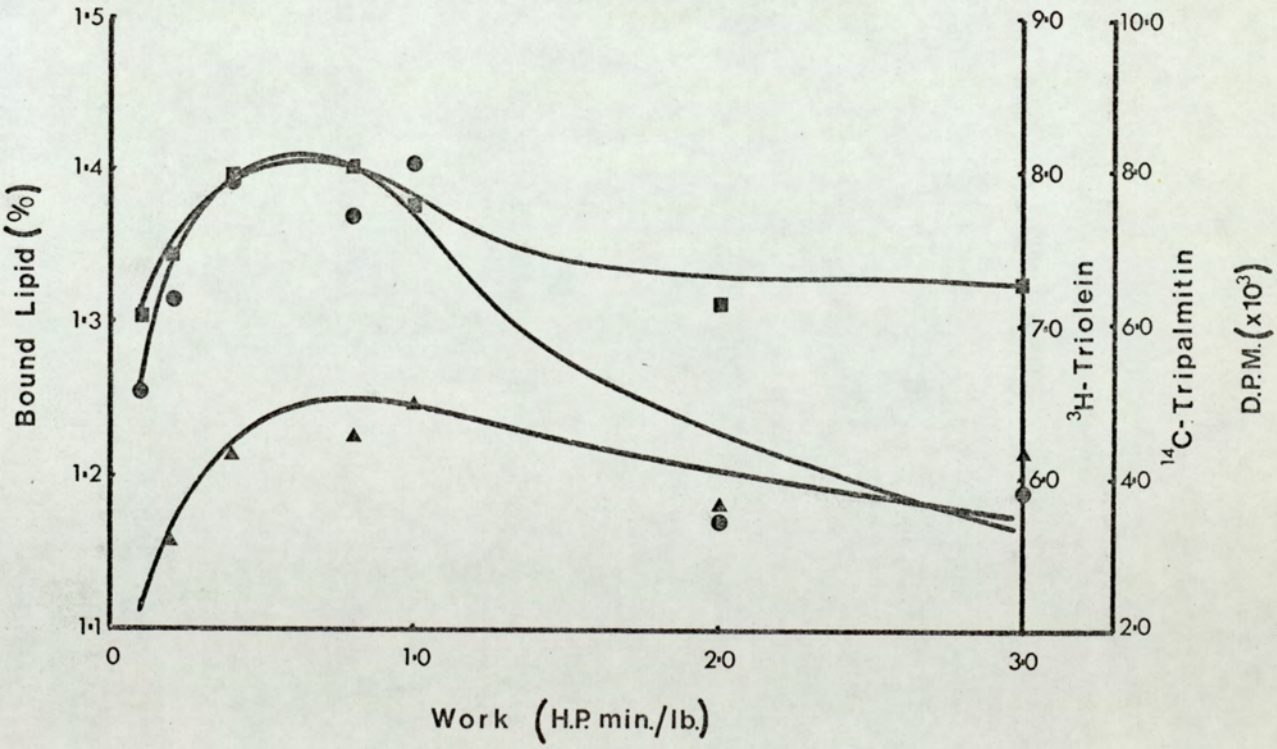
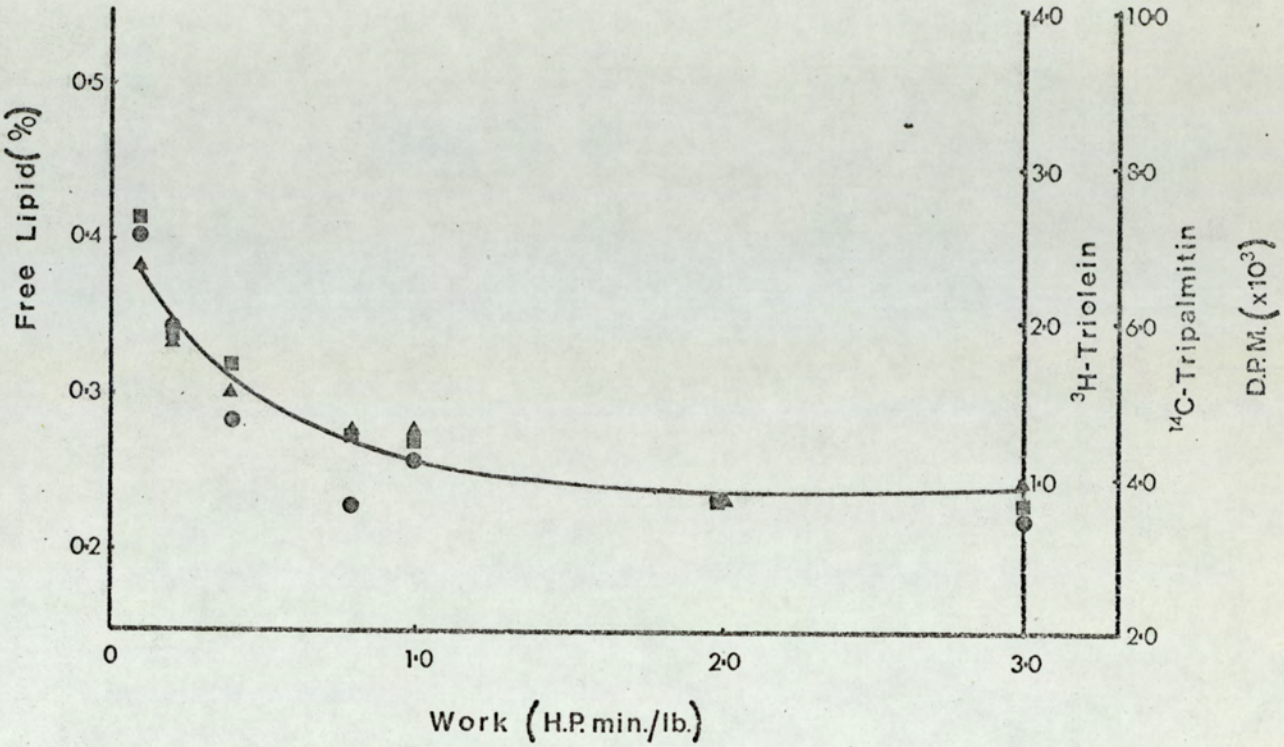
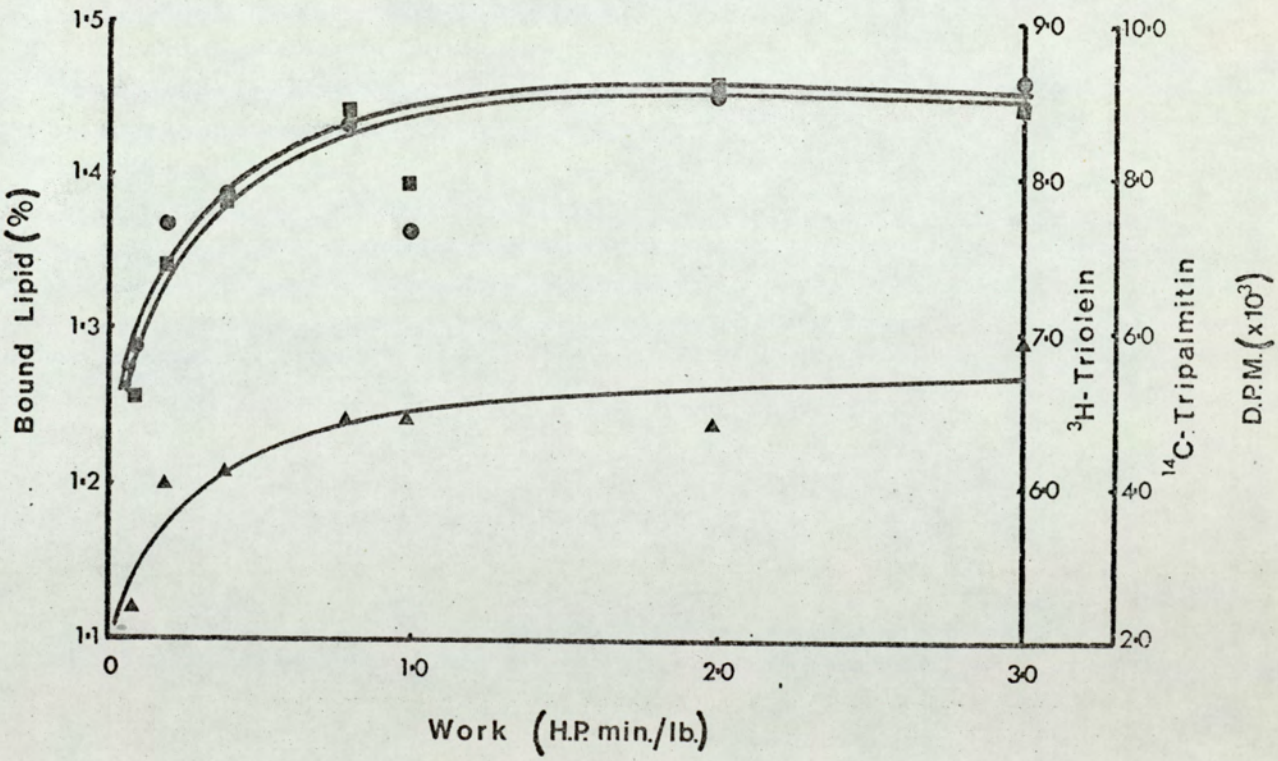


FIG.5.9 The binding of ^{3}H-triolein, ^{14}C-tripalmitin and flour lipid in doughs containing soya flour and mixed at 0.2 H.P.min./lb./min. in nitrogen. Radiotracers added to the dry ingredients.

Symbols: ● flour lipids; ▲ ^{3}H-triolein; ■ ^{14}C-tripalmitin





(ii) Results.

In these experiments flour C was used. The radioactivity, on glass beads, was added to the dry ingredients. The results are presented in Figs. 5.8 (air) and 5.9 (nitrogen).

In both air and nitrogen it was found that the unsaturated liquid ^{3}H-triolein and the saturated solid ^{14}C-tripalmitin had overall binding patterns very similar to the total flour lipids. Whilst there may be slight differences in the relative amounts of each triglyceride in the free and bound extracts these were not considered significant. Certainly there was nothing to suggest that either triglyceride may be more preferentially bound than the other.

5.6 DISCUSSION.

The experiments described in this section have demonstrated unequivocably that during dough mixing the flour lipids are in a dynamic equilibrium between the free and bound states. Moreover this dynamic interchange in the free and bound lipid occurs equally in the presence of air and nitrogen atmospheres. Thus it has been shown that irrespective of whether the gross effect is lipid binding or lipid release, radioactive lipid added to the dough rapidly appears in the bound lipid. Similarly the evidence suggests that added "bound lipid" equally rapidly appears in the free form.

As a result of this dynamic interchange the added radioactive lipids quickly attained the same stage of binding as the total lipids had reached. That is, even if lipid release was occurring overall at the time of addition of the labelled lipids, these would be bound to reach approximately the same stage of binding as the total lipids within thirty seconds of addition.

Thereafter the added radioactive triglyceride binding curves would follow a similar pattern to the total dough lipids.

Whilst ^{14}C-palmitic acid behaved quite differently from ^{14}C-tripalmitin and ^{3}H-triolein becoming preferentially bound, there are indications that it was also involved in the dynamic interchange affecting the triglycerides. For example on addition to the dough it was initially bound as rapidly as the ^{3}H-triolein and it was also released on rehydration of the fat-extracted freeze-dried dough powder as were the triglycerides.

A further demonstration can be obtained by comparing the ratios of ^{3}H-triolein, ^{14}C-palmitic acid and lipid in the free and bound states after 2.0 H.P,min./lb. mixing. This data derived from Figs. 5.1 to 5.7 is presented in Table 5.1.

TABLE 5.1.

The ratio of free to bound, ^{3}H-triolein, ^{14}C-palmitic acid and flour lipids after 2.0 H.P.min./lb. mixing at 0.2 H.P.min./lb./min.

Fig.No.	Atmosphere	Activity added after (H.P.min./lb.)	Ratio: Free/bound		
			^{3}H-triolein	^{14}C-palmitic acid	Flour Lipids
5.1	air	0	2.5	0.23	0.49
5.4	..	0.1	1.9	0.31	0.50
5.5	..	0.4	1.35	0.20	0.44
5.6	..	1.0	1.23	0.28	0.38
5.2	N_2	0	0.45	0.13	0.16
5.3	..	0.2	0.37	0.13	0.16
5.7	..	0 (Radioactive bound lipid")	0.30	0.13	0.16

The most important comparison in this table is between the binding

of ^{14}C-palmitic acid in air and nitrogen. It shows quite conclusively that the binding of ^{14}C-palmitic acid is greater in nitrogen indicating that the fatty acid must be affected to some extent by the lipoxygenase system. The observation leads to the conclusion that at least some of the ^{14}C-palmitic acid was bound by the same mechanism as the triglycerides.

A comparison of the binding of an unsaturated triglyceride, ^{3}H-triolein, with a saturated triglyceride, ^{14}C-tripalmitin, demonstrated that there were no basic differences in their binding patterns. Whilst this result would indicate that unsaturation plays no part in determining the binding of triglycerides it should be recalled that small dispersed amounts of triglycerides were used in these experiments. In the commercial dough system where larger amounts of shortenings are added, the solid crystalline nature of saturated fats may prevent their becoming bound.

By the use of radiotracers a fuller knowledge has been acquired of the way in which mechanical work affects lipid binding. Armed with this new technique it should be possible to attack the problem of lipid binding at a different level. The extra knowledge so gained should in turn lead to a deeper understanding of the part played by the enigmatic lipid-protein complex in dough mixing and bread manufacture.

SECTION 6

DISCUSSION

DISCUSSION.

The binding of lipids in wheat flour doughs has been shown by Daniels et al.[121] to be dependent upon both the work rate and work level during mixing in nitrogen. Whilst this observation implicates mechanical work in the promotion of lipid binding other investigations have demonstrated that raising the moisture content of a "work-free" dough also caused a progressive increase in the amount of lipid bound.[125,163]

The investigation reported in this thesis set out to examine in greater detail the mechanism involved in lipid binding resulting from the introduction of mechanical work. An early hypothesis (Section 3.1) was that increased binding was due to the more efficient distribution of water throughout the dough system during mixing. Was hydration of the dough constituents the mechanism responsible for both work-free and work-induced lipid binding?

The function of mixing in promoting lipid binding.

By introducing small amounts of mechanical work to "work-free" doughs prepared by the method of Davies et al.[163] it was demonstrated (Fig.3.3) that above 29% moisture work-induced lipid binding was independent of moisture content although still influenced by various other parameters, such as atmosphere and work rate.

This "critical" moisture content was shown to coincide with the development of a gluten structure (Fig.3.5). As the moisture content was increased above 29% the resistance to mixing of the doughs began to increase. A similar relationship has been demonstrated by Davies et al.[163] who reported that cylinders of wetted flour began to shrink at this moisture content. Further evidence for a critical relationship between lipid binding and

gluten development was provided by the sensitivity of the wetted (above 29%) "work-free" system to small amounts of mechanical work. As little as one minutes mixing at 15 r.p.m. produced a large drop in free lipid. It was concluded that gluten development was the prime requirement for lipid binding to occur although gluten development in itself required the presence of free water. Mechanical work promoted lipid binding by increasing the development of the gluten structure which had taken place to some extent spontaneously on wetting.

This conclusion necessitated a reappraisal of the results (Fig.3.4) of Davies et al.[163] who reported that bound lipid increased over the moisture range 20% to 35% even though dough shrinkage (i.e. gluten development) did not occur until moisture content was in excess of 28%. These findings may now be regarded as an artifact caused by the limitations of the "work-free" wetting procedure. Their method depends on the grinding of ice to a fine powder which was then intimately mixed with the flour by slurrying in liquid nitrogen. The subsequent melting of the larger ice crystals could raise local areas of themixture above the "critical" moisture content, even though the total moisture content was below this level. The consequent spontaneous development of a local gluten network would result in some lipid binding. As the moisture content was raised the number of such areas would increase and the net effect would be an apparent steady increase in lipid binding.

Confirmation of these conclusions was provided by experiments involving rewetting of worked doughs. It was shown that the increase in bound lipid noted by Davies et al.[163] when the moisture content was increased, did not take place if work-induced lipid binding had already occurred (Table 3.2) even though free lipid was still available for binding in the freeze-dried powder.

The overall conclusions to be drawn were therefore that the relationship between moisture content and lipid binding was indirect. Water was vital to the process, in that the interaction of the protein chains to form a gluten network could not take place without it, and its very presence would force lipids with a low affinity for water into the hydrophobic areas of the gluten. However the extent to which lipid binding occurs must be regarded as solely dependent upon the extent to which the gluten network is developed rather than upon the presence of free water when the critical moisture level is exceeded.

Lipid binding in air.

Lipid binding is not the only lipid interaction which can occur when doughs are mixed. If a dough containing lipoxygenase is mixed in air then lipid release rather than lipid binding will take place.[23,100,121] However it was shown in Section 4.3 that this lipid release occurs only after a rapid initial binding of lipids has taken place at the onset of mechanical mixing. (Fig.4.1) The onset of lipid release was shown to be both time (Fig.4.2) and atmosphere dependent (Fig.4.3) and this was seen as further evidence of the involvement of the lipoxygenase enzymes in promoting lipid release.

The speed at which the doughs were mixed appeared to have little influence upon the effects of the lipoxygenase system. That is, higher mixing speeds did not promote a faster release of bound lipids. On the contrary higher mixing speeds led always to higher levels of bound lipid irrespective of atmosphere composition. Such observations invited speculation concerning the likelihood of lipid binding continuing even during mixing in air. Was the observed distribution of free and bound lipid in air-mixed doughs the net result of two competing systems, namely work-

-induced lipid binding and lipoxygenase-linoleate coupled lipid release?

Rapid interchange of triglycerides between free and bound states.

In an attempt to answer this question radiotracers were used to follow the movement of individual lipid classes during dough development. The use of specifically labelled lipids proved to be particularly effective in this work. It was demonstrated that the actual involvement of lipids in dough mixing was somewhat more complex than had been envisaged above. Whilst the binding of triglycerides and fatty acids was clearly demonstrated during mixing in air, (Fig.5.6) the rate at which these lipids were incorporated into the bound lipid suggested some mechanism must be involved, other than that proposed earlier. A rapid interchange of lipid between the free and bound states caused by the action of mixing, irrespective of atmosphere, was proposed as the most likely explanation of the observed effects.

Such a theory required not only that lipid binding should occur concurrently with lipid release during mixing in air, but that lipid release should also occur concurrently with lipid binding during mixing in nitrogen. The results of experiments designed to test this theory demonstrated that a constant equilibrium ratio between the amount of free and bound lipid of any class was attained (Table 5.1), the value of which depended on the conditions of dough mixing.

Overall effects.

From the above observations it is now possible to summarize the effects leading to lipid binding during dough mixing. As the flour components are hydrated the appearence of free water allows interaction between protein chains and, as a consequence, there is spontaneous

rearrangement to form a rudimentary gluten network. This causes or requires the binding of some lipids, probably those in close proximity to the protein chains. The introduction of mechanical work to the system promotes a more extensive rearrangement of the proteins leading to further lipid binding. In nitrogen this process continues with further work but in air or oxygen the lipoxygenase system becomes activated and causes release of lipids.

However, whilst the total amount of lipid bound increases in nitrogen and decreases in air, it is now realised that the lipids of certain classes rapidly interchange between the free and bound states under the influence of mechanical work. This interchange appears to continue throughout mixing even though after a certain level of mechanical work is reached the overall lipid ratio may remain constant. The effect of varying the atmosphere appears to shift this equilibrium ratio. The mechanisms which control these effects will now be considered in more detail.

What is lipid binding?

The effect known as lipid binding is usually measured in a purely arbitrary way and is related to the amount of lipid which can be extracted with various solvents. The more polar the solvent used the more lipids are extracted. It is clear, therefore, that the term "lipid binding" is an oversimplification of lipid behaviour during dough mixing, and it is probably more true to say that dough lipids exist in a wide "spectrum" of states. These states may vary from completely free droplets of oil to fully bound lipid in lipoproteins. Furthermore it should be remembered that the actual lipids involved are a complex mixture ranging from neutral hydrocarbons to highly polar phospholipids. Therefore it is unlikely that any one single kind of binding is involved and probable that several kinds may

take part in the binding of even a single molecule.

Types of bond involved in lipid binding.

(i) Electrostatic binding.

Electrostatic bonding occurs as a result of the mutual Coulombic attraction or repulsion of the net charges or electric moments carried by two interacting molecules. As an example Salem[182] has demonstrated an arrangement whereby both charged groups of a lecithin molecule interact simultaneously with two charged protein side-chain groups, the positive ammonium ion of a lysine residue and the negative carboxylate ion of an aspartic acid residue.

The involvement of such bonds in lipid binding in dough has been implicated by several workers,[104,136] and there is considerable evidence for these suggestions. The polar lipids, including lecithin, are strongly and preferentially bound during mixing[23,128,130,179] and solvents of high dielectric constant are required to extract the strongly bound phospholipids.[136] The addition of salt to doughs reduces the amount of lipid bound which is indicative of electrostatic bonding[127,128,136] although Pomeranz[126] has reported that the reduction in binding observed is due mainly to neutral lipid. The addition of hexane to doughs promotes binding of lipid phosphorus,[183] again indicative of electrostatic bonding.

Considerably more evidence is provided by investigations on the structure of natural membranes.[138,181] The main theories of membrane structure[153,154,155,156] have been based on the electrostatic binding of phospholipids to proteins. It is worth remembering at this point that a considerable proportion of the proteins of flour originate from membranes within the wheat grain endosperm.[5]

(ii) Binding of fatty acids.

Throughout the experiments involving radiotracers it was observed that palmitic acid became preferentially bound. Whilst it is possible that this was due to covalent bonding, Chapman[174] reports that this kind of bond is not usually considered to play an important role in lipid-protein interactions. The most likely explanation would seem to be that the observed binding of palmitic acid is due to ionic interactions with the dough proteins.

The presence of excessive quantities of fatty acids in flour has been shown to be detrimental to bread quality by several early workers.[80,81,82,83] It may be that the fatty acids compete with the phospholipids or the dough proteins themselves for available binding sites. Modification of the lipid composition of membranes is known to affect their functionality and in an analagous manner excess fatty acids may modify gluten structure.

(iii) Hydrophobic bonding.

Whilst the binding of polar lipids probably involves electrostatic bonds, such interactions could not explain triglyceride binding. A more feasible explanation of many of the observed effects is hydrophobic bonding. Hydrophobic bonding, or more correctly interaction, occurs as a result of the low affinity for water that non-polar hydrocarbon side-chains exhibit. If the hydrocarbon chains come together and in this way are removed from the aqueous environment a more stable configuration will result. It has been suggested[180] that hydrophobic bonds are stabilized by the large entropy gain occurring when hydrocarbon chains leave the aqueous environment. A considerable amount of evidence for such interactions has been provided by studies of other biological systems.[140,141,142,143]

The feasibility of such bonds in dough has been demonstrated on theoretical grounds[175] and several authors have speculated upon their involvement in the lipid-protein complex.[104,138] Daniels et al.[100] have suggested that the release of lipids which occurs during mixing in air may be due to the inversion of the hydrophobic binding sites. This was envisaged as being brought about by oxidation of disulphide bonds within hydrophobic areas of the protein, by unstable transient intermediates formed by the linoleate-lipoxygenase system. The consequent change in the tertiary structure of the protein was seen as leading to an inversion of the binding areas resulting in the release of lipid.

(iv) Triglyceride binding.

Such a system involving hydrophobic bonding would explain many of the results observed in this work. The formation of hydrophobic areas within the protein would be dependent initially on the presence of free water and subsequently upon the rearrangement of the protein to allow a more stable configuration. Mechanical work would promote this process and at the same time bring the free lipids into the proximity of these areas. The low affinity for water of the triglycerides would encourage their migration to these hydrophobic areas.

Two-way interchange.

Whilst mechanical work would promote the formation of hydrophobic areas a further effect would inevitably be the destruction or inversion of some of these areas due to work-distortion of the protein network. Thus, as lipid was becoming bound in freshly formed hydrophobic areas, previously bound lipid would be released following the inversion or distortion of hydrophobic areas by the mixing action. It can be seen

that within such a system the overall effect would be a rapid interchange of lipids between the free and bound states. Such a theory is fully compatible with the observations made during the course of this work.

It was evident in the experiments with radiotracers that palmitic acid as well as triolein was involved in this dynamic interchange (Table 5.1). Since polar lipids never completely disappear from the free lipid[23] it may also be suggested that other lipid classes are involved as well.

The binding of lipids within hydrophobic areas as envisaged here would also explain why work-induced lipid binding is not an exhaustive process. When doughs are mixed in nitrogen it is observed that, even after continued mixing at high work rates, the dough lipids never become totally bound, even though the gluten is capable of binding three times as much lipid as is normally present in flour.[125] The binding of lipid within areas which were constantly being disrupted and reformed would ensure that there was always some free lipid.

Effect of atmosphere.

A further question concerns the mechanism by which a change of atmosphere shifts the equilibrium ratio of free to bound lipid. The gradual development of the optimum number of binding sites in nitrogen has been explained above, but the attainment of an equilibrium in air is more difficult to explain.

The observations of Frazier et al.[178] on the rheological changes in dough as a result of the lipoxygenase-linoleate system are relevant in this context. They found that an increase in dough relaxation time, consistent with improvement, brought about by the lipoxygenase system, proceeded to a

maximum at 3.0 H.P.min./lb. However, lipid release, which was brought about by the same system, was at a maximum by 1.0 H.P.min./lb. Obviously if the same oxidation mechanism caused both dough improvement and lipid release, it continued long after the overall steady state in lipid binding had been established. Thus we come back to the process suggested in Section 4.7 in which the development of new hydrophobic binding sites as a result of mechanical work is offset by their oxidation by the lipoxygenase-linoleate system.

This system would explain the results of Frazier et al.[178] since the release of lipids caused by the oxidative system producing rheological improvement would continue after 1.0 H.P.min./lb. However beyond this work level the rate of lipid release would approximate to the rate of lipid binding and an equilibrium would be attained.

When experiments were conducted to test this hypothesis the previously unsuspected rapid interchange of lipids, caused solely by mechanical work, was revealed. This had the effect of masking any slower dynamic equilibrium and the mechanism must remain unconfirmed.

Other forms of binding.

Some consideration must now be given to the involvement of other forms of lipid-protein bond in wheat flour doughs. Hydrogen bonding between lipids and proteins has been suggested by several workers although the only lipids likely to be involved are the glycolipids. In fact Hosenay[135] has suggested that the glycolipids play a vital role in dough by linking glutenin to gliadin. On the basis of n.m.r. and i.r. studies he proposed that the glycolipids were bound to the gliadin by hydrogen bonds and to the glutenin by hydrophobic bonds. Obviously one could also

imagine them as forming a similar interface between the proteins and neutral lipids.

Another form of bonding, suggested by Fullington[138] has been the formation of mixed chelates of lipid and protein on a divalent cation. Flour of 70-72% extraction has been reported to contain 18mg/100g of calcium[183] so that even in the untreated flours under investigation here such bonds might occur. However this sort of bond cannot be implicated in the binding of triglycerides being essentially a form of phospholipid binding.

The involvement of double bonds in the lipid-protein complex has been suggested by Schulerud[137] and preferential binding of polyunsaturated lipids has been noted by other workers. Such conclusions have usually failed to recognize that the preferentially bound polar lipid contained considerably more linoleic acid than the shortening fats added to the dough. The comparison of a saturated with an unsaturated triglyceride (Section 5.5) showed no obvious differences in binding pattern, although complete confirmation of non-preferential binding of triglycerides would require experiments to be carried out quantitatively using a poly-unsaturated triglyceride.

A summary of the lipid binding system.

The proposed mechanisms controlling lipid binding as discussed above are summarized schematically in Fig.6.1. As the moisture content of the ingredients (Fig.6.1a) is raised above 29% (Fig.6.1b), the presence of free water promotes the formation of hydrophobic bonds between the dis-organised protein chains. The consequent formation of a rudimentary gluten network (Fig.6.1b) causes some lipid binding, probably of lipids in close

FIG. 6.1 A schematic illustration of the proposed model for lipid binding in wheat flour doughs. The areas representing lipid and water are approximations to the relative proportions in the various states e.g. free or bound, polar or neutral.

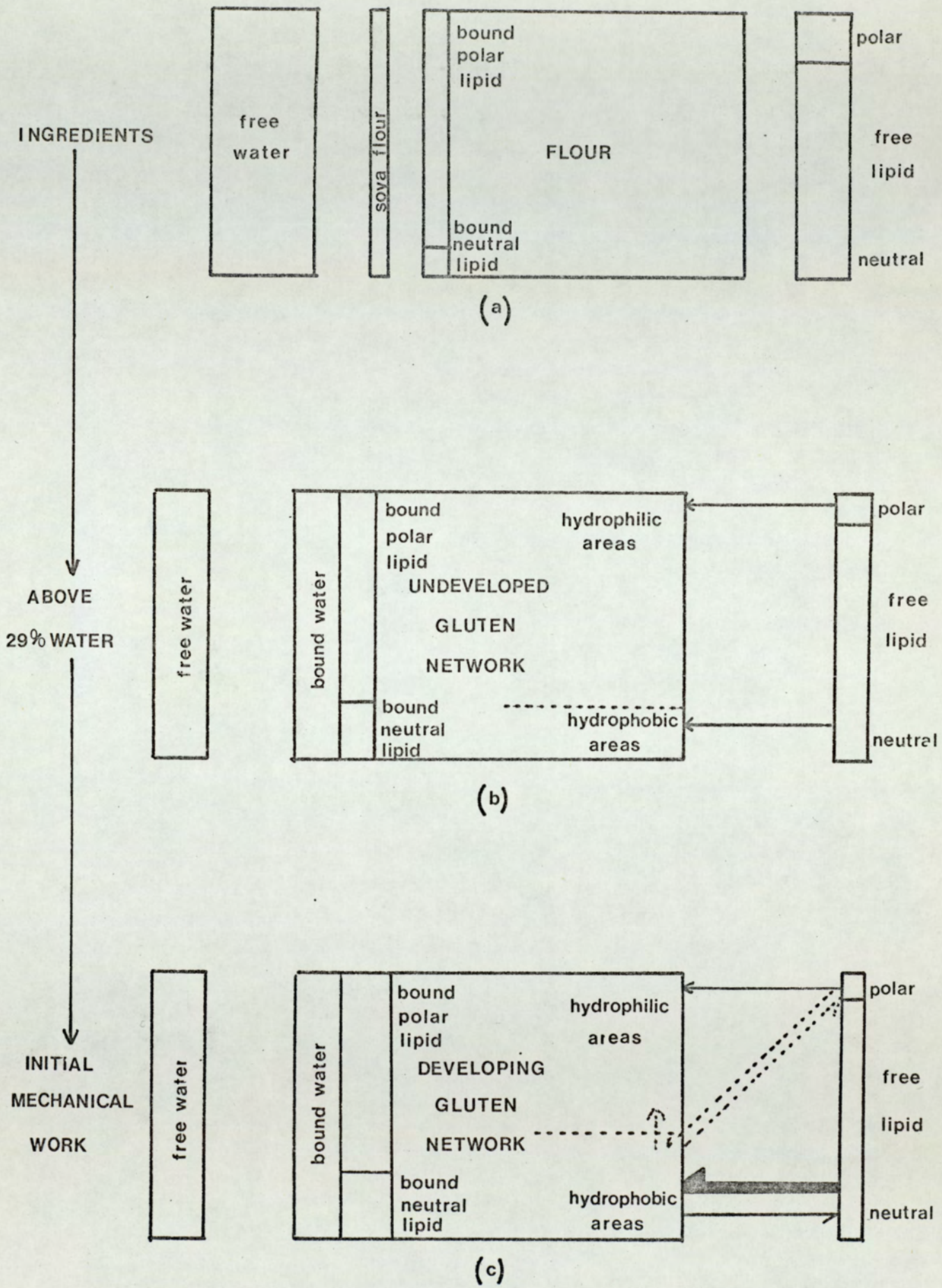
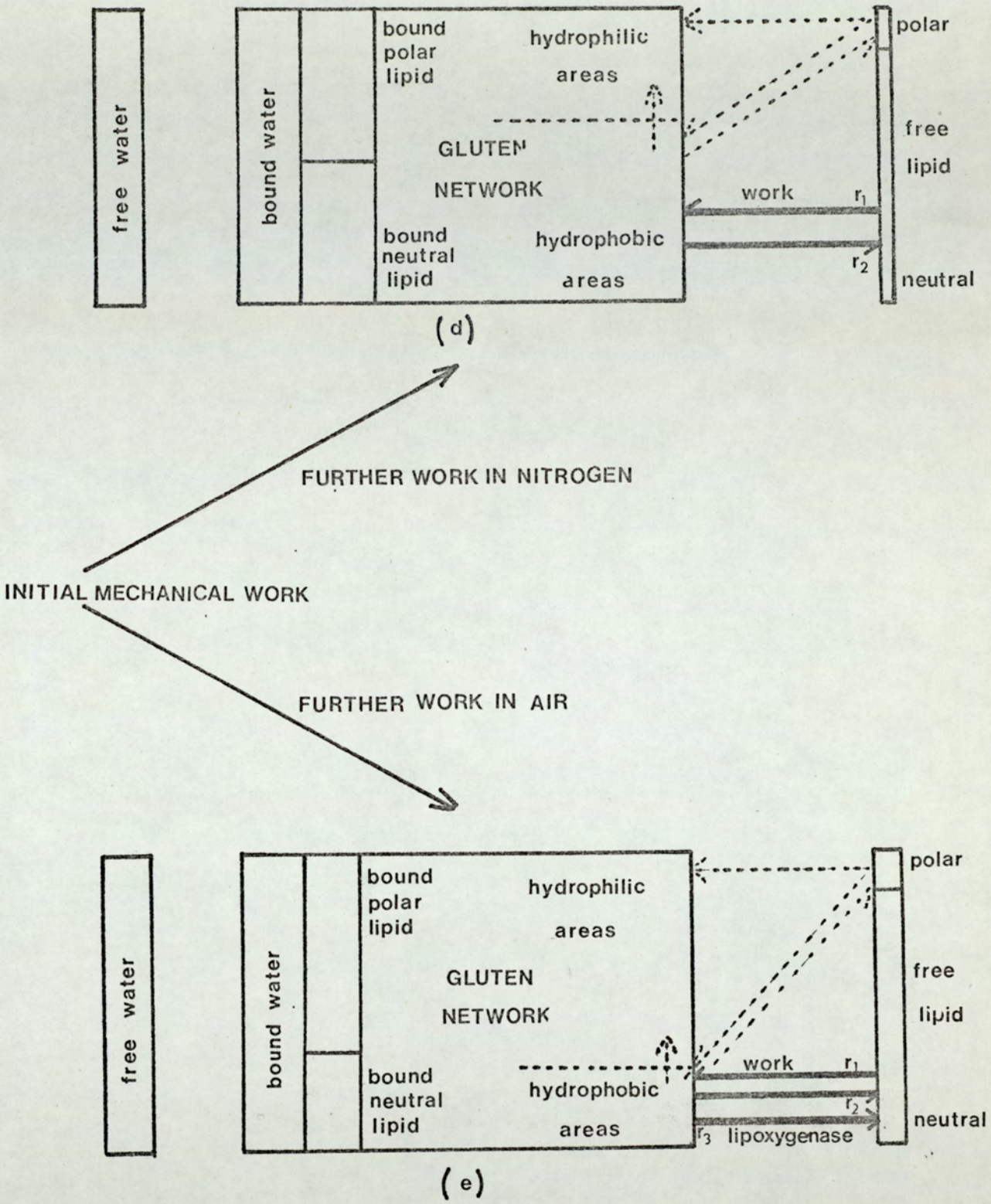


FIG. 6.1 (cont.) A schematic illustration of the proposed model for lipid binding in wheat flour doughs. The areas representing lipid and water are approximations to the relative proportions in the various states e.g. free or bound, polar or neutral.



proximity to the proteins. This may be hydrophilic binding of polar lipids or hydrophobic binding of neutral lipids due to entropy effects.

The introduction of mechanical work to the underdeveloped dough leads to an immediate rearrangement of the proteins to more stable configurations in which the hydrophobic areas would be extended and increased (Fig.6.1c). As a result of the formation of these areas further free lipid would become hydrophobically bound. This lipid could possibly include polar lipid which might subsequently become more firmly bound by hydrophilic bonds. Whilst the overall effect of mechanical work would be to increase the hydrophobic areas within the protein network it would also inevitably distort or invert some of these areas and as a result there would be some release of hydrophobically bound lipid as indicated by the reverse arrows. Thus, the net result would be the rapid interchange of lipids between the free and bound states as demonstrated in Section 5.

Further mixing of the dough system in nitrogen (Fig.6.1d) would continue this process to the point where the slowing down in the formation of new hydrophobic areas would be exactly balanced by the increasing rate of release of bound lipid. As this point the rates of binding and release (r_1 and r_2) would be equal and the ratio free/bound would be small(Table 5.1).

During mixing in air the systems described above would still be operative but a further factor would influence the amount of lipid bound. This would be the release of lipid caused by the inversion of hydrophobic binding sites due to the coupled linoleate-lipoxygenase system. Upon activation of this system, the rate of release of lipids (r_3) would initially be high since there would be a large amount of hydrophobically bound lipid (Fig.6.1c). However as the level of bound lipid fell so would the rate of release (r_3) until the total rate of release (r_2 and r_3) was

equal to the rate of binding (r_1) and the ratio free/bound would be large (Table 5.1).

Future Work.

The reactions and interactions in which fats and flour lipids are involved during dough mixing are now becoming well documented. However the mechanisms by which fats, both natural and added, cause such beneficial results in bread are not so well understood. The use of radiotracers as demonstrated in this investigation could aid in remedying the situation. For example, the binding of phospholipids, which are a major component of wheat flour lipids, might be investigated in more detail. Similarly the other major polar lipid class, the glycolipids, which are known to be beneficial to bread quality and could be involved in hydrogen bonding might be studied by radioactive techniques.

The important oxidative improving reactions involving lipids offer considerable scope for research. As has been stated above the site of coupled oxidation of the proteins by the linoleate-lipoxygenase system is thought to be the thiol and disulphide groups although most of the evidence is circumstantial. Further research might be directed towards confirming the exact site of protein oxidation. In this context a study of the work-sparing improver L-cysteine might be rewarding. L-cysteine is known to react with the thiol groups of gluten and its effect on lipid binding might be investigated. Such studies might help in solving the technologically important question which is raised by the oxidative release of lipids during aerobic mixing. That is, does lipoxygenase improve bread by reducing the level of bound lipid or is this effect merely incidental to the oxidation of the gluten network?

All these possible investigations would be particularly relevant to commercial practice since soya flour, L-cysteine and added fats, such as glycolipids, are of wide interest as bread improvers and additives. A fuller understanding of the way in which such additives modify the gluten network will aid considerably in formulating future commercial bread improvers.

The results outlined in this thesis have thrown new light on the complex interactions during hydration of flour, gluten formation and dough development in two atmospheres. The use of radioactive tracers has demonstrated the dynamic nature of lipid distribution in dough, a discovery of importance in understanding fully the behaviour of fats during dough mixing. It is hoped that these ideas and speculations will aid future workers in their studies on the function of fats in breadmaking.

APPENDICES.

APPENDIX A.1 (see Fig. 3.2)

Effect of low levels of work on lipid binding at various moisture contents in flour-water doughs mixed under nitrogen.

Moisture %	Time min.	Mixing Speed r.p.m.	Work H.P. min./lb.	Free Lipid %	Bound Lipid %	Total Lipid %
24.5	0	0		1.05	0.55	1.60
	0.5	15	0.0013	1.05	0.55	1.60
	1.0	15	0.0024	1.05	0.51	1.56
	5.0	15	0.0120	1.04	0.60	1.64
	5.0	45	0.0655	0.94	0.60	1.54
27.3	0	0		1.02	0.53	1.55
	0.5	15	0.0012	1.08	0.51	1.59
	1.0	15	0.0023	1.03	0.54	1.57
	5.0	15	0.0120	1.01	0.56	1.57
	5.0	45	0.047	0.98	0.58	1.56
29.9	0	0		0.98	0.58	1.56
	0.5	15	0.0047	0.95	0.61	1.56
	1.0	15	0.0110	0.96	0.59	1.55
	5.0	15	0.1090	0.91	0.64	1.55
32.5	0	0		0.97	0.55	1.52
	0.25	15	0.0047	0.89	0.58	1.47
	0.5	15	0.0094	0.86	0.64	1.50
	1.0	15	0.020	0.77	0.69	1.46
35.4	0	0		0.95	0.61	1.56
	0.5	15	0.0218	0.78	0.77	1.55
	1.0	15	0.053	0.72	0.77	1.49
	5.0	15	0.258	0.70	0.78	1.48
37.0	0	0		0.82	0.67	1.49
	0.5	15	0.016	0.72	0.77	1.49
	1.0	15	0.03	0.66	0.85	1.51
	5.0	15	0.16	0.69	0.81	1.50
40.0	0	0		0.89	0.66	1.55
	0.5	15	0.01	0.78	0.78	1.56
	1.0	15	0.019	0.71	0.78	1.49
	5.0	15	0.10	0.71	0.81	1.52

APPENDIX A.2 (see Fig. 3.3)

Effect of low levels of work on lipid binding at moisture levels in the range 25-33%: flour-water doughs mixed under nitrogen.

Moisture %	Time min.	Mixing Speed r.p.m.	Work H.P. min./lb.	Free Lipid %	Bound Lipid %	Total Lipid %
25.6	0	0	0	1.02	0.56	1.58
	1	15	0.004	1.00	0.53	1.53
	5	15	0.023	0.99	0.57	1.56
	10	15	0.046	0.98	0.59	1.57
	15	15	0.056	0.99	0.58	1.57
27.4	2	15	0.006	1.03	0.57	1.60
	5	15	0.023	0.98	0.56	1.54
	10	15	0.030	0.98	0.59	1.57
	15	15	0.041	1.01	0.59	1.60
29.2	0	0	0	1.00	0.54	1.54
	0.5	15	0.003	1.00	0.60	1.60
	1	15	0.006	0.96	0.60	1.56
	2	15	0.012	0.94	0.63	1.57
	5	15	0.031	0.90	0.66	1.56
	10	15	0.062	0.87	0.72	1.59
30.0	0	0	0	0.99	0.58	1.57
	0.5	15	0.006	0.97	0.59	1.56
	1	15	0.012	0.92	0.67	1.59
	2	15	0.042	0.79	0.78	1.57
	5	15	0.151	0.69	0.87	1.56
31.2	0	0	0	1.01	0.56	1.57
	0.5	15	0.008	0.95	0.59	1.54
	1.0	15	0.012	0.94	0.63	1.57
	2	15	0.030	0.83	0.73	1.56
	5	15	0.151	0.72	0.82	1.54
31.6	0	0	0	0.99	0.57	1.56
	0.5	15	0.008	0.87	0.68	1.56
	1	15	0.018	0.85	0.64	1.49
	2	15	0.06	0.72	0.77	1.49
	5	15	0.21	0.70	0.84	1.54

APPENDIX A.3 (see Fig. 3.4)

The variation of lipid distribution with moisture content in unworked doughs.

Moisture Content %	Free Lipid %	Bound Lipid %
14.3	1.1	0.49
15.5	1.08	0.54
21.2	1.02	0.53
24.8	1.05	0.55
25.1	1.05	0.53
25.6	1.02	0.56
27.5	1.02	0.53
28.5	0.99	0.59
29.2	1.00	0.63
30.0	0.98	0.58
30.0	0.99	0.58
31.2	1.01	0.56
32.0	0.99	0.57
32.5	0.97	0.55
33.0	0.90	0.64
35.0	0.95	0.61
36.2	0.95	0.59
37.0	0.82	0.67
39.0	0.88	0.67
40.0	0.89	0.66
40.0	0.85	0.72
41.2	0.89	0.68
50.2	0.83	0.75
56.0	0.80	0.75

APPENDIX A.4 (see Fig. 3.5)

Effect of moisture content on resistance to mixing of flour-water doughs after ½ minute mixing time at 15 r.p.m. in the Brabender Do-corder.

Moisture Content %	Torque m kg
24.7	0.125
25.6	0.10, 0.14
27.3	0.075
27.4	0.10
28.2	0.16
29.2	0.15, 0.20
29.5	0.25
30.0	0.30, 0.40, 0.27
31.2	0.50
32.0	0.50
32.4	0.60
33.8	1.0
34.6	1.75
35.4	1.40
35.7	1.23
37.7	0.98
39.7	0.62
40.3	0.63

APPENDIX A.5 (see Fig. 4.1)

Influence of mixing speed on lipid binding in air.

Mixing Conditions						
Speed r.p.m.	Time min.	Work H.P. min./lb.	Moisture Content %	Free Lipid %	Bound Lipid %	Total Lipid %
0	0		44.0	1.03	0.63	1.66
15	1	0.010	42.7	0.74	0.90	1.64
	2	0.021		0.74	0.88	1.66
	5	0.056		0.78	0.81	1.59
	10	0.115		0.82	0.77	1.59
	20	0.187		0.83	0.73	1.56
	40	0.362		0.81	0.73	1.54
30	0.25	0.004	44.0	0.80	0.79	1.59
	0.50	0.010		0.78	0.84	1.62
	1	0.020		0.74	0.89	1.63
	2	0.052		0.76	0.84	1.60
	5	0.144		0.82	0.83	1.65
	10	0.312		0.80	0.76	1.56
50	0.25	0.010	40.5	0.75	0.85	1.60
	0.50	0.021		0.74	0.90	1.64
	0.75	0.032		0.69	0.94	1.63
	1	0.042		0.70	-	-
	2	0.125		0.71	0.89	1.60
	5	0.218		0.75	0.84	1.59
100	0.166	0.014	45.7	0.73	0.82	1.55
	0.25	0.021		0.69	0.88	1.57
	0.33	0.029		0.64	0.91	1.55
	0.66	0.058		0.62	0.98	1.60
	1.33	0.145		0.60	0.97	1.57
	2.5	0.260		0.63	0.94	1.57

APPENDIX A.6 (see Fig. 4.3)

Influence of mixing speed on lipid binding in oxygen and nitrogen.

Mixing Conditions		Nitrogen			Oxygen		
Speed r.p.m.	Time min.	Work H.P. min./lb.	Free Lipid %	Moisture %	Work H.P. min./lb.	Free Lipid %	Moisture %
15	1	0.005	0.76	45.9	0.007	0.83	45.5
	2	0.009	0.71	45.3	0.013	0.76	45.9
	5	0.023	0.65	46.7	0.036	0.80	45.5
	10	0.194	0.67	39.3	0.081	0.81	45.0
	15	0.131	0.67	42.8	0.117	0.78	44.5
	20	-	-	-	0.312	0.78	41.2
30	0.25	0.004	0.82	45.2	0.005	0.80	44.8
	0.50	0.008	0.79	44.0	0.011	0.79	44.7
	1	0.016	0.72	44.9	0.020	0.78	44.9
	2	0.031	0.68	45.0	0.045	0.81	44.5
	5	0.090	0.66	44.7	0.119	0.81	42.2
	10	0.200	0.72	45.0	-	-	-
50	0.25	0.006	0.76	44.0	0.012	0.76	44.2
	0.50	0.013	0.71	46.2	0.019	0.78	44.0
	0.75	0.023	0.68	44.5	0.030	0.76	44.0
	1	0.034	0.57	-	0.043	0.81	44.1
	2	-	-	-	0.086	0.82	44.1
	5	0.210	0.65	-	0.260	0.77	42.2
100	0.166	0.008	0.73	44.3	0.014	0.78	44.0
	0.25	0.016	0.69	44.8	0.020	0.75	45.1
	0.33	0.021	0.68	44.0	0.026	0.75	45.2
	0.66	0.049	0.66	45.0	0.054	0.72	45.5
	1.33	0.117	0.56	44.5	0.105	0.74	44.1
	2.50	0.272	0.52	42.3	-	-	-

APPENDIX A.7 (see Fig. 5.1)

The binding of ^{3}H-triolein, ^{14}C-palmitic acid and flour lipids in doughs containing soya flour and mixed at 0.2 H.P.min./lb./min. in air. Radiotracers added to the dry ingredients.

Work Level H.P.min./lb.	Free Lipid			Bound Lipid		
	Lipid %	^{14}C D.P.M.	^{3}H D.P.M.	Lipid %	^{14}C D.P.M.	^{3}H D.P.M.
0.1	0.495	1230	45600	1.140	2640	19500
0.2	0.430	1050	38900	1.180	2720	23400
0.4	0.385	910	37400	-	-	-
0.6	0.370	910	36300	1.210	4430	37400
1.0	0.430	960	44600	1.055	2930	27300
2.0	0.460	890	51000	0.970	2870	25000
3.0	0.510	750	50000	0.965	2900	25000

APPENDIX A.8 (see Fig. 5.2)

The binding of ^{3}H-triolein, ^{14}C-palmitic acid and flour lipids in doughs containing soya flour and mixed at 0.2 H.P.min./lb./min. in nitrogen. Radiotracers added to the dry ingredients.

Work Level H.P.min./lb.	Free Lipid			Bound Lipid		
	Lipid %	^{14}C D.P.M.	^{3}H D.P.M.	Lipid %	^{14}C D.P.M.	^{3}H D.P.M.
0.1	0.420	1120	36160	1.14	3820	32200
0.2	0.360	960	32760	1.20	4120	36450
0.4	0.280	780	28400	1.24	4230	37250
0.6	0.290	740	25860	1.32	3870	43900
0.8	0.265	670	24130	1.27	-	37500
1.0	0.225	550	20880	1.35	3800	43550
2.0	0.230	480	22260	1.36	3510	-
3.0	0.245	520	21510	1.31	3500	43300

APPENDIX A.9 (see Fig. 5.3)

The distribution of ^{3}H-triolein and ^{14}C-palmitic acid in doughs containing soya flour and mixed at 0.2 H.P.min./lb./min. in nitrogen. Radiotracers added after one minute mixing.

Work Level H.P.min./lb.	Free Lipid			Bound Lipid		
	Lipid %	^{14}C D.P.M.	^{3}H D.P.M.	Lipid %	^{14}C D.P.M.	^{3}H D.P.M.
0.4	0.300	760	26400	1.38	4650	43400
0.6	0.305	759	23200	1.31	4330	37700
1.0	0.260	614	23200	1.39	4410	41000
2.0	0.240	502	20700	1.42	3670	41600

APPENDIX A.10. (see Fig. 5.4)

The distribution of ^{3}H-triolein and ^{14}C-palmitic acid in doughs containing soya flour and mixed at 0.2 H.P.min./lb./min. in air. Radiotracers added after half a minute

Work Level H.P.min./lb.	Free Lipid			Bound Lipid		
	Lipid %	^{14}C D.P.M.	^{3}H D.P.M.	Lipid %	^{14}C D.P.M.	^{3}H D.P.M.
0.2	0.370	1110	37600	1.155	3590	34500
0.3	0.330	950	32600	1.160	3610	35700
0.5	0.335	920	32200	1.215	3630	39500
0.7	0.375	980	35200	1.175	4020	39600
0.9	0.360	820	33200	1.210	3560	38400
1.1	0.420	910	37800	1.110	3200	32000
2.1	0.505	950	48500	0.975	2920	28400

APPENDIX A.11 (see Fig. 5.5)

The distribution of ^{3}H-triolein and ^{14}C-palmitic acid in doughs containing soya flour and mixed at 0.2 H.P.min./lb./min. in air. Radiotracers added after 2 minutes mixing.

Work Level H.P.min./lb.	Free Lipid			Bound Lipid		
	Lipid %	^{14}C D.P.M.	^{3}H D.P.M.	Lipid %	^{14}C D.P.M.	^{3}H D.P.M.
0.6	0.360	1070	35000	1.650	3460	37600
1.0	0.400	1050	40700	1.350	3750	34200
1.4	0.430	960	44200	1.010	3230	31100
2.0	0.425	760	41900	0.985	3910	31300

APPENDIX A.12 (see Fig. 5.6)

The distribution of ^{3}H-triolein and ^{14}C-palmitic acid in doughs containing soya flour and mixed at 0.2 H.P.min./lb./min. in air. Radiotracers added after 5 minutes mixing.

Work Level H.P.min./lb.	Free Lipid			Bound Lipid		
	Lipid %	^{14}C D.P.M.	^{3}H D.P.M.	Lipid %	^{14}C D.P.M.	^{3}H D.P.M.
1.1	0.355	880	29300	1.175	2740	31300
1.2	0.400	990	32000	1.160	2840	29400
1.4	0.410	830	25200	1.150	3140	29000
2.0	0.450	910	32900	1.125	3320	2580

APPENDIX A.13. (see Fig. 5.7)

The distribution of ^{3}H-triolein and ^{14}C-palmitic acid in doughs containing soya flour and mixed at 0.2 H.P.min./lb./min. in nitrogen. Radiotracers added to the dry ingredients as "bound lipid" in fat-extracted freeze-dried dough powder.

Work Level H.P.min./lb.	Free Lipid			Bound Lipid		
	Lipid %	^{14}C D.P.M.	^{3}H D.P.M.	Lipid %	^{14}C D.P.M.	^{3}H D.P.M.
0	-	5160	17800	-	-	-
0.1	0.355	900	25400	1.300	4170	90500
0.2	0.305	900	28000	1.355	5050	91400
0.4	0.245	670	27200	1.400	4770	89400
0.6	0.255	650	27300	1.390	4280	90900
1.0	0.250	660	28900	1.345	4210	86400
2.0	0.220	620	27000	1.415	4840	90100

APPENDIX A.14 (see Fig. 5.8)

The binding of ^{3}H-triolein and ^{14}C-tripalmitin and flour lipids in doughs containing soya flour and mixed at 0.2 H.P.min./lb./min. in air. Radiotracers added to the dry ingredients.

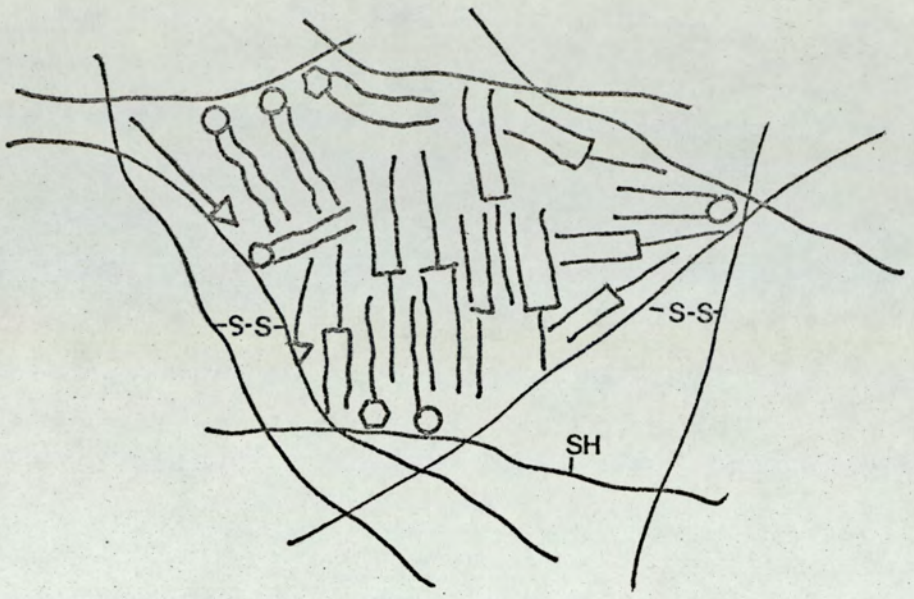
Work Level H.P.min./lb.	Free Lipid			Bound Lipid		
	Lipid %	^{14}C D.P.M.	^{3}H D.P.M.	Lipid %	^{14}C D.P.M.	^{3}H D.P.M.
0.1	0.495	8040	3210	1.255	6090	3300
0.2	0.430	6590	2480	1.315	6900	5570
0.4	0.415	5100	1860	1.390	7910	6140
0.8	0.450	5060	1960	1.370	8030	6250
1.0	-	5170	1930	1.405	7530	6470
2.0	0.440	6500	3070	1.170	6230	5800
3.0	0.405	6230	2820	1.190	6510	6160

APPENDIX A.15 (see Fig. 5.9)

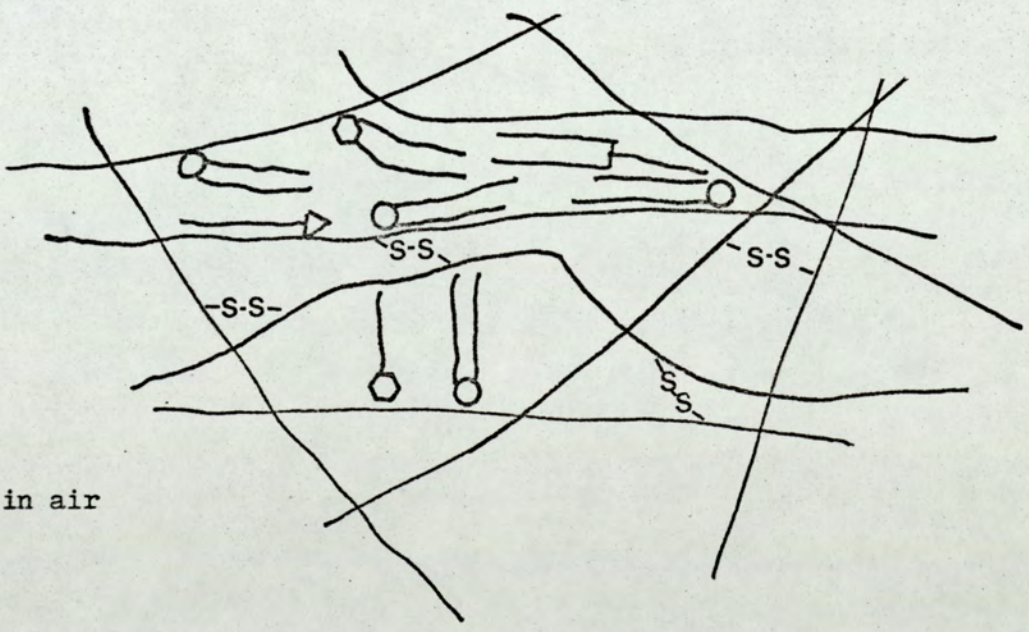
The binding of ^{3}H-triolein, ^{14}C-tripalmitin and flour lipid in doughs containing soya flour and mixed at 0.2 H.P.min./lb./min. in nitrogen. Radiotracers added to the dry ingredients.

Work Level H.P.min./lb.	Free Lipid			Bound Lipid		
	Lipid %	^{14}C D.P.M.	^{3}H D.P.M.	Lipid %	^{14}C D.P.M.	^{3}H D.P.M.
0.1	0.405	7300	2370	1.285	5070	5220
0.2	0.345	5790	1870	1.365	6740	5950
0.4	0.285	5450	1540	1.385	7600	6030
0.8	0.230	4490	1290	1.430	8730	6330
1.0	0.260	4440	1290	1.360	7810	6350
2.0	0.235	3700	840	1.450	9150	6320
3.0	0.220	3680	970	1.460	8850	6810

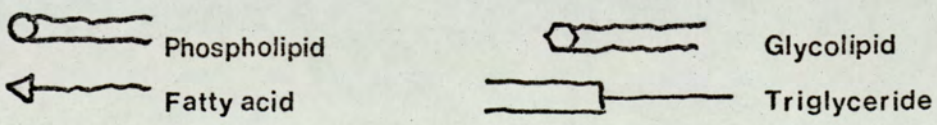
A schematic illustration in molecular terms of the proposed model for lipid bindings. Lipid molecules are bound within hydrophobic areas of the gluten network. Polar lipids are bound to polar sites on the protein network (see Appendix A.17) thus acting as an interface between the hydrophilic areas of the protein and the hydrophobic binding sites. Oxidation of sulphydryl groups causes distortion of the binding sites resulting in release of most of the neutral lipids.



a) in nitrogen

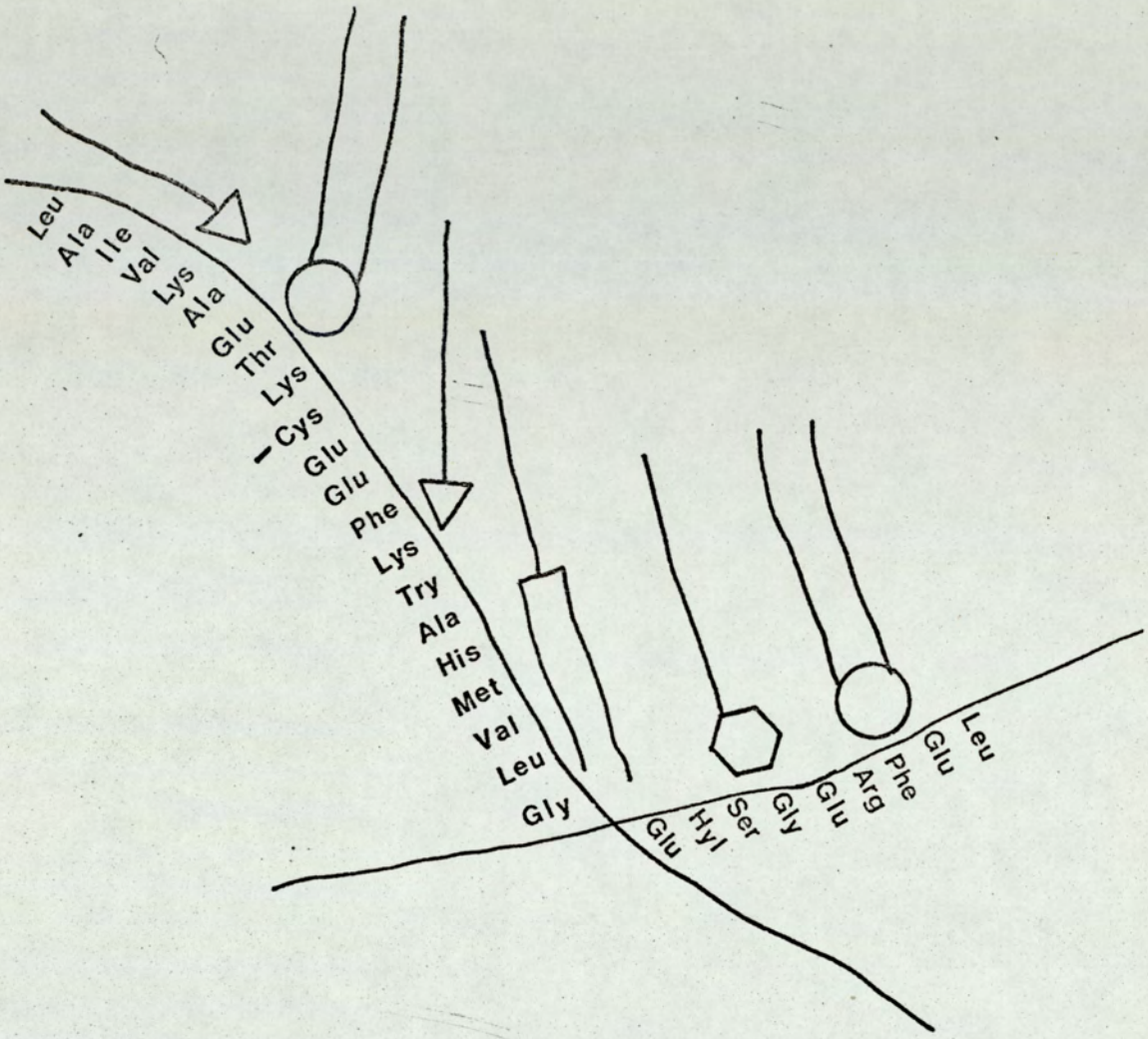


b) in air

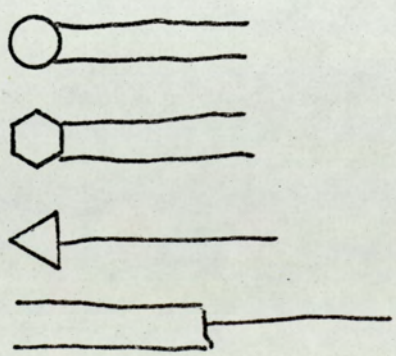


APPENDIX A.17

A schematic example of the interface between protein chains and the hydrophobic binding site. Polar molecules are bound to appropriate amino acid residues whilst the hydrocarbon chains of the lipids are bound to hydrophobic sequences of amino acid residues. The detail of the intermolecular interactions is illustrated for specific examples in Appendix A.18



Symbols



Phospholipids

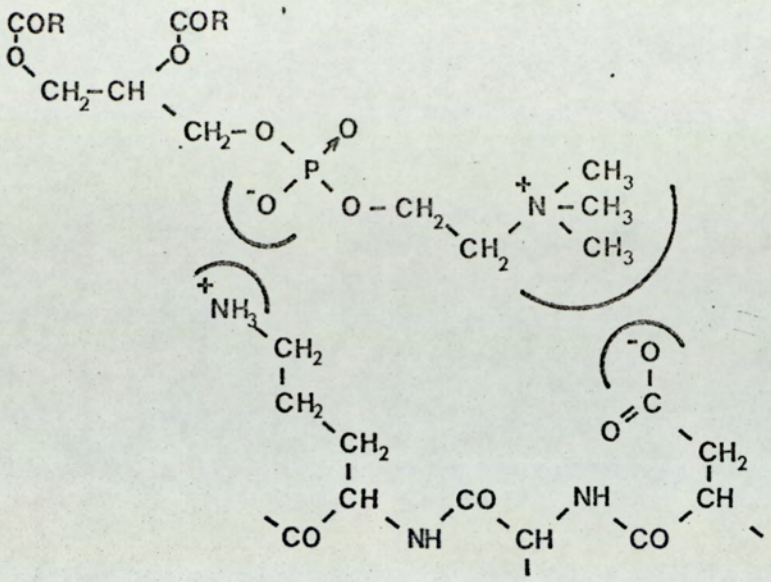
Glycolipid

Free fatty acid

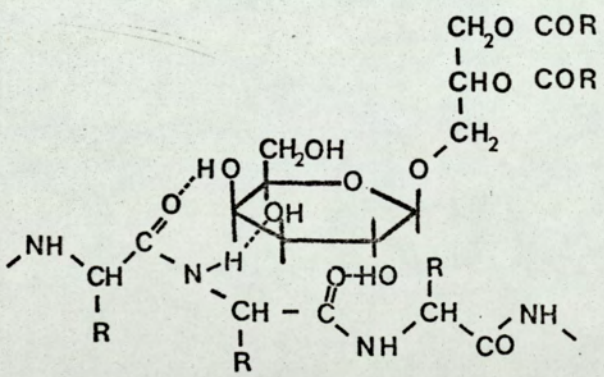
Triglyceride

APPENDIX A.18

Examples of the binding forces which are probably involved in the proposed model for lipid binding.

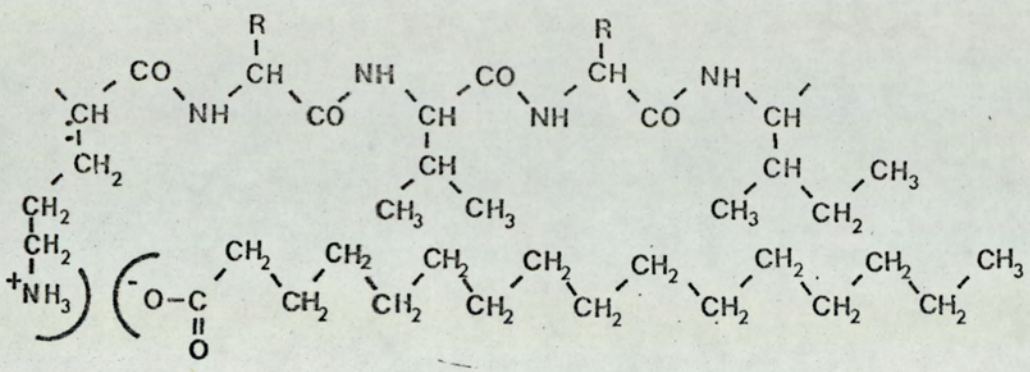


1) Electrostatic binding of phosphatidyl choline to lysine and aspartate residues on a protein chain.



2) Hydrogen bonding of galactosyl diglyceride to a protein chain.

Examples of the binding forces which are probably involved in the proposed model for lipid binding.



3) Electrostatic and hydrophobic bonding of palmitic acid to a protein chain.

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The effect of water on lipid binding in doughs mixed to low work levels

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Summary

Wheat flour doughs of various moisture contents were mixed to low work levels. It was shown that there was a critical moisture content below which mechanical work does not induce lipid binding. The onset of gluten development occurs at a similar moisture content. The quantity of bound water in doughs was determined by differential scanning calorimetry. It was shown that work caused no significant change in the amount of bound water in the doughs.

Introduction

Many early workers observed that the lipids and protein of flour form a complex when the flour is made into a dough. The importance of water in the formation of this complex was first noted by Olcott & Mecham (1948). They showed that as the water content of a high protein flour (15·3% protein) was increased the amount of lipid extractable with a non-polar solvent decreased. The introduction of mechanical work reduced even further the level of 'free' lipid in the dough. This was subsequently confirmed by other workers and Davies, Daniels & Greenshields (1969) showed that the largest decrease in free lipid occurred as the moisture content was increased from 20 to 35% (wet weight basis) in work-free systems. Daniels *et al.* (1967) demonstrated that lipid binding in commercial doughs was also dependent upon work rate and total work input, the level of free lipid falling rapidly during the initial stages of mixing in nitrogen. In air, bound lipid was released during mixing, an effect subsequently claimed to be coupled to enzymic lipid oxidation (Daniels *et al.*, 1970).

However, these effects were observed at work levels above 0·2 HP min/lb whilst at 0·1 HP min/lb there appeared to be little difference in lipid binding in either atmosphere. In both air and nitrogen approximately 1·1% lipid (on a flour dry weight basis) was bound. The commercial dough formula employed contained 2·5% total

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lipid. In the unworked system studied by Davies & Webb (1969) approximately 0·7% of lipid (on a flour dry weight basis) was bound when the flour was wetted to 45% moisture calculated on a wet weight. The total lipid content of the flour used by Davies & Webb (1969) was only 1·65% but they also showed that additional shortening (as used in Daniels' system) was not bound during work-free wetting. These results would suggest, therefore, that, after the initial increase in bound lipid due to work free wetting, there is a very rapid increase in bound lipid during the initial stages of mixing.

In this study factors responsible for this rapid initial increase have been investigated.

Materials and methods

An untreated, unbleached commercial bread flour containing 11·7% protein (Nitrogen × 5·7) and 14·2% moisture was used in these experiments.

The flour was wetted to the required moisture content without the introduction of mechanical work by the method of Davies *et al.* (1969). In this method the flour is slurried in liquid nitrogen and the required amount of water added as ice powder, prepared by previously grinding water under liquid nitrogen in a steel mortar and pestle.

Doughs were mixed in a 500-g Farinograph bowl, clad in stainless steel and attached to a modified *Brabender* Do-corder. The frozen flour/ice mixture (300 g) was allowed to thaw for 1½ hr in the Farinograph bowl which was held at 30 ± 1°C. Although it was unlikely that air would have any effect in lipid binding at the low work levels studied, lipid oxidation was minimized by flushing the bowl with oxygen-free nitrogen when the powder was introduced and again during the mixing. Since it was impracticable at these low work levels to measure the rate of work input accurately, all doughs were mixed at a constant speed of 15 rpm. The time of mixing was adjusted to give the required work level as calculated from the torque curve. Unworked doughs were allowed to thaw on the bench for 2 hr.

All the doughs were frozen, freeze-dried and ground to pass a 5XX silk screen. The proportions of 'free' and 'bound' lipid were determined by the procedures adopted by Daniels *et al.* (1969), and are presented on a flour or dough dry weight basis. Free lipid was defined as that removed by a 7 hr Soxhlet extraction with 40–60°C petroleum-ether. Bound lipid was then subsequently removed by the solvent system of Tsen, Levi & Hlynka (1962).

Moisture contents were determined by the vacuum oven method of the American Association of Cereal Chemists. (A.A.C.C. Approved Methods, 1969), and are presented on a wet weight basis.

Bound water was determined by the method of Davies & Webb (1969) on doughs of 30% moisture content, which had been mixed to various levels of work input. A *Dupont* 900 Differential Thermal Analyser fitted with a Differential Scanning Calori-

metry (D.S.C.) cell was used. The samples (15 mg) were accurately weighed (± 0·01 mg) into aluminium pans using a torsion balance. An empty pan was used as the reference in the D.S.C. cell. Distilled water was used to calibrate the instrument. In order to obtain as homogeneous a sample of the dough as possible, the dough was first frozen in liquid nitrogen before being ground to a powder in a steel mortar and pestle which was cooled below 0°C.

Results and discussion

Figs. 1a and b show the influence of mechanical work on lipid distribution at various moisture contents. The curves for 25·6 and 27·4% moisture and for 30·0, 31·2, and 31·6% moisture coincided and for the sake of clarity have been superimposed.

It will be seen that above 30% moisture there was an increase in the level of bound lipid with the introduction of mechanical work. This initial increase levelled off after 0·1 HP min/lb and further work produced very little increase in bound lipid at the work levels examined. Below 27·4% moisture mechanical work had only a small

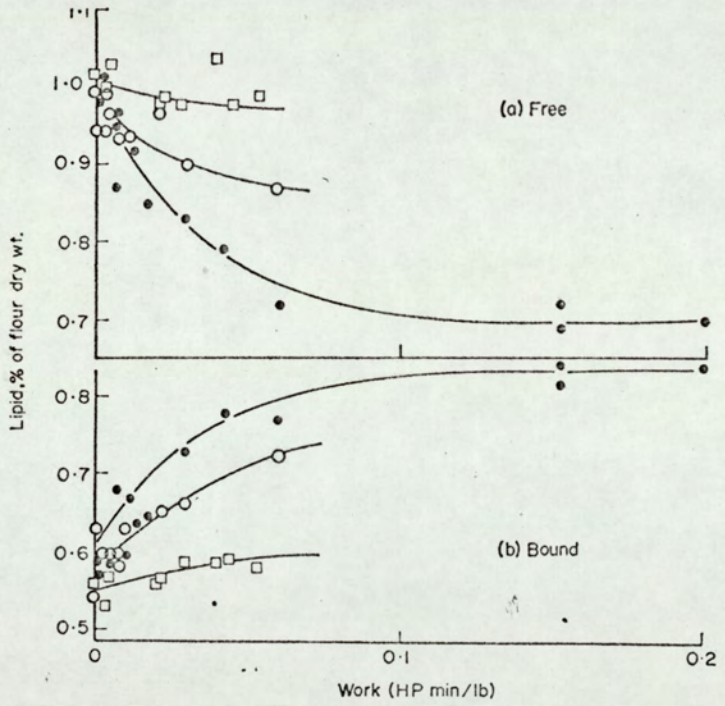

FIG. 1. Variation of lipid distribution with mechanical mixing at various moisture contents. □, 25·6% and 27·4% moisture; ○, 29·2% moisture; ●, 30·0% ,31·2% and 31·6% moisture.

effect on lipid binding causing a slight steady fall in the level of free lipid. At 29·2% moisture, mechanical work produced a slight increase in lipid binding but not so pronounced as at 30% moisture. It would appear, therefore, that the water requirement for the onset of work-induced lipid binding is about 29% and may be critical to within ± 1%.

One of the main objects of this investigation was to discover the cause of the rapid increase in lipid binding at the onset of dough mixing. If this was solely due to a more efficient distribution of water brought about by mechanical mixing then it could explain the equilibrium in lipid binding in unworked, 40% moisture doughs noted by Davies *et al.* (1969). That is, the maximum possible diffusion of the water in the absence of work may well have been reached resulting in no further binding of lipids. The results in Fig. 2 are in sensible agreement with the findings of Davies *et al.* (1969), and also Olcott & Mecham (1948), and show that there is a steady increase in the proportion of bound lipid as the moisture content is increased from 20 to 50%, levelling off thereafter. If water dispersion was the main factor influencing lipid binding then one might expect that, above 30%, an increase in water content would cause the increase in lipid binding to occur at a lower work level. The coincidence of

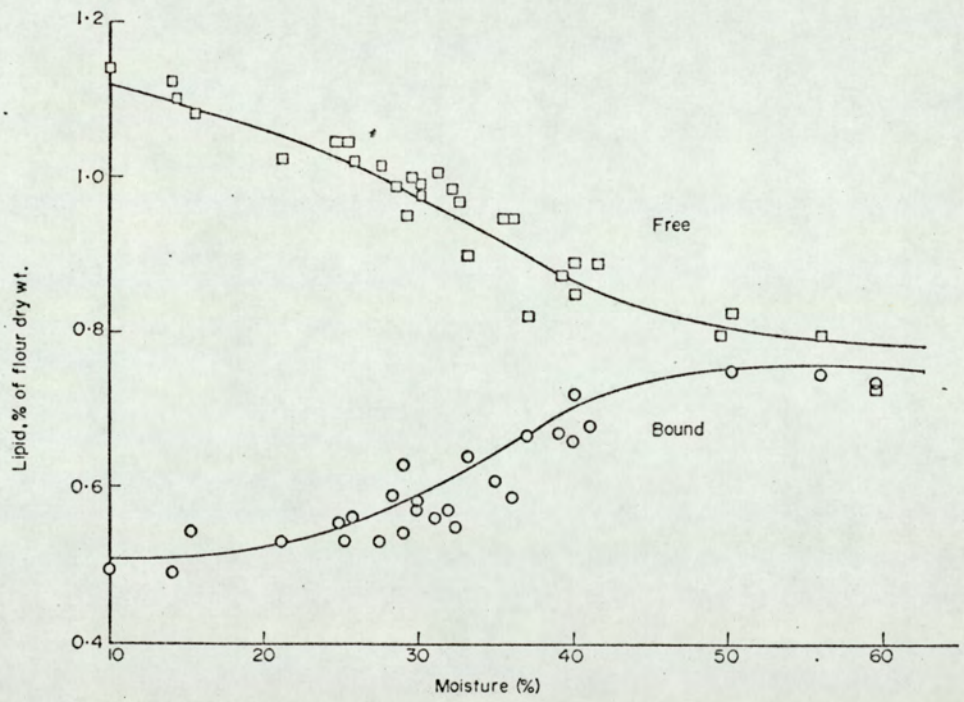

FIG. 2. Variation of lipid distribution with moisture content in unworked doughs. □, free lipid; O, bound lipid.

the lipid binding curves for doughs containing more than 30% moisture (Fig. 1) does not support this proposition, and some mechanism other than water dispersal must be involved in promoting lipid binding.

As a result of rheological studies on worked doughs, Webb *et al.* (1970) have suggested that work produces an increase in the amount of free water in the dough system. If this increase occurred during the initial stages of mixing it might be related to the phenomenon of lipid binding.

In an attempt to ascertain if this were the case, a series of doughs containing 29·8% water were mixed to various work levels. The free water in the doughs was determined by a D.S.C. method, and the results are presented in Table 1. It can be seen that there was no significant difference between any of the doughs and it would appear that at these low work levels mixing does not alter the proportions of free and bound water. The discrepancy between this result and the findings of Webb *et al.* (1970) may be due to the much higher work levels they investigated.

On the basis that there was no significant difference between the doughs, statistical evaluation of the results as a whole gives a free water content of 5·3 ± 0·08%. This corresponds to a bound water content of 24·5% which agrees with the 24·8% found by Davies & Webb (1969) in worked doughs. Other workers have found 22·5% bound water in wet flour using different methods (Toledo, Steinberg & Nelson, 1968; Vail & Bailey, 1940; Lee, 1970.) As noted by these workers all water added to the flour enters the bound state until the water requirement is satisfied. Thereafter further additions of water remain in the free state.

The results presented here show that the proportion of bound water in a dough containing 30% moisture was not altered by mixing. Since the lipid distribution in a similar (Fig. 1) dough was altered by mixing there would appear to be no direct correlation between the two effects, although, as demonstrated in Figs. 1a and b, at least 5% of free water is required for work-induced lipid binding to occur.

TABLE 1. Effect of work on freezable water content of doughs containing 29·8% total water

Time of mixing at 15 rpm (Min)	% Freezable water	Least significant difference at $P = 0{\cdot}05$			
		Min 1	2	5	10
0	5·56	0·554	0·598	0·609	0·570
1	5·23	—	0·542	0·562	0·518
2	5·64	—	—	0·686	0·653
5	5·38	—	—	—	0·669
10	5·58	—	—	—	—

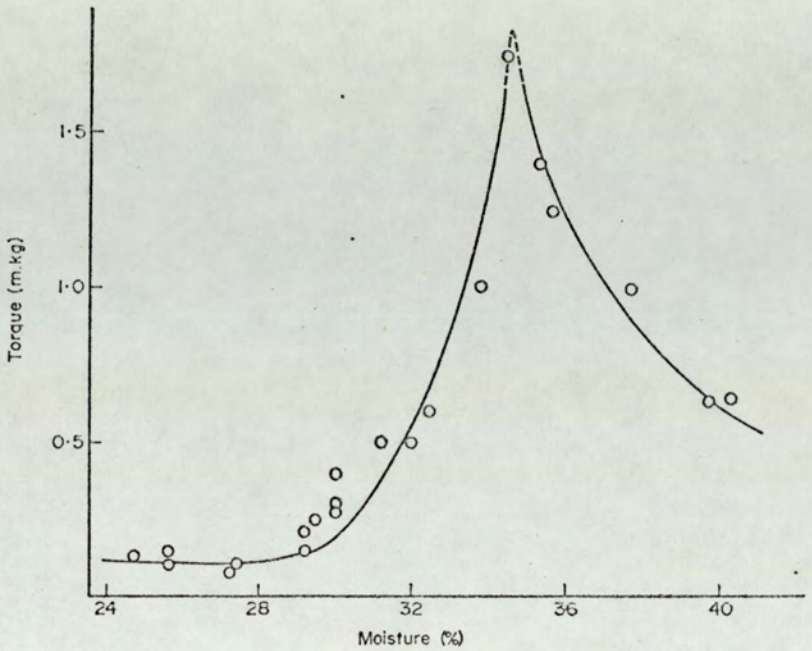

FIG. 3. Effect of moisture content on resistance to mixing of doughs after 0·5 min mixing time at 15 rpm in the *Brabender* Do-corder.

It was observed throughout these experiments that the flour/water mixtures began to show evidence of gluten formation at about 30% moisture. Below this moisture content the doughs were never more than lumpy powders whilst above 30% moisture a solid dough mass was formed. Davies & Webb (1969, Fig. 2) have illustrated this effect in a photograph showing cylinders of wetted flour which began to shrink above 28–30% moisture content. The coincidence of this moisture content with the onset of gluten development is further demonstrated in Fig. 3 in which the resistance to mixing, after 0·5 min mixing at 15 rpm, is plotted against moisture content. The Do-corder torque measurements were consistently low up to 28% moisture. Thereafter they increased very rapidly to a maximum at 35% moisture, indicating the increasing stiffness of the doughs as the proteins chains began to interact. The decrease in torque above 35% moisture can probably be attributed to the lubricating action of the water (Webb *et al.*, 1970). Thus it can be seen that the onset of gluten formation as measured by both dough cylinder shrinkage and an increase in Do-corder torque coincides with the critical moisture content for work-induced lipid binding. It is concluded from this evidence that it is gluten formation and development which is the prime requirement for lipid binding to occur rather than the presence of free water. During gluten hydration it would appear that conformational changes in the wheat proteins, under

the influence of water, cause or require the binding of flour lipids to the gluten matrix. Mixing the dough accelerates the rate of gluten development and it is this which gives rise to the observed increase in lipid binding.

The binding of lipids in non-worked systems, and particularly the rapid increase in binding in the moisture range 25–40% may well occur entirely as a result of the spontaneous re-arrangement of the wheat proteins caused by water during the work-free development of a gluten structure.

It is clear from the findings in Figs. 1a and b that the development of a critical gluten structure requires at least 5% of free water in the dough. Only when this level of free water is available is the protein capable of mechanical development leading to further incorporation of lipids into the structure.

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