THE FREE FATTY ACIDS OF WHEAT FLOUR

With reference to their contribution to chemical changes which occur during the mixing od doughs.

by

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Summary

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SUMMARY

It is known that, during the storage of wheat flour, lipase activity leads to an increase in the amounts of free fatty acids present in the flour. It has been shown by indirect means that the free fatty acids are associated with the development and character of the rheological properties of flour-water doughs. The strengthening (improvement) of doughs is an oxidative process and can occur naturally or due to the addition of oxidising agents (flour improvers). Improvement is generally thought to involve oxidation of sulphydryl groups of the structural proteins of dough (gluten) to intermolecular disulphide groups. Improvement during the mixing of flour-water doughs is accompanied by the absorption of atmospheric oxygen. Free fatty acids, and in particular linoleic and linolenic acids, are involved in the absorption of oxygen, which is largely, if not entirely, an enzymic process. It has been suggested that the mechanism of these processes is lipoxidase-catalysed oxidation of free linoleic and linolenic acids of the flour, with coupled oxidation of gluten sulphydryl groups.

The thesis describes a direct study of the fatty acids of flour and flour-water mixtures.

Methods were developed for this study, and, after elimination of all phospholipids and galactolipids, the lipids were separated into free and esterified fatty acids, and their compositions determined by gas chromatography.

A reappraisal of data published by other authors showed that there was reason to believe that lipoxidase activity was not the complete explanation of the absorption of oxygen by flour. Examination of three flours confirmed this, since it was found that there were not enough linoleic and linolenic acids present to account for the known oxygen absorptions.

Analysis of the free and esterified fatty acids recovered from flour-water mixtures showed that losses had occurred only in the free fatty acids, and these were due to enzymic oxidations. The losses were shown to be due to oxidation of linoleic and linolenic acids by lipoxidase, or a similar enzyme, and to general oxidation of all free fatty acids by enzymes of the type involved in β-oxidation. The latter is a novel finding in flour-water mixtures.

The theoretical oxygen absorption of the flour was calculated on the basis of these two types of fatty acid oxidation, and was found to agree with published values. Lipoxidase activity was calculated to account for 1 volume in 17.6 volumes of the oxygen absorbed, and this was shown to agree with the known levels of lipoxidase-catalysed coupled oxidations in flour.

Experiments in which oxidising, reducing and antioxidant conditions were present during the mixing process were described, and the results discussed in relation to the two types of fatty acid oxidation.

The thesis has a supplementary section containing a description of rheological dough testing experiments, and reprints

of three published papers relevant to the thesis.

ON THE FREE FATTY ACIDS OF WHEAT FLOUR

With Reference to Their Contribution to Chemical Changes Which Occur During the Mixing of Doughs

by

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A thesis submitted in accordance with the requirements of the Faculty of Science of the University of Glasgow for the Degree of Doctor of Philosophy.

May, 1962

BAKING

Wheat-flour contains in one hundred parts, starch 68, gluten 24, gummy sugar 5, and vegetable albumen 1.5. Gluten is a substance chiefly of vegetable nature, ... it contains azote; in putrefying it exhales an odour of putrescent animal matter: a fatty matter has even been discovered in it after it has undergone putrefaction.

When gluten is worked as described, not in water, but in a large quantity of alcohol ..... a part dissolves .... called gliadin: .... the residue is called zimomin .... its most remarkable property is that of forming a blue colour when mixed with powdered guaiacum and as much water as will form them into a paste. The contact of air is necessary to the change of colour.

The journals of Holland have for some time announced that sulphate of copper, or blue vitriol, was employed in that country to assist in the fermentation of bread (Brande's Journal, 1829).

New flour never makes good bread; it should lie over for three months before it will be fit for the baker. On the other hand, too much age damages flour. The summer of 1816 was exceedingly wet; the quality of the grain was far below average; and it was almost universally malted. It is chiefly the glutinous part which is altered in corn which has been exposed to humidity. The gluten almost entirely loses its adhesive powers, and dissolves.

from the Cabinet Cyclopaedia, Useful Arts,
Domestic Economy, Vol. I, Chapter X.
by M. Donovan.

Longman, Rees, Orme, Brown, and Green; and John Taylor,
London, 1830.

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C O N T E N T S

PREFACE

PREFACE

Wheat, and the products derived from it by grinding and milling processes, have long been of dietary significance to man. Much of this significance is due to the fact that when wheat flour is mixed with water a cohesive elastic dough is formed, and as a result wheat flour can be used to make an enormous range of products, most of which are part of the bakers' craft. In this respect wheat differs from all other cereals.

These unique properties can be measured by rheological means, but the underlying physico-chemical systems which produce them are still incompletely understood. Moreover, as would be expected from any system derived from a biological source, large variations occur in the balance of these properties among wheat varieties, and even in the same variety grown under different conditions. It has not yet proved possible to detect any significant chemical difference which might account for these differences, and this problem has been a long-standing challenge to cereal chemists. There is also the additional incentive that a better understanding of the factors involved would almost certainly lead to a greater degree of control over a rather variable raw material. The economical and technological significance of this can hardly be exaggerated when it is remembered that wheats of good breadmaking quality can only be grown in a few countries, and are consequently relatively expensive in countries such as Great Britain.

It has long been known that the properties of a dough are largely those of the protein constituents, known collectively as gluten. Gluten can be recovered from a dough by washing away the non-cohesive starch, cellulosic material and soluble materials. The resulting mass is found to be hydrated protein to which most of the flour lipids are fairly firmly bound, and it has been described as a mixture of gliadin and lipoglutenin. The forces binding the protein molecules together are believed to act through disulphide, thiolester, sulphydryl and indole groups, with hydrogen bonding and salt linkages also playing a part. Without doubt the most important linkage so far discovered is the intermolecular disulphide group, and much attention has been given to its chemistry during the past decade.

Dough quality depends on the balance of plastic and elastic properties of the dough mass, and may be improved by the absorption of atmospheric oxygen during mixing, or by very small amounts of oxidising agents known commercially as "flour improvers". A characteristic feature of flour improver action is the large change in rheological properties brought about by a few p.p.m. of improver. Flour lipids appear to be indirectly involved in the action of at least one improver (potassium bromate), and the free linoleic and linolenic acids of flour are of importance in the enzymic absorption of atmospheric oxygen. Much research effort is being expended on the phospholipids and galactolipids of flour, but the dough chemistry

of even the simpler lipids is not yet fully understood.

In this thesis the literature on the free fatty acids and other simple lipids has been critically reviewed, together with the literature on the uptake of oxygen by doughs during mixing. It has been concluded that the published facts do not form a satisfactory chain of evidence, and that the free fatty acids are probably involved in some, as yet, unknown chemical reactions. The experimental part of this thesis is a preliminary investigation of the chemistry of the free fatty acids of flour during mixing, and includes parallel observations on the other simple flour lipids which have been grouped together under the term "esterified fatty acids". Some additional experimental work which was necessary in the development of the main work has also been included.

INTRODUCTION

INTRODUCTION

This thesis has been divided into five parts, each of which is a stage in the development of the whole work. Part I is a review of the relevant literature with a critical re-appraisal of some of the published results. Part II describes the materials and methods used in the main work. Part III contains the main experimental results, and a discussion of the results. Part IV describes the experimental work required to develop the methods given in Part II to a sufficiently reliable level for routine analytical work. Part V describes some past and present work which is supplementary to the main work of the thesis.

Throughout this thesis it is necessary to refer frequently to several words and terms which can be conveniently abbreviated, and these are listed below.

SH	=	the sulphydryl or thiol group
SS	=	the disulphide group
FFA	=	free or unesterified long chain fatty acids
EFA	=	free essential fatty acids (linoleic and linolenic)
Estd.FA	=	esterified fatty acids (as glyceryl or sterol esters, all galactolipids and phospholipids are absent)
GLC	=	gas-liquid chromatography (gas chromatography)
16:0	=	palmitic acid
17:0	=	margaric acid
18:0	=	stearic acid
18:1	=	cis 9 oleic acid

18:2 = all cis 9, 12 linoleic acid

18:3 = all cis 9, 12, 15 linolenic acid

20:0 = arachidic acid

20:1 = cis 9 gadoleic acid

20:4 = all cis 5, 8, 11, 14 arachidonic acid

PART I

LITERATURE REVIEW

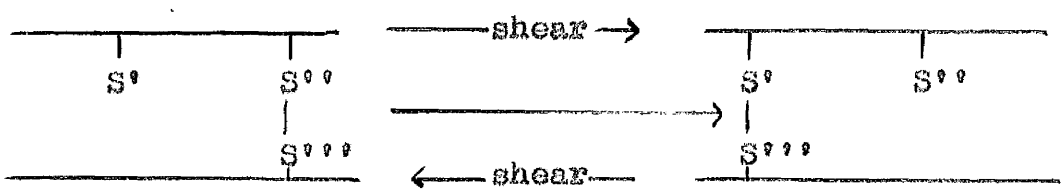
LITERATURE REVIEW

(a) General

The unique property of forming a dough from mixing wheat flour and water is due to the gluten-forming capacity of flour proteins. Wheat is the only cereal with this property, although rye can form a poor gluten under special circumstances (94), and in the non-cereals carob germ flour is also said to have this property (136, 139).

It is generally accepted that intermolecular SS links are of major significance in gluten structure (111, 157), but the simple theory whereby oxidation of SH to SS increases dough strength and reduction of SS to SH decreases it does not fit all the observations of dough behaviour. There does not appear to be any correlation between the total SH and SS content of flour and dough quality (5, 149), but this may be due to some SH being unavailable for reaction (31, 34). There is a rough correlation between SH losses during mixing and dough strength (149). The fact that specific SH blocking agents have an effect similar to oxidising agents is contrary to what would be expected from the above theory (52, 62, 149, 155).

A more recent theory postulates an exchange mechanism (52, 62, 111, 155) as shown.



When dough is subjected to a deforming stress an SS under strain can be reduced by an adjacent SH, with the formation of a new SH and a new SS which is relatively strain-free. When free SH are specifically blocked no exchange can take place, and enough work eventually leads to rupture of the SS. Oxidising agents have the same effect rheologically since they remove SH by oxidation to SS. It is known (52) that in model systems the oxidation of protein SH by iodate can proceed anywhere between the limits,

$$IO_3^- + 6RSH = 3RSSR + 3H_2O + I^-$$

and

$$IO_3^- + RSH = RSO_3H + I^-$$

The more oxidised sulphinic and sulphonic acids could have an effect like specific SH blocking agents (52), and could form salts and esters. In doughs, oxidising agents are not absolutely specific for SH (42, 98), but the rheologically significant action is most probably that leading to the formation of SS links between protein molecules (52). Since gluten proteins are well orientated (65, 74, 161), intramolecular SS are likely to be much less frequent and important than intermolecular SS. Reducing agents, which increase the amount of SH in the dough, facilitate exchange reactions and thus soften the dough. Oxidising agents decompose in resting dough, but their improving action is only noted when the dough has been mechanically worked (16).

In studies of gluten composition it has been found that

nearly all the flour lipids are associated with the protein in varying degrees of firmness(resistance to solvent extraction) (10, 29, 30, 63, 74, 75, 76, 102, 114, 116, 129, 130, 138, 143, 152, 161, 164), and it has been found by X-ray analysis that the phospholipids are present as platelets orientated perpendicular to the main protein axis (65, 161). This association is quite different from that in flour, and appears to be formed during mixing (74A). It has been suggested that the lipids act as a lubricant in the gluten structure by being orientated to form lipid surfaces on the gluten which act as slip planes (65). It has also been suggested that lipid polymerisation could have an intermolecular protein bonding effect (148).

The role of flour lipids in these chemical changes has remained obscure, but it seems that FFA are particularly active, and Estd.FA may also have some effect (17, 41, 64, 95, 142, 144, 145, 158). This is reviewed in detail below, but at this point it may be noted that there is no direct relationship between the iodine value of the total lipids and dough properties (68).

Thioctic acid is a lipid-type co-enzyme which has recently been identified in flour (125, 154), and its role in flour oxidation has been the subject of speculation (154). Since thioctic acid is not directly extracted from flour by lipid solvents it has not been included in the main work of this thesis; experiments directed towards its extraction, identification and quantitative estimation are described in Part V of this thesis.

Wheats and flours are arbitrarily divided into strong and soft types according to whether they form strong elastic doughs or not. There has so far been no chemical means of distinguishing such wheats, and since the problem is linked with that of the formation of dough structure their solutions probably lie in the same direction. Because of this lack of knowledge on which to base experiments this investigation was largely confined to one flour of good breadmaking quality, as has been customary with many other investigators. All references hereinafter to flour refer to this type of flour, unless otherwise stated.

(b) Distribution, Extraction and Analysis of Flour Lipids

The distribution of lipids in the main parts of the wheat grain is given in Table 1.

Table 1.

The Distribution of Lipids in the Wheat Grain

Tissue	Lipid, wt.%	Tissue	% of Total Lipids	
			Phospholipids	Non-phospholipids
Bran	3 - 5.6	Pericarp, testa	25	31
Germ	6 - 15	Embryo, scutellum	10	19
Scutellum	30	Outer endosperm	7	7
Endosperm	0.75	Inner endosperm	58	43

References 8, 14, 27, 73, 83, 153.

In flour the lipid content depends on the milling extraction rate, that is the yield of flour expressed as a percentage of the wheat fed into the mill. As the extraction rate falls and the proportion of lipid-rich bran, germ and scutellum falls, the flour lipid content also falls. The relationship is complicated by the fact that about 30% of flour lipids are from lipids expressed from the germ during milling, without the germ itself being retained in the flour (150). The relationship between flour extraction rate and lipid content is shown in Table 2.

Table 2

Lipid Content of Flours of Varying Extraction Rate (103)	
Extraction Rate, %	Wt. % Lipids
100	2.49
85	1.70
80	1.43
75	1.32
70	1.16
42	0.86

In conducting analyses such as those in Table 2 the figures depend on the solvent used to extract the lipids (38, 73). In order of effectiveness in flour these are: light petroleum, diethyl ether, ethanol-ether 3/1 (v/v) (Bloor's solvent), methanol

followed by methanol-chloroform 1/1 (v/v) (38), and water-saturated n-butanol (113).

The complete analysis of all the classes of lipid present has not been made, but the major sub-divisions are FFA 10 - 14%, glyceryl esters 35%, sterol esters 4.8%, galacto- and phospholipids 57% approximately (49, 116, 167). The conventional lipid analyses are given in Table 3.

Table 3

Analyses of Wheat Lipids	
Saponification value	164 - 187
Iodine value	105 - 146
Unsaponifiable matter %	4 - 5.6
FFA %, new flour	5 - 10
FFA %, old flour	up to 72
References 8, 12, 13, 17, 37, 38, 41, 59, 64, 66, 85, 95, 108, 142, 156, 159, 169.	

In recent years GLC has enabled accurate fatty acid analyses to be made, and Table 4 shows values which are representative of the composition of both phospholipids and non-phospholipids. The relatively large amount of linoleic acid is of significance in the experimental work to be considered later.

Table 4

Fatty Acid Composition of Flour Lipids	
Acid	Mole %
16:0	14 - 24
18:0	0.6 - 2.5
18:1	8 - 16
18:2	58 - 66
18:3	1 - 3.5
20:1	1 - 1.4
others	up to 0.2 each
References 50, 98, 99, 108	

(c) The Effect of Oxygen on Doughs

For the study of the uptake of oxygen by doughs during the process of mixing it is often more convenient to use sponges (water = 1 to 10 times flour weight) rather than doughs (water = 0.55 to 0.65 times flour weight). In either system the chemical processes appear to be the same, although the rates of reaction are altered. Sponges were used in the present work for ease of freeze-drying, and more precise termination of enzymic reactions by sudden deep freezing.

A complete survey of the literature on the role of oxygen in the mixing of bread doughs has been published recently by Hawthorn (70)

and those aspects which are of particular relevance to this thesis are reviewed and discussed below.

The fact that doughs show a response to atmospheric oxygen has been recognised since about 1912, and has been used in commercial processes (6, 7, 54, 55, 56, 70, 71, 86, 87). The "Oxygen Effect" results in an improvement of dough strength (similar to the effect of oxidising agents, q.v. infra) and in partial bleaching of the flour pigments (67, 71). The rate at which strain is relaxed in stretched doughs is proportional to the oxygen tension. The uptake of oxygen is pH dependent (39, 144), having an optimum in the same region as wheat lipoxidase (141). When the uptake of oxygen by doughs is plotted against the FFA content of the flour a linear relationship is found above ca. 16% FFA, and is calculated to be equivalent to the absorption of 1 mole oxygen per mole of FFA (144). This and similar evidence strongly suggests that oxygen is absorbed by an FFA-lipoxidase system, and it seems certain that coupled oxidative reactions cause pigment bleaching and oxidation of SH to SS, resulting in dough improvement (39, 40, 71, 96, 97, 144, 145). (It is known that pea lipoxidase can oxidise the SH of glutathione in a coupled reaction) (106). It has also been found that catalase can promote pigment bleaching during aerobic dough mixing (71).

Lipoxidase is an enzyme which contains no metallic prosthetic group, and from most sources (including wheat and soya) it is not SH dependent (25). Lipoxidase acts by oxidising fatty

acids containing a methylene-interrupted double bond system (EFA) to conjugated hydroperoxides, and can cause coupled oxidation of secondary substrates (25, 70). Secondary substrates subsequently added to lipoxidase and EFA hydroperoxides are not oxidised; this would not be a coupled oxidation since it would take place after the EFA oxidation (70). The term lipoxidase is used in this review and elsewhere in this thesis as if it was the only enzyme capable of catalysing the oxidation of EFA with coupled oxidations of other substrates (70). It is however recognised that unsaturated fat oxidases would be a more general and accurate term to use, since there is no proof that lipoxidase is the enzyme responsible for the observed phenomena. There is also the possibility that iron-porphyrin compounds (haematins) could exert a similar effect if some preformed EFA hydroperoxide was present (25, 70).

Since there is no known inhibitor of lipoxidase which could be used to test this theory the best approach has been to remove the lipid substrate by solvent defatting the flour before mixing (71). Experiments with defatted flours (using light petroleum or ether) have shown both response (71) and no response to oxygen (97, 148), according to different workers. It has therefore been concluded that (i) oxygen improvement as distinct from bleaching is not dependent on lipoxidase, but is due to direct oxidation of protein (71) or to another enzyme system (70), (ii) since all the lipids cannot be removed by defatting with light

petroleum or ether, lipoxidase is still active using the residual lipid (60, 97, 148). In support of the latter argument it has been found that defatted flours, with or without a supplement of soya lipoxidase, show an oxygen response only when EFA is added (96).

Since the criticism of these experiments is based on completeness of light petroleum defatting it is important to consider what is extracted. From published data (38, 73) it seems that non-polar solvents remove FFA, Estd.FA and small amounts of polar lipids, leaving in the flour only the polar lipids. It has also been found that FFA support 10 times the oxygen uptake of Estd.FA (144), and that the dough resistance to mixing of defatted flour can be almost fully restored to normal by FFA, but not at all by Estd.FA or phospholipids (145). In a model system lipoxidase was found to use FFA, Estd.FA and polar lipids from flour as substrates in the ratio 5 : 1 : 0, despite their fatty acid compositions being similar (124). When the water-solubles of flour are boiled and returned to the flour it is found that the oxygen uptake is very slight (39, 144). There does not appear to be any correlation between flour strength and lipoxidase activity (144).

On re-examining the published graphs of oxygen uptake/g. flour fat against %FFA or %EFA (144), it is seen that on extrapolation to 0% FFA or EFA there is a residual uptake of 3 - 6 ml./50 g. flour. This was not pointed out by the authors, but it

suggests that there is an uptake not due to the lipoxidase/EFA system. Table 5 shows the oxygen uptake of flours calculated to a common basis from other authors' results. Cross mixed 18 g. flour in 450 ml. 1/8M KCl in the presence of air, and measured the oxygen uptake polarographically. Smith and Andrews measured the uptake of doughs mixed under oxygen by a manometric method. Cosgrove mixed flour and water sponges under nitrogen, and after 5 minutes changed the atmosphere to air over a now static sponge; he then measured the oxygen uptake manometrically, but because the method relied on diffusion of oxygen into a previously mixed sponge it is not truly comparable although the trend of the results was comparable. In flours of the type used there would be not more than 0.8% lipid, containing about 10% FFA, so that in 30 g. flour there would be not more than 18 mg. EFA. If all this EFA was totally consumed by lipoxidase in 20 minutes (which is most unlikely since oxygen uptake continues well past that time) the absorbed oxygen on a mole/mole basis would be 1.44 ml. It is therefore reasonable to deduce that there must be some other FFA oxidation(s) to account for the discrepancy in oxygen uptakes.

Table 5

A Comparison of the Published Oxygen Uptakes of Flours

Reference	Reaction Time, Minutes	Measured Uptake ml./30g. Flour	EFA Required Theoretically, on Mole/Mole Basis, mg.
Cross (40)	10	3.45	43.1
	20	3.88	48.5
Smith (144)	10	3.60	45.0
and	20	4.20	52.5
Andrews	30	4.80	61.7
Cosgrove (39)	10	0.60	7.5
	60	1.70	21.0

There is a measurable loss of SH in doughs made from defatted flours, and in these doughs the oxygen uptake is too small to be measurable (145). Atmospheric oxygen inhibits bromate decomposition according to the oxygen tension (33, 36, 42). Anti-oxidants such as nordihydroguaiaretic acid and α-tocopherol acetate partially inhibit pigment bleaching, but do not affect loaf volume (70, 71). Other antioxidants inhibit the rate of bromate decomposition in dough only in the presence of oxygen (36).

The picture is thus very confused, but the following points summarise the more important facts on which the experimental

part of this thesis is largely based:

1. Atmospheric oxygen acts as a dough improver.

2. Most of the uptake of oxygen during dough mixing can be attributed to enzymic lipid oxidation.

3. There is some evidence of residual uptake of oxygen which is independent of unsaturated fat oxidase action.

4. SH groups are very oxygen sensitive in defatted flours (i.e. where unsaturated fat oxidase activity is likely to be absent).

5. There is no evidence to show that all oxygen improvement is enzymic.

6. There is evidence that lipoxidase oxidation is responsible for some of the improving effect of oxygen by causing secondary oxidation of SH.

7. The bleaching of flour pigments is due to their acting as a secondary substrate in lipoxidase catalysed oxidation of EFA.

8. There is reason to believe that lipid oxidation during dough mixing involves enzymic activity, other than lipoxidase, which uses more oxygen than would normally be attributed to lipoxidase activity.

(d) Oxidising Agents Added to Doughs

There is a considerable list of substances which have a flour improving effect (88). Most of these are oxidising agents, and the others include ascorbic acid which acts in the form of dehydroascorbic acid to oxidise SH (105, 112, 115), and Cu^{++} which could act as a pro-oxidant catalyst or as a SH blocking agent with the same effect (33-37). The effect of the presence or absence of flour lipids on the dough chemistry of two oxidising agents, bromate and persulphate, has been examined (42, 100). Commercial flour bleaching agents such as chlorine dioxide also have an improving effect, and they are found to cause some loss of tocopherols and slight lipid peroxidation in the dry flour, which could have an improving effect (45, 53, 121, 122).

Potassium bromate and iodate are used commercially at ca. 15 - 30 ppm. of flour weight, and persulphate at ca. 160 ppm. (71, 89, 148). In terms of available oxygen these bromate and persulphate levels are similar (71). Improvers are decomposed in the process of exerting an improving effect in doughs. Iodate, which is a fast-acting improver, is largely decomposed in a matter of minutes (31), but the decomposition of bromate takes over 5 hours (32, 33). It is customary to measure the rate at which bromate or other improvers decompose in doughs, and to study the effect of experimental conditions on this rate. Defatting flour with water-saturated n-butanol has the effect of greatly lowering the

rate of bromate decomposition, and the rate cannot be restored to normal by returning the lipids to the dough (42). Persulphate is not affected by this treatment (100). Defatting flour with light petroleum has a smaller effect on bromate decomposition, and the effect can be reversed by returning the lipids to the dough (42). A proposed explanation of these results is that the butanol removes tightly bound lipids from specific sites in the gluten, and, since this structure is not recoverable, specific bromate oxidation is not possible (42). Since the orientation of proteins and lipids in flour is different from that in dough (74, 75, 129) and since water-saturated n-butanol can considerably inactivate enzymes (124) and gluten proteins (20), it seems to this author that enzyme damage is an equally plausible explanation. This would require an enzyme intermediate in the decomposition of bromate, and in support of this suggestion it has been shown that peroxidase can use bromate as a substrate (58). Persulphate presumably acts by a different mechanism.

From the above considerations it might be thought that bromate reacts directly with the lipids, but no evidence of any reaction is found when they are mixed in dioxane solution (99).

The rate of bromate decomposition is decreased by increasing oxygen tension during mixing (33, 36, 42), and by the presence of hydroperoxides, iodate, benzoyl peroxide and chlorine dioxide (33, 36, 39, 98). The antioxidants butylated hydroxyanisole and n-propyl gallate decrease bromate decomposition only in the

presence of oxygen (36). Since light petroleum removes some tocopherol antioxidants along with lipids it might be expected to increase the rate of bromate decomposition, but the increased oxygen-sensitivity of the flour exerts a more powerful inhibitory effect (34, 39).

Oxidising agents are said to be non-specific in their action (36, 42, 98), but on the other hand bromate and iodate are said to be virtually specific for SH oxidation (31, 33).

In yeasted doughs there is a marked loss of ether-extractable lipids only if bromate is present (157).

In summary it can be said that there is some indirect connection between the mechanism of bromate action and the presence of flour lipids, but other improvers seem to be independent of the flour lipids.

(c) Free Fatty Acids in Flour and Doughs

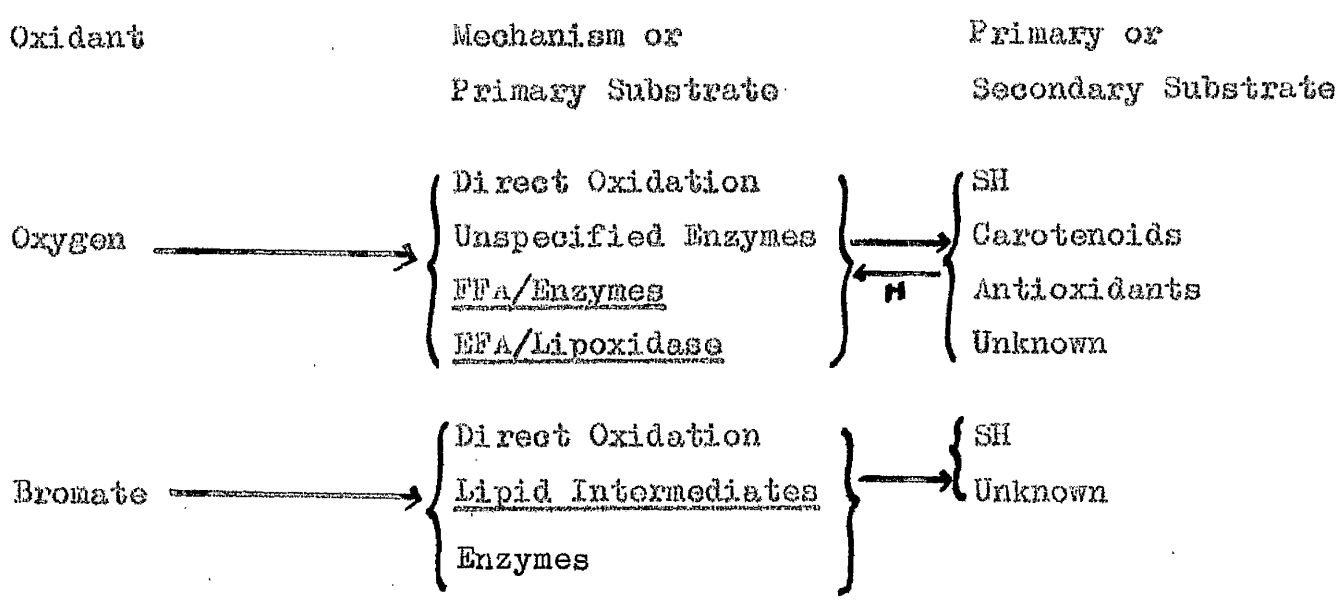
Much of the information concerning FFA has already been mentioned in connection with oxygen uptake, but is repeated here for completeness. In high grade flour there is about 0.8% lipid which contains from 10% FFA (fresh flour) to 30 - 72% FFA (very old flour). The increase in FFA content is due to lipase action during storage (17, 41, 61, 64, 95, 142). Old flour exhibits typical overoxidation properties on aerobic mixing (152), and this has been attributed to the increased FFA content (95), or to FFA hydroperoxides (153). Autoxidised FFA added to defatted flour

mixed under nitrogen give a dough equivalent to that obtained from aerobic mixing of undefatted flour (145). This ability of dough to utilise autoxidised EFA for oxidative purposes indicates that a coupled oxidation by lipoxidase is not necessary for the oxidative effects. This does not however mean that coupled oxidations are not the normal mechanism when EFA are enzymically oxidised and give rise to the normal oxidative effects. Oxidised triglycerides do not affect dough quality, as measured by the volume of a loaf baked from the dough (119). Estd.FA can account for some of the oxygen absorbed during mixing (144), but the same authors found that Estd.FA alone cannot restore a defatted flour to its undefatted quality as judged by aerobic mixing tests, although EFA did (145). There is no correlation between the iodine value of the total flour lipids and the flour strength (68), and it seems unlikely that FFA would show a correlation, although this has not been demonstrated. There is no evidence that FFA or any other particular lipid is involved in the mechanism of bromate action, but it is assumed that the EFA/lipoxidase system does compete with bromate for the same substrates (36).

It is significant that there have been no published results of experiments in which the FFA of flours and doughs have been examined to demonstrate changes in their composition in conformation with the above statements. Since nearly all these statements have been arrived at by addition and/or subtraction of lipids from flour before

mixing, they can only be accepted as evidence in support of the particular theory being examined. It has already been shown that there is a fault in the deductions from the experimental evidence, since the oxygen uptake of high grade flours cannot be reasonably accounted for in terms of EFA/lipoxidase activity, and other FFA oxidation mechanisms must therefore be involved. There is no suggestion from the information above to indicate the nature of the mechanisms. The purpose of the main part of this thesis is to make a preliminary examination of the FFA oxidations of a high grade flour, by analysis of FFA before and after mixing experiments, to clarify this point.

In the light of current knowledge it is possible to draw up a scheme of oxidative reactions which are likely to occur in doughs mixed aerobically, but this does not exclude the possibility of other mechanisms which may be more significant. It is assumed that other reactions involving FFA are insignificant, but this point is dealt with in the experimental part of the thesis.



Persulphate ⟶ Direct Oxidation ⟶ {SH, Unknown}

EFA Hydroperoxide ⟶ Probably Enzymes ⟶ As for Oxygen

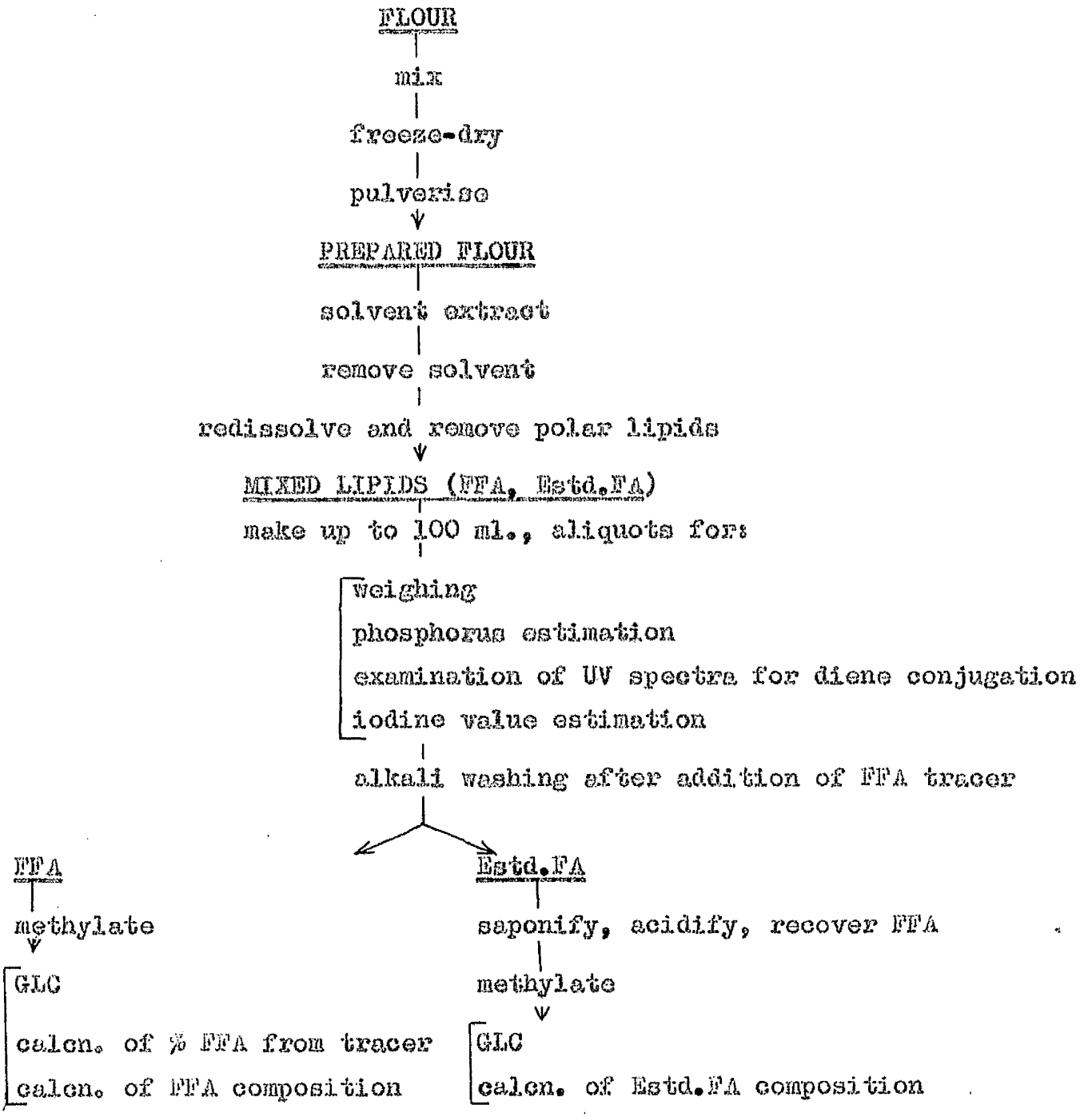
There is no order of priority suggested, but one must exist if, for example, oxygen can decrease the rate of bromate decomposition. The investigations reported in Part III are concerned with the three underlined mechanisms and the associated substrates.

PART II

EXPERIMENTAL PLAN, MATERIALS, METHODS

EXPERIMENTAL PLAN

The purpose of the investigation was to examine the role of the FFA of a single high grade spring wheat flour in the oxidative changes which occur during sponge or dough mixing. The Estd.FA were examined at the same time. From the literature survey it was anticipated that FFA changes due to lipoxidase activity would be found, and other FFA oxidative changes might also be expected. To further clarify results obtained from mixing of flour and water aerobically, and to ascertain the role of FFA oxidations in flour containing additives which affect dough rheology, additional experiments were designed in the light of those results already obtained, the details of which are given later. The general scheme of analysis which was thought suitable for this purpose is shown on the following page.


FLOUR
mix
freeze-dry
pulverise
PREPARED FLOUR
solvent extract
remove solvent
redissolve and remove polar lipids
MIXED LIPIDS (FFA, Estd.FA)
make up to 100 ml., aliquots for:
weighing
phosphorus estimation
examination of UV spectra for diene conjugation
iodine value estimation
alkali washing after addition of FFA tracer
FFA
methylate
GLC
calcn. of % FFA from tracer
calcn. of FFA composition
Estd.FA
saponify, acidify, recover FFA
methylate
GLC
calcn. of Estd.FA composition

At the outset it was clear that existing analytical methods were not always directly applicable to this scheme of analysis for technical reasons, and much development work was required before routine analysis of flour and dough lipids was placed on a reliable basis. This development work is given in some detail in Part IV.

MATERIALS

The flour used throughout most of the work was from a single sample of unbleached, untreated high grade spring wheat flour from a mixed Manitoban grist. It was stored in glass jars at ca. 0^{o}, and was found to be only slightly altered during storage (q.v. Part III). In one experiment two flours used by Cross were examined since their oxygen uptakes during mixing had been measured (40). They had been stored in the same manner as the flour mentioned above; but for much longer periods, and were of slightly lower grade - i.e. they were of higher extraction rate and contained more lipids.

All chemicals and solvents were of reagent grade where obtainable, and the nitrogen was the commercially available oxygen-free quality without further purification.

METHODS

(a) Flour Preparation, Mixing and Freezing

The control flour, against which all lipid changes were assessed, was used straight from storage, but allowed to warm to room temperature before solvent extraction. The moisture content was measured with a Marconi moisture meter.

Flour was mixed according to the following procedure. 40 g. of flour were weighed and allowed to warm to room temperature. 40 ml. water were added to a 250 ml. M.S.E. Atomix mixer, followed by the flour, then a further 40 ml. of the water, and mixing started at once. Mixing for 10 seconds on half-speed followed by 5 seconds on full speed gave a clear aerated sponge. Chemical additives, when used, were dissolved in the water or added to the water in 1 - 2 ml. of a solvent such as ethenol to form an emulsion. An atmosphere enriched with oxygen was obtained by blowing oxygen at ½ p.s.i. into the Atomix mixer by a tube through the lid. Where an atmosphere of nitrogen was required, dry flour was put into the mixer and the container flushed with nitrogen for 20 minutes, the dry flour being disturbed at intervals by switching on the mixer for a second. Water flushed with nitrogen was then added, maintaining a positive internal nitrogen pressure, and mixing carried out as usual with a constant flow of nitrogen. The temperature of the water was adjusted to give a sponge at 20°.

The sponge was allowed to lie in the mixer for a given reaction time under the appropriate atmosphere, and then transferred

to a 1 litre round-bottom flask and frozen to form a shell within the flask, using an acetone/solid carbon dioxide mixture. For the ½ minute reaction time the flask was previously immersed in a bucket of freezing mixture and the sponge poured in as soon as it was mixed. After freezing the sponge was stored at -30° until it could be vacuum dried. Vacuum drying was done using an Edwards Speedivac model 1SC50B vacuum pump at full vacuum with an acetone/solid carbon dioxide cooled water trap. The drying flask was periodically bathed with cold water to remove ice and hasten drying which took about 2½ to 3 hours.

(b) Flour Moisture-Equilibration, Solvent Extraction

To make the solvent extraction of prepared flour as comparable to that of the original flour as possible, the flour was ground and sieved through a nylon sieve, spread out as a thin layer on paper and allowed to come into equilibrium with atmospheric moisture overnight. This gave a moisture content of 12 to 14%. The danger of lipid changes occurring was considered slight since flour of 14% moisture content is stable in air for weeks. A bigger source of error would have been the lowered yield of lipid from a dry flour (72).

To extract flour, 30 g. were slurried with 120 ml. methanol and allowed to lie for ½ hour to complete swelling. Glass columns 3 cm. diameter and drawn to a neck at the foot were fitted with a plug of defatted cotton wool in the neck. The methanol-flour slurry

was poured into the column and rinsed in with a few ml. of methanol. When the methanol level had dropped to the flour surface the column was refilled with 200 ml. of 1:1 (v/v) methanol-chloroform, taking care not to disturb the flour surface. The total percolated extract was collected, evaporated to dryness under vacuum with a nitrogen leak at a temperature about 40°, and redissolved in ca. 20 ml. of diethyl ether.

(c) Removal of Phospholipids and Galactolipids

10 g. silicic acid (Mallinckrodt, 100 mesh, suitable for analysis by the method of Ramsey and Patterson) were prepared by heating at 130° for 2 to 3 hours and cooling in a desiccator. The silicic acid was then added to the ether solution of crude lipid which boiled due to the heat of solvent adsorption. After swirling to ensure complete mixing the mixture was filtered through a No. 44 Whatman filter paper into a 100 ml. graduated flask. It was necessary to cover the filter with a watch-glass to reduce solvent evaporation and to thoroughly wash the filter paper with ether. This procedure effectively dried the lipids in solution and removed phospholipids and galactolipids.

(d) Weighing of Aliquots

Two 10 ml. aliquots were pipetted into previously equilibrated and tared weighing bottles. The solvent was removed by a stream of nitrogen with the bottles immersed in a water bath

at ca. 40°. The bottles were then dried and placed in a desiccator. The desiccator contained calcium chloride desiccant and shavings of hard paraffin wax to remove solvent vapour. The bottles were left under vacuum overnight, then allowed to equilibrate with the balance atmosphere for at least 1 hour before weighing. The procedure was repeated, and replicate weighings usually agreed to within 0.02 mg. Two blanks were always run concurrently to correct for humidity fluctuations of the atmosphere in the balance.

(e) Phosphorus Determination

This method combined features of the methods of Harvey (69), Fogg and Wilkinson (51), and Beveridge and Johnson (18). The following reagents were required:

Water - deionised quality,

Sulphuric acid, S.G. 1.84,

Hydrogen peroxide, 33% ("100 volumes")

Sodium sulphite - a 33% (w/v) aqueous solution of $Na_2SO_3 \cdot 7H_2O$

Molybdate reagent - 10 g. crystalline ammonium molybdate were dissolved in 100 ml. water and added to a cooled mixture of 60 ml. sulphuric acid and 150 ml. water, and finally made up to 360 ml. with water.

Ascorbic acid - solid

Standard phosporus solution - an aqueous solution containing 0.10069 g. $NaH_2PO_4 \cdot 2H_2O$ per litre (20γ phosphorus per ml.)

Procedure

A suitable aliquot of lipid solution (containing up to 10 mg. solids) was evaporated to dryness in a 30 ml. Kjeldahl flask, 1 ml. of sulphuric acid added, and the flask heated over a small microburner flame for 5 minutes to char and partially oxidise the organic matter. 2 - 6 drops of hydrogen peroxide were added, shaking thoroughly between addition of each drop, until the solution became colourless. It was essential to shake very thoroughly otherwise low phosphorus yields were obtained through incomplete oxidation of organic matter. Heating was continued for 10 minutes, the flask was then cooled and the contents transferred to a 100 ml. beaker with water to give a final volume of 30 - 35 ml. 1 ml. sulphite solution was added, then 4 ml. molybdate reagent and 0.1 g. ascorbic acid. The beaker was swirled to dissolve the ascorbic acid and then boiled for 1 minute. After cooling the solution was made up to 50 or 100 ml. with water and the optical density at 822 mμ measured against distilled water using 1 cm. glass cells. The heteropoly blue colour formed by this method was very stable, and obeyed Beer's Law, the equation

being,

Optical Density = 0.0176 x γ phosphorus.

The method was sensitive to phosphorus content but stable to variations of reagents or time of boiling of at least ± 50%. Reagent blanks were always run concurrently with samples in duplicate.

(f) Nitrogen Determination

The A.O.A.C. microkjeldahl procedure (2a) was used for some exploratory studies mentioned in Part IV, but was not part of the routine analysis.

(g) Ultraviolet Spectrophotometry of Lipids

1 ml. of the total lipid solution or of Estd.FA solution was evaporated to dryness under nitrogen and redissolved in 4 or 8 ml. of spectroscopically pure cyclohexane respectively. The absorption over the range 220 mμ to 300 mμ against cyclohexane was measured using 1 cm. quartz cells. The instruments used were an Optika CF4 spectrophotometer with automatic recording, and a Hilger Uvispek spectrophotometer with manual operation.

(h) Iodine Value Determination

The A.O.A.C. method using Hanus' iodine reagent (2b) was scaled down to 1/5th. The procedure was:

10 ml. aliquots of lipid were evaporated to dryness under nitrogen in 100 ml. flat-bottom flasks with B24 ground necks.

The dry lipid was redissolved in 1 ml. chloroform, and 2 ml. Hanus' iodine solution were pipetted into each flask allowing exactly 20 seconds draining time. The flasks were prepared at 5 minute intervals so that each could have 30 minutes reaction in the dark and be titrated before the next flask was due for titration. After the 30 minute reaction time 1 ml. 15% (w/v) aqueous potassium iodide was added, then 10 ml. water and the excess halogen titrated with N/20 sodium thiosulphate using starch indicator. The iodine value was calculated in the usual manner from the difference between blank and sample titrations (in duplicate).

This was not a true micromethod but sufficed for the present work, and was probably much simpler and more accurate than a true micromethod (147), judging by published results.

(i) Alkali Washing to Remove Free Fatty Acids from Lipids

The method of Mattick and Lee (110) was used to remove and recover FFA and Estd.FA as two relatively pure fractions, the whole process being scaled down to 1/10th. Before removing the FFA a 5 ml. aliquot containing 1.5 mg. margaric acid (17:0) in light petroleum (B.Pt. 40°-60°) was pipetted into a 35 ml. aliquot of lipids. The margaric acid served as a quantitive yardstick from which the percentage FFA in the lipid or flour, and the degree of removal of FFA from Estd.FA was calculated from GLC analyses.

The 40 ml. of lipids were evaporated to dryness under nitrogen over a water bath at 40°, and redissolved in a solvent

comprising 70 parts light petroleum (B.Pt. 40°- 60°), 70 parts diethyl ether and 25 parts ethanol and transferred to a 50 ml. separatory funnel using 16.5 ml. solvent mixture. 5 ml. 1% (w/v) aqueous sodium carbonate were added and the stoppered funnel shaken for 30 seconds. The lower layer was run off and retained. To the funnel a further 0.5 ml. ethanol and 3 ml. sodium carbonate were added, the funnel shaken for 30 seconds and the lower layer run off and retained with the previous extract. This was repeated using 0.5 ml. ethanol and 2 ml. sodium carbonate, and finally with 2 ml. water. The ethereal layer in the funnel contained the Estd.FA, and the aqueous layer the soaps of the FFA.

The FFA were recovered by acidifying with 2 ml. 10% (v/v) hydrochloric acid (to pH 1 to 2) and extracting three times with a 1:1 (v/v) mixture of light petroleum and diethyl ether. The combined ethereal extract was washed once with 2 ml. water and dried with anhydrous sodium sulphate. The Estd.FA was likewise dried.

(j) Saponification of Esterified Fatty Acids, Recovery of the Fatty Acids.

Fresh alcoholic potassium hydroxide was prepared for each saponification by dissolving 0.7 g. solid potassium hydroxide in less than 1 ml. water and making up to 25 ml. with 95% ethanol. This solution is about $\frac{1}{2}$N and did not appear to affect the EFA (44) during saponification. The Estd.FA solution was evaporated to dryness under nitrogen in a 25 ml. pear-shaped flask in a water

bath at 40°. When dry the Estd.FA were covered immediately with 10 ml. alcoholic potash and refluxed under nitrogen for 30 minutes. The soaps were transferred to a 50 ml. separatory funnel with 15 - 20 ml. water, acidified to pH 1 to 2 with dilute hydrochloric acid (sulphuric acid was not used because potassium sulphate is not very soluble in ethanolic solution) and the fatty acids extracted with petroleum ether (B.Pt. below 40°) till the aqueous phase was clear. The lower layer was run off and the petroleum layer dried with anhydrous sodium sulphate.

(k) Methylation of Free Fatty Acids

It was originally intended to prepare fatty acid methyl esters using diazomethane, but it was found that artifacts were being formed which interfered with GLC of the esters. Further work showed that the boron fluoride-methanol (118) reagent was quite suitable and very convenient for this purpose (127), and it was used in this work (see Part IV).

The reagent was prepared by slowly bubbling boron fluoride gas into chilled methanol until it had gained 12.5% weight. The reagent was kept stoppered and dry, and was stable for months. To methylate FFA the solution of acids was evaporated to dryness under nitrogen and 1.5 ml. boron fluoride - methanol reagent added to the acids in a test tube. The test tube was heated in a boiling water bath for 2 minutes and immediately cooled by adding 10 ml. cold water

and 20 ml. of light petroleum (B.Pt. below 40°). The mixture was transferred to a separatory funnel and shaken till the aqueous layer was clear, and it was then run off and the petroleum solution of esters dried with anhydrous sodium sulphate.

(l) Purification of Methyl Esters by Vacuum Distillation

Methyl esters from FFA contain Estd.FA entrained in the separation, and those from Estd.FA contain unsaponifiable matter which should be removed before GLC, to prevent build-up of material on the GLC column which might subsequently decompose and give rise to spurious peaks or base-line wandering. Methyl esters of long chain fatty acids (C_{14} - C_{18}) can be distilled and a 5 mg. load quantitatively recovered in 60 minutes at 60° ± 2° and 0.2 ± 0.15 mm. mercury pressure (151). In practice it was found that 62° - 65° and 0.03 mm. mercury for 30 minutes gave complete recovery of loads of esters up to 60 mg. in the particular pot still used. The design is given in Part IV. The distilled esters were washed off the condenser with ca. 1 - 2 ml. light petroleum (B.Pt.40° - 60°), via a filter funnel with a drawn-out stem, into 2 ml. ampoules. The ampoules were concentrated, sealed under vacuum and stored at ca. 0° till required for GLC analysis.

(m) Gas Chromatography of Fatty Acid Methyl Esters

A Pye gas chromatograph with an argon ionisation detector was used. The column consisted of a 4 foot glass column of 4 mm.

internal diameter. The packing was 80 - 100 mesh Celite coated with 15% (w/w) LAC-2-R446 (a linear polyester of ethylene glycol and adipic acid cross-linked with pentaerythritol) and 2% (w/w) orthophosphoric acid. The phosphoric acid was intended to stabilise the column packing at higher temperatures (200° - 230°) for GLC of FFA (117) but at the temperatures for methyl esters (170° - 185°) it has the effect of giving the column a prolonged life. In practice the column was run at 182° with an argon flow rate of 40 ml./minute and a detector voltage of 1000 or 1500 volts at x 10 amplification. Detector response was linear with molar concentration of eluted peak material.

When esters were to be analysed the ampoule was opened and the volume of solvent reduced under nitrogen to achieve the necessary concentration. The opened ampoule was then carefully manipulated to wash the inside walls with the remaining solvent, and placed in a stoppered weighing bottle containing a small amount of the solvent to saturate the enclosed space. This prevented changes in concentration of the esters, or fractional crystallisation of the saturated esters. The esters were stable at room temperature, if kept away from light, for several days as judged by identical GLC analyses.

The method of Bartlet and Smith (11) was used for quantitative analysis of the chromatograms, in which the area of a Gaussian curve is given by the formula:

$$\text{area} = \text{standard deviation} \times \text{height} \times \sqrt{2\pi}$$

The standard deviation is the peak breadth at 0.885 height, ½ peak breadth at 0.607 height, or 1/3 peak breadth at 0.332 height. A plot of standard deviations against peak retention times gave a linear relationship of the type $y = mx + c$. Calibration plots were made from numerous peak measurements, and for routine calculations the standard deviation was derived from measurement of retention time converted graphically, or by the equation describing the graph. Details and typical chromatograms with full calculations are given in Part IV.

(n) Calculation of Percentage Free Fatty Acids in Lipids

By measuring the amount of margaric acid present as a percentage of the total natural acids present, the percentage FFA in the lipids was given by:

$$\%FFA = \frac{1.5 \times 10^4}{3.5 \times w \times s}$$

where w = the weight of a 10 ml. aliquot in milligrams and s = the percentage margaric acid.

If s' = the percentage residual margaric acid in the Estd.FA esters then the percentage recovery of FFA by alkali washing was given by:

$$\% \text{ recovery} = 100 - \frac{s' \times 100}{s}$$

It was generally found that recoveries were of the order 98 - 99% and at this level residual FFA would hardly affect the Estd.FA analyses.

PART III

EXPERIMENTAL AND RESULTS, AND DISCUSSION

EXPERIMENTAL AND RESULTS

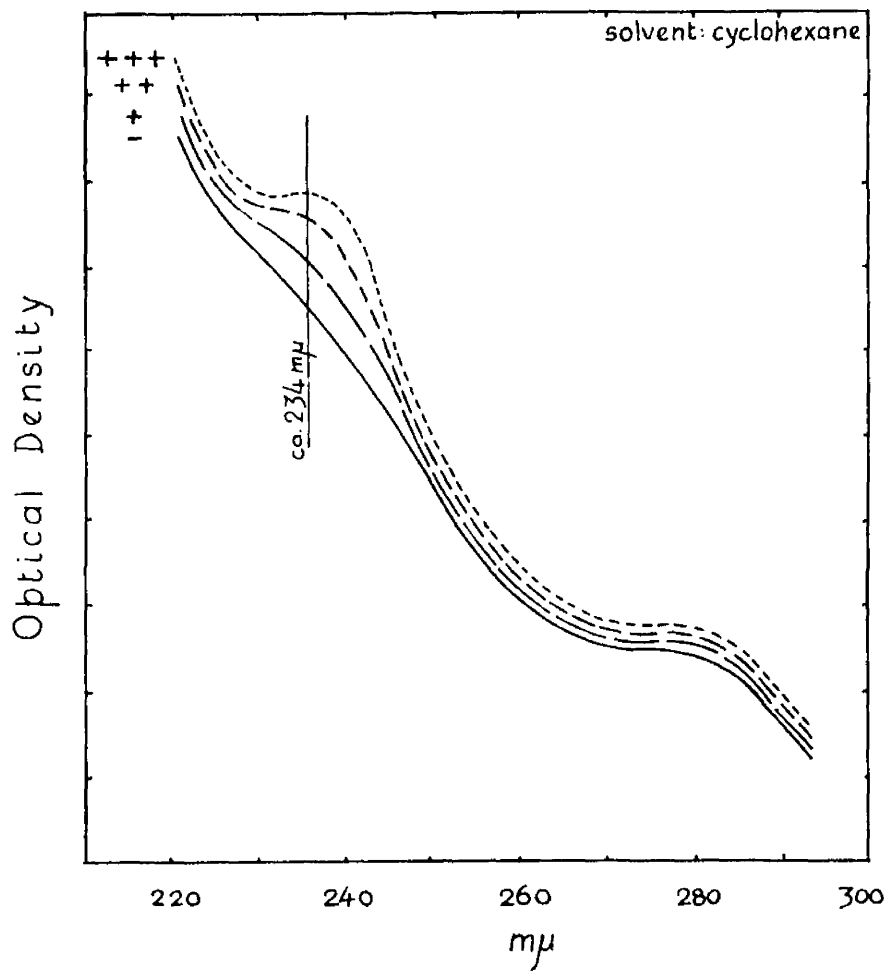
All lipid samples were routinely analysed for phosphorus and found to contain less than 0.02% phosphorus, the normal figure being below 0.01 %. This would correspond to not more than 0.25% phospholipid calculated as lecithin. The samples were therefore assumed to be free from phospholipids and other polar lipids, and no further mention of their phosphorus content is made below.

The ultraviolet spectra of the recovered lipids showed a typical absorption from 215 to 260 mμ in all samples (Fig. 1). The relatively small absorption due to diene conjugation superimposed on this could not be accurately measured. The four spectra opposite (Fig. 1) show the effect of the observed increases of diene conjugation, with the magnitude indicated by a number of + signs against each curve.

It was confirmed that the main fatty acids in wheat lipids are palmitic, stearic, oleic, linoleic, linolenic and gadoleic (cf. Table 4). Although gadoleic acid was found in normal amounts in Estd.FA it was found to be virtually absent from FFA. For convenience gadoleic acid and all other trace components were omitted from GLC calculations without in any way altering the trends of the results.

All mixing experiments were done 2 - 6 times, and each GLC sample 3 - 4 times, in order to obtain adequate statistical data.

Figure 1



UV Spectra of lipids showing degrees of conjugation.

Comparison of Lipids of Flours

Table 6 shows the results of several flour analyses which were carried out to determine whether the composition of FFA differs sufficiently from that of the Estd.FA to account for their different effects in doughs mixed aerobically. The "Normal" flour was the one used throughout the main experimental work, and flours R.C.I and R.C.II were slightly lower grade flours which had been used for studies of oxygen uptake during mixing (40). All flours were unbleached and untreated with improvers.

Flour Mixed in Air

Table 7 shows the results of experiments in which flour - water sponges were mixed in air and allowed to lie for varying periods of time (reaction times) before freezing and drying. It is a common observation that if flour and water are efficiently mixed, the typical rheological properties are well developed within 10 minutes, and presumably the associated chemical changes have mostly taken place within the same time. Reaction times of 0(no mixing), ½, 2, 10 and 60 minutes were given. In another experiment flour was autoclaved in an attempt to inactivate the enzymes without altering the lipids. The experiment failed in that there appeared to have been accelerated enzyme activity before inactivation. The result is thus akin to infinite reaction time and has been included out of interest.

Table 6

Analysis of Lipids from Spring Wheat Flours

Flour	Age in Months	Lipid, % of Flour	FFA, % of Lipid	Mole % Composition of FFA					Mole % Composition of Estd. FA				
				16:0	18:0	18:1	18:2	18:3	16:0	18:0	18:1	18:2	18:3
Normal	1	0.55	10.9	21.7	1.7	8.9	65.4	2.1	20.5	0.8	14.4	61.8	2.5
Normal	6	0.58	12.0	20.4	1.8	10.2	64.9	2.7	19.8	1.1	13.9	62.5	2.8
R.C.I	10	1.33	14.4	19.0	1.6	14.0	62.4	2.9	19.2	0.9	16.0	61.0	2.9
R.C.II	23	0.78	25.0	17.2	1.3	12.4	66.2	3.1	21.3	0.9	14.6	60.1	3.1

TABLE 7

Analysis of Lipids from Sponges Mixed in Air with Various Reaction Times

Reaction Time, Minutes	Lipid, % of Flour Weight (14% Moist)	Iodine Value	FFA, % of Lipid	Diene Conjn.	Mole % Composition of FFA					Mole % Composition of Estd.FA				
					16:0	18:0	18:1	18:2	18:3	16:0	18:0	18:1	18:2	18:3
0 (August '61)	0.554 $_{0.014}$	117.8 $_{5.9}$	10.9 $_{0.89}$	-	21.7 $_{1.18}$	1.7 $_{0.19}$	8.9 $_{0.38}$	65.4 $_{1.14}$	2.1 $_{0.27}$	20.5 $_{0.70}$	0.8 $_{0.10}$	14.4 $_{0.31}$	61.8 $_{0.64}$	2.5 $_{0.33}$
0 (January '62)	0.580 $_{0.032}$	117.9 $_{1.3}$	12.0 $_{0.71}$	-	20.4 $_{0.45}$	1.8 $_{0.09}$	10.2 $_{0.31}$	64.9 $_{0.44}$	2.7 $_{0.26}$	19.8 $_{0.49}$	1.1 $_{0.14}$	13.9 $_{0.43}$	62.5 $_{0.78}$	2.8 $_{0.24}$
$\frac{1}{2}$	0.611 $_{0.030}$	115.3 $_{3.3}$	11.1 $_{0.43}$	+	22.9 $_{1.41}$	1.5 $_{0.20}$	9.6 $_{0.61}$	64.0 $_{1.79}$	1.9 $_{0.26}$	21.3 $_{1.09}$	0.8 $_{0.18}$	14.0 $_{0.75}$	61.1 $_{0.76}$	2.3 $_{0.17}$
2	0.580 $_{0.029}$	115.7 $_{0.5}$	11.7 $_{0.56}$		21.3 $_{1.10}$	1.9 $_{0.19}$	10.7 $_{0.59}$	63.9 $_{0.89}$	2.1 $_{0.31}$	20.2 $_{0.44}$	1.1 $_{0.18}$	14.0 $_{0.27}$	62.5 $_{0.79}$	2.6 $_{0.18}$
10	0.563 $_{0.029}$	114.5 $_{0.5}$	6.5 $_{0.29}$	++	25.4 $_{1.26}$	2.7 $_{0.28}$	12.1 $_{0.61}$	58.4 $_{0.70}$	1.2 $_{0.38}$	20.2 $_{0.51}$	0.8 $_{0.10}$	14.6 $_{0.38}$	62.0 $_{0.67}$	2.5 $_{0.29}$
60	0.545 $_{0.032}$	114.0 $_{0.4}$	6.2 $_{0.44}$	-	24.3 $_{1.40}$	3.1 $_{0.26}$	12.2 $_{0.55}$	58.0 $_{1.10}$	2.0 $_{0.10}$	21.1 $_{0.67}$	1.0 $_{0.07}$	13.8 $_{0.48}$	61.2 $_{0.79}$	2.6 $_{0.12}$
Autoclaved	0.404	120+	0.22	-	21.8	6.8	18.2	52.0	2.1	20.6 $_{0.31}$	1.0 $_{0.05}$	14.0 $_{0.08}$	61.5 $_{0.00}$	2.9 $_{0.06}$

Small subscript numerals = 1 standard deviation.

Table 8

The Effect of Oxygen, Nitrogen, Methanol and Additives on the Analysis of Lipids from Sponges

Treatment	Reaction Time, Minutes	Lipid, % of Flour Weight (14% moist)	Iodine Value	FFA, % of Lipid	Diene Conjn.	Mole % Composition of FFA					Mole % Composition of Estd.FA				
						16:0	18:0	18:1	18:2	18:3	16:0	18:0	18:1	18:2	18:3
Oxygen	10	0.560 $_{0.250}$	110.9 $_{1.0}$	8.0 $_{0.25}$	+++	25.1 $_{0.99}$	2.0 $_{0.24}$	12.2 $_{0.22}$	59.2 $_{0.78}$	1.6 $_{0.40}$	19.9 $_{0.50}$	0.9 $_{0.10}$	14.3 $_{0.24}$	62.1 $_{0.76}$	2.7 $_{0.20}$
Nitrogen	10	0.554 $_{0.013}$	117.8 $_{5.45}$	10.0 $_{1.04}$	-	21.7 $_{1.23}$	2.3 $_{0.19}$	10.2 $_{0.40}$	63.3 $_{1.40}$	2.6 $_{0.22}$	20.5 $_{1.07}$	1.1 $_{0.16}$	14.2 $_{0.33}$	61.5 $_{0.73}$	2.7 $_{0.23}$
Sulphite	10	0.599 $_{0.024}$	112.2 to 117.0	8.8 $_{0.51}$	++	22.9 $_{1.09}$	2.9 $_{0.35}$	12.3 $_{0.53}$	59.1 $_{1.10}$	2.3 $_{0.37}$	20.6 $_{0.84}$	1.0 $_{0.08}$	12.9 $_{0.33}$	62.3 $_{1.21}$	3.1 $_{0.61}$
NDGA	10	0.534 to 0.615	-	7.5 to 8.7		31.5 $_{1.34}$	3.0 $_{0.47}$	14.3 $_{1.67}$	50.2 $_{1.87}$	1.8 $_{0.19}$	20.4 $_{1.00}$	1.1 $_{0.12}$	14.0 $_{0.50}$	61.9 $_{1.14}$	2.7 $_{0.37}$
NEMI	10	0.585 $_{0.027}$	113.6 $_{1.3}$	8.8 $_{0.26}$	+	22.8 $_{0.41}$	2.7 $_{0.17}$	12.3 $_{0.19}$	60.2 $_{0.50}$	2.2 $_{0.17}$	20.3 $_{0.16}$	1.1 $_{0.14}$	14.2 $_{0.35}$	61.4 $_{0.86}$	2.9 $_{0.38}$
Bromate	60	0.581 $_{0.012}$	110.1 to 114.5	6.1 $_{0.37}$	- to ++	24.1 $_{0.94}$	3.2 $_{0.45}$	12.4 $_{1.02}$	58.8 $_{0.74}$	2.0 $_{0.29}$	20.0 $_{0.58}$	1.1 $_{0.13}$	14.0 $_{0.07}$	62.1 $_{0.50}$	2.8 $_{0.34}$
Methanol	0	0.543 $_{0.024}$	107.5 to 117.8	12.1 $_{1.61}$	+	18.3 $_{0.46}$	2.3 $_{0.28}$	11.3 $_{0.49}$	65.6 $_{1.35}$	2.6 $_{0.24}$	21.2 $_{1.19}$	1.0 $_{0.20}$	13.4 $_{0.69}$	62.2 $_{1.70}$	2.6 $_{0.27}$
Methanol	10	0.545 $_{0.013}$	117.8 $_{5.45}$	10.0 $_{1.04}$	+	18.6 ~~21.7~~ ~~1.23~~ $_{0.32}$	2.3 $_{0.11}$	10.7 $_{0.40}$	65.9 ~~63.3~~ ~~1.40~~ $_{0.51}$	2.6 $_{0.14}$	20.4 ~~20.5~~ $_{0.13}$	1.1 $_{0.15}$	14.0 $_{0.32}$	61.8 ~~61.5~~ ~~0.73~~ $_{0.11}$	2.8 $_{0.29}$

Subscript numerals = 1 standard deviation

Flour Mixed Under Varying Conditions

Table 8 shows the results of a series of experiments designed to study the effects of conditions known to affect dough rheology and lipid oxidation, and to help in the interpretation of the results in Table 7 on the previous page.

Oxygen and Nitrogen

The well known effects of oxygen on flour - water systems have been discussed in the literature review, and it was pointed out that the mechanisms are unknown, although much of the evidence favours uptake largely by enzymic lipid oxidations. By mixing sponges in atmospheres of oxygen or nitrogen the changes which may occur in the lipids due to the increased or decreased oxygen effect can be studied. In practice neither of the atmospheres was quite pure, but an adequate concentration of each gas was obtained to demonstrate the effects. The sponges showed changes in pouring characteristics comparable to doughs mixed under these gases, and in fact this observation applied to all sponges mixed with additives which affect dough rheology.

Enzyme Inactivation

In order to determine the contribution of enzyme activity to the FFA changes during mixing, it was decided to prepare flour in which the enzymes were inactivated but the lipids unaltered. This flour if used for mixing experiments would almost conclusively

establish whether the observed FFA changes were enzymic or not. As previously noted a first attempt to prepare the flour by autoclaving failed in that the lipids were altered. In a second attempt, flour was added to boiling methanol under nitrogen, and the methanol then evaporated off. The flour was well mixed, sieved and re-equilibrated with atmospheric moisture, and although slight changes in the lipids had occurred, as shown by the ultra violet spectra, the flour was adequate for the purpose intended. One group of mixing experiments with 10 minutes reaction time was done. This flour was peroxidase negative at all times, and it was assumed that all other enzymes had also been inactivated.

Sulphite

Sulphite or bisulphite is a reducing agent which has a drastic softening effect on doughs (78, 109, 126, 145), and is presumed to act by reducing intermolecular SS in gluten to sulphydryl and thiosulphonic acid (109). It is possible that sulphite could act as a preferential substrate for lipoxidase secondary oxidation since it has been found that the effect of sulphite in dough can be offset by addition of linoleic acid and mixing in the presence of oxygen. Sulphite was therefore added at a level which had previously been found to have a drastic softening effect on doughs made from the same quality of flour (126), that is 2 ml. 0.03M sulphite were added to 40 g. flour which was mixed with 78 ml. water.

N-ethyl Maleimide

SH blocking agents have an effect in dough similar to that of fast-acting improving agents such as iodate, and to some extent this has been explained by theories which centre on the SS and SH groups of gluten (52, 62, 111, 149, 155). It was expected that blocking SH groups would remove a secondary substrate for lipoxidase action, and at the same time inhibit all SH-dependent enzymes. Most workers have used N-ethyl maleimide (NEMI) or other SH blocking agents at a level corresponding to the SH content of flour which is about 1 - 1.5 μEq./g. flour (52, 111, 155). For the maximum effect on the rate of bromate decomposition about 10 times this level was required (33), and in this work it was decided to use this large excess to be certain of the maximum effect. 80 mg. NEMI/40 g. flour were dissolved in the mixing water just prior to mixing by the usual method.

Nordihydroguaiaretic acid

There is no known inhibitor of lipoxidase, but there are reports that nordihydroguaiaretic acid (NDGA) has the greatest effect (40 - 60% maximum inhibition) (24, 71). The optimum amount of NDGA for flour as measured by carotene bleaching corresponds to 240 mg./ 80 ml. mixing water (71), and this was prepared by dissolving in 2 ml. ethanol and adding to the water to form an emulsion. NDGA had a strong ultraviolet absorption with a maximum at 283.5 mμ in ethanol, and all absorption due to lipids was swamped

so that no routine spectra were obtained. An approximate measure of the NDGA in the aliquots was obtained spectroscopically, and by subtraction the weight of the lipids in the aliquots obtained. NDGA was also oxidised non-stoichimetrically by Hanus's iodine reagent in the method used in this work, and hence no iodine values were obtained from these experiments.

Bromate

Potassium bromate appears to be unique among flour improvers in that it is indirectly involved with lipids (42, 90). It was thought that FFA were possibly involved, so experiments with bromate were included. The rate of bromate reaction is slow, so 60 minutes reaction time was given to sponges mixed in air, containing bromate dissolved in the mixing water at the normal level of 30 ppm. of flour weight.

Model Systems Containing Oxidised Linoleic Acid.

Since the results in tables 7 and 8 show that there was a loss of FFA during the mixing experiments, and since the literature review suggests that lipoxidase activity could be a cause of this, it was considered necessary to study the behaviour of oxidised linoleic acid in the methylation and GLC stages of analysis. Since the first product of lipoxidase catalysed oxidation of linoleic acid is the same as that from autoxidation, namely conjugated linoleic acid hydroperoxides (48, 132, 133), autoxidation was used as the simpler method of preparing the

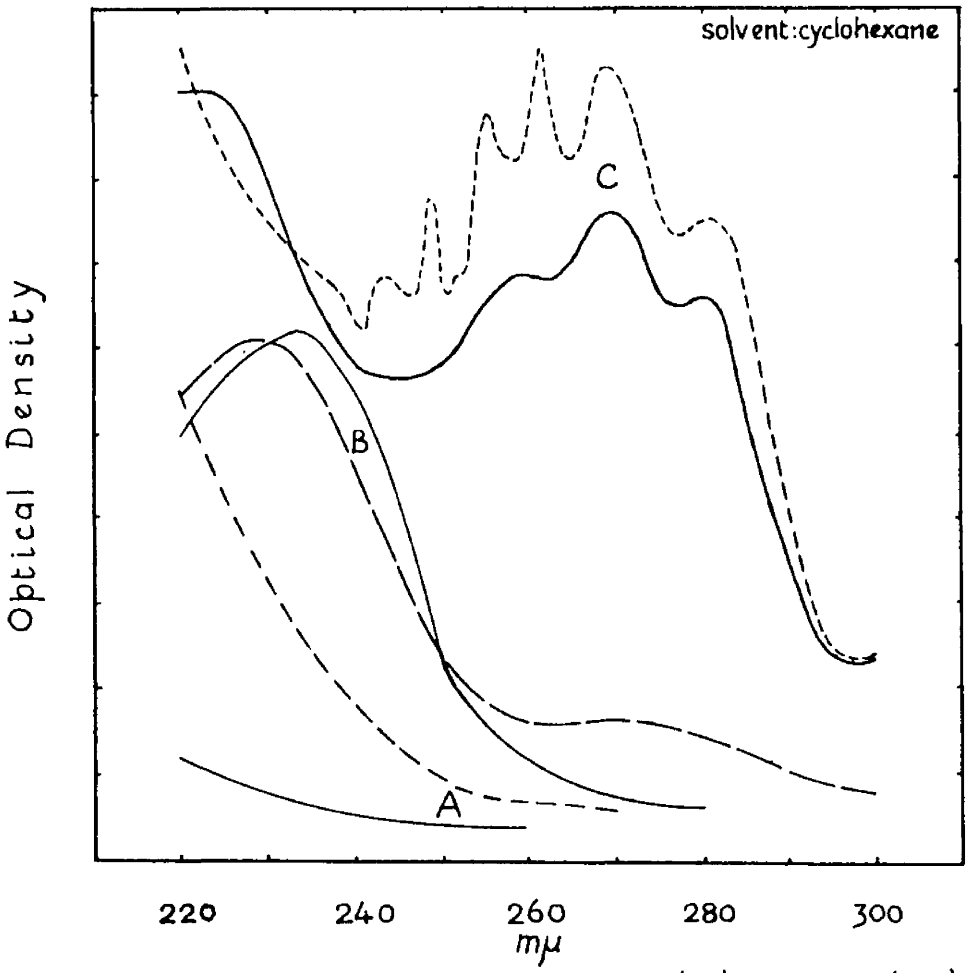
hydroperoxide. The behaviour of other oxidation products of linoleic acid will be discussed later.

A range of synthetic methyl esters of fatty acids (16:0, 17:0, 18:0, 18:1, 18:2) was prepared, and part of the mixture was allowed to autoxidise at room temperature till appreciable diene conjugation had developed, as shown by an absorption maximum in the UV spectrum at 232 - 234 mμ (Fig. 2).

Some of the autoxidised esters were treated with the boron fluoride-methanol reagent in order to determine the effect of this reagent on the hydroperoxide groups, as distinct from the carboxyl group which it is normally used to methylate. The treated esters were found to have a complex UV spectrum (Fig. 2), and there was no evidence of diene conjugation. Part of the complex spectrum was due to conjugated triene (131), which was presumably formed by boron fluoride catalysed dehydration of the conjugated linoleic acid hydroperoxides, with the formation of a new double bond conjugated to the other ones.

GLC of the two mixtures of autoxidised esters showed, by comparison with the original mixture, identical patterns of peaks in the chromatograms, with the addition of two small peaks due to conjugated trienes (mixed cis-trans and all trans) which appeared long after the unaltered esters (120, 123). It appears that the conditions in the GLC column also dehydrated the hydroperoxides to the same products as the boron fluoride-methanol reagent. It

Figure 2



UV Spectra of synthetic acids(—) & esters(----),
A: before autoxidation.
B: autoxidised.
C: autoxidised, boron fluoride/methanol effect.

was also shown experimentally that treatment of unoxidised linoleic acid with the strength of alkali used to saponify Estd.FA does not give rise to diene conjugation, in contrast to the effects of stronger alkali (44).

A similar series of experiments was conducted using free acids instead of esters. The free acids were found to autoxidise more readily, and in all cases gave similar UV spectra (Fig. 2), except that the conjugated triene spectrum was pure, as judged by three peaks at 260, 270, and 280 mμ which are identical in pattern to published spectra of conjugated trienes (123, 131). After methylation the esters formed from the acids were analysed by GLC, and comparison with the unoxidised mixture showed that a loss of ca. 4% linoleic acid had occurred, which is close to the content of conjugated linoleic acid hydroperoxide calculated from the UV spectrum. It was also confirmed that the GLC detector response was proportional to the molar concentration of esters. The GLC tracings showed peaks due to conjugated trienes, as in the previous experiments, but there were also two peaks due to cis-trans and trans-trans conjugated dienes which appeared after the linolenic acid, and these presumably were formed by autoxidation of oleic acid followed by dehydration of the hydroperoxide(s) in the same way as with linoleic acid.

The action of lipoxidase on linoleic acid is thought to produce a conjugated diene structure and free radicles, including

hydroperoxide radicles, which are oxidised in coupled reactions (25). The final products are linoleic acid hydroperoxides and a variety of other products which are conjugated, polymeric or degradation products (48). From the above experiments it was concluded that any lipoxidase activity giving rise to conjugation would be reflected in a loss of cis-cis linoleic acid in GLC tracings, and that there might be peaks due to conjugated dienes or trienes which would appear much later. Polymeric materials would not be expected to distil during the purification of the esters, and degradation products, such as C_9 aldehydes and ketones, would not be condensed along with the fatty acid esters. In GLC these products would not be expected to interfere, and would appear as a loss.

As will be shown later, there was also a loss of saturated acids through enzymic oxidations, and it is sufficient to say that the loss of more than 4 carbon atoms from the chain would result in acids which would not appear in the GLC tracings, or would give other products which would be most unlikely to interfere.

Thus, all but the most superficial enzymic oxidation of any FFA would appear as a loss of FFA in the GLC tracings in the method of analysis used in this work.

There is no published evidence of the effect of the boron fluoride-methanol reagent on long chain fatty acids containing conjugated unsaturation (110). The above results indicate that while hydroperoxide groups adjacent to unsaturated groups are

dehydrated with the formation of a new double bond conjugated to the original one(s), the resulting conjugation does not seem to be readily altered by the reagent, and is probably relatively stable.

DISCUSSION

In the following discussion enzyme activity is accepted as the prime cause of all the observed FFA changes. Since flour represents a biological system which has been almost completely disorganised, it is reasonable to expect enzyme behaviour in flour and water sponges to have some similarities to that in germinating grain, but on the other hand there will be considerable differences. The differences will include altered rates of reaction, and stray reactions which would be slight or absent in the grain. Further, certain reactions might not occur in the disorganised system. A true understanding of the enzymic processes can be had only by studying the flour itself, and since such data for lipid-oxidising enzymes in high grade flour is non-existent, close analogies with well defined enzyme systems cannot be drawn.

(a) The Analysis of Free and Esterified Fatty Acids in Flours

It should be noted when comparing the results in Tables 6 and 7 that minor differences between the "normal" flour figures are due to the use of two GLC columns. This does not apply to the later experiments.

The levels of FFA in fresh flour are in agreement with

published values (Table 3), and the higher levels in the older flours show that the storage temperature is not sufficiently low to prevent slow lipase action. There is a remarkable similarity in composition between FFA and Estd.FA, and from this it is concluded that the marked effect of FFA in doughs, compared with that of Estd.FA, is due to their being unesterified, and not to compositional differences.

Since the FFA and Estd.FA are very similar in composition, it seems likely that flour lipase(s) are non-specific in the position of the esters they hydrolyse, or the distribution of fatty acids in the hydrolysed esters is nearly random.

It is now possible to calculate the theoretical oxygen uptake of these flours, assuming that it is entirely due to lipoxidase activity using 1 mole oxygen/mole EFA (144). 30 g. flour contain 0.5 - 1.0% lipid, of which 10 - 15% is FFA, or 7 - 12% EFA. If all the EFA are consumed, and if there is no lipase action (which is the conclusion reached later in this discussion), the required uptake is 0.8 - 2.4 ml. oxygen/30 g. flour. This is well below the values found by experiment at even 10 minutes reaction time (40, 144), and since oxygen uptake continues for at least 30 minutes (39, 40, 144), it is concluded that this evidence supports the proposal that there are other lipid oxidations taking place concurrently. This is more conclusively shown by the analyses of FFA from mixing experiments, and is discussed in the next pages.

(b) Flour Lipid Constants

Tables 7 and 8 show that the iodine values of the lipid samples followed the trend of decreasing unsaturation shown by GLC analysis of the FFA. This is proof that the losses are real, or that they are due to considerable conjugation of diene and triene (which give low iodine values compared with theoretical values). Since the lipid samples showed no evidence of such conjugation (Fig. 1), it is concluded that there was a proportionately greater loss of unsaturated acids.

Taking round figures, the iodine value of the lipids was found to be 118, and the iodine value calculated from GLC data was 128. From this it is deduced that the unsaponifiable matter was 7.8%. This is somewhat higher than the figures given for crude flour lipids (Table 3), and is probably due to the fact that the lipids consisted of FFA, glycerol esters and sterol esters only, whereas the other lipids would also contain polar lipids in varying amounts.

Using the GLC data, it was also calculated that the mean molecular weight of the fatty acids was 275, and the saponification value of the lipids was 180. Using a microsaponification procedure, the saponification value was found to be 169 - 175, which compares with published values - (Table 3).

(a) Esterified Fatty Acids in Mixing Experiments

The GLC analyses of the Estd.FA in tables 7 and 8 are,

within 1 standard deviation, identical. This means that the Estd.FA were qualitatively unaltered during the experiments. When the weight of the FFA is deducted from the weight of lipid extracted from the samples, it is found that the remaining Estd.FA remained constant at 0.512 - 0.547%, at the 1 standard deviation level. From these considerations it is concluded that during mixing experiments with a reaction time of up to 60 minutes, and in some cases in the presence of abnormal conditions due to additives or altered oxygen tension, Estd.FA are not significantly altered, and can be presumed inert.

(d) Free Fatty Acids During Mixing Experiments

To obtain a better interpretation of the results they have been recalculated to show (i) FFA as a percentage of the total lipid, (ii) each acid as a percentage of the total lipid, and (iii) the ratio of the weight of each acid relative to the weight palmitic acid as unity. These figures are given in Table 9.

An approximate measurement of the diene conjugation, expressed as linoleic acid was made from the UV spectra of the lipids, taking 22,800 as the molar extinction co-efficient of cis-trans conjugated linoleate (133), and this is included in Table 9.

For the purpose of simplifying the calculations which follow, linoleic acid data are used to represent all the EFA, and the error of ca. 3% which is incurred is considered negligible compared with the magnitude of 1 standard deviation of the linoleic acid figures alone.

Treatment	Reaction Time, Minutes	FFA as Wt.% of Lipid	Diene Conjn. at 234 mμ		Weight % FFA in Total Lipid					Weight Ratio of FFA to Palmitic Acid (=1)				
			Optical Density	As % of 18:2 in FFA	16:0	18:0	18:1	18:2	18:3	16:0	18:0	18:1	18:2	18:3
Oxygen	10	8.0 $_{0.25}$	0.44	3.62	1.87 $_{0.14}$	0.168 $_{0.03}$	1.00 $_{0.05}$	4.84 $_{0.49}$	0.128 $_{0.04}$	1	0.09	0.535	2.59	0.069
Nitrogen	10	10.0 $_{1.04}$	-	-	2.02 $_{0.36}$	0.230 $_{0.05}$	1.05 $_{0.11}$	6.43 $_{0.82}$	0.250 $_{0.05}$	1	0.114	0.520	3.18	0.124
Sulphite	10	8.8 $_{0.51}$	0.20	2.17	1.90 $_{0.21}$	0.264 $_{0.03}$	1.12 $_{0.11}$	5.34 $_{0.56}$	0.206 $_{0.05}$	1	0.139	0.590	2.81	0.108
NEMI	10	8.8 $_{0.26}$	0.10	0.73	1.86 $_{0.09}$	0.246 $_{0.03}$	1.11 $_{0.05}$	5.39 $_{0.19}$	0.193 $_{0.02}$	1	0.132	0.597	2.90	0.104
NDGA	10	7.46 $_{0.54}$	-	-	2.17	0.224	1.10	3.83	0.135	1	0.103	0.507	1.76	0.062
Bromate	60	6.1 $_{0.37}$	-	-	1.37 $_{0.15}$	0.201 $_{0.04}$	0.774 $_{0.12}$	3.74 $_{0.19}$	0.134 $_{0.11}$	1	0.147	0.565	2.73	0.098
Methanol Flour	-	12.1 $_{1.61}$	0.08	0.7	2.06 $_{0.34}$	0.29 $_{0.08}$	1.398 $_{0.29}$	8.06 $_{0.24}$	0.314 $_{0.08}$	1	0.141	0.68	3.91	0.152
Methanol, Air	10	11.7 $_{1.32}$	0.08	0.7	2.01 $_{0.28}$	0.28 $_{0.07}$	1.274 $_{0.24}$	7.83 $_{0.99}$	0.292 $_{0.05}$	1	0.139	0.634	3.89	0.145

Subscript numerals = 1 standard deviation

(Continuation sheet)

Table 9

Changes in the Free Fatty Acids of Flour Mixed Under Varying Conditions

Treatment	Reaction Time, Minutes	FFA as Wt.% of Lipid	Diene Conjn. at 234 mμ		Weight % FFA in Total Lipid					Weight Ratio of FFA to Palmitic Acid (=1)				
			Optical Density	As % of 18:2 in FFA	16:0	18:0	18:1	18:2	18:3	16:0	18:0	18:1	18:2	18:3
Flour Control	-	10.9 ±0.89	-	-	2.19 ±0.33	0.195 ±0.04	0.99 ±0.12	7.25 ±0.73	0.228 ±0.05	1	0.089	0.451	3.34	0.104
" "	-	12.0 ±0.71	-	-	2.28 ±0.24	0.228 ±0.01	1.25 ±0.11	7.91 ±0.56	0.324 ±0.05	1	0.100	0.550	3.47	0.142
Air	½	11.1 ±0.43	0.16	1.45	2.37 ±0.26	0.177 ±0.03	1.10 ±0.11	7.25 ±0.45	0.21 ±0.03	1	0.075	0.454	3.06	0.089
"	2	11.7 ±0.56	-	-	2.33 ±0.25	0.234 ±0.03	1.29 ±0.12	7.62 ±0.47	0.246 ±0.05	1	0.100	0.554	3.27	0.105
"	10	6.5 ±0.29	0.16-0.24	1.45-2.17	1.55 ±0.16	0.182 ±0.03	0.812 ±0.08	3.88 ±0.21	0.079 ±0.03	1	0.117	0.524	2.50	0.051
"	60	6.2 ±0.44	-	-	1.41 ±0.20	0.198 ±0.03	0.786 ±0.09	3.68 ±0.33	0.124 ±0.01	1	0.140	0.557	2.61	0.088
Autoclaved Flour	0	0.22	-	-	0.045	0.0154	0.0407	0.1497	0.0046	1	0.342	0.905	3.32	0.102

(i) Mixing experiments in air.

The results in Table 9 show that there is no significant difference between the control and the experiments at ½ and 2 minutes reaction time, taking the 1 standard deviation level as the criterion of significance. The results at 10 and 60 minutes reaction time are significantly different from the control (but not from each other), and are the basis of this discussion and the later comparison of the effect of additives and oxygen tension. Unless otherwise stated, all mixing experiments referred to hereinafter were of 10 minutes reaction time, and all the information required for the calculations is derived from Table 9 or from references.

The methanol treated flour, which was enzymically inactive, and the same flour mixed in air, showed no significant difference in quantitative or qualitative analyses of their FFA. The same experiments with normal enzymically active flour showed large significant differences, and it is concluded that all the FFA changes observed in mixing experiments were enzymic in origin. Since oxygen uptake is almost entirely enzymic, and is mostly directed towards lipid oxidation, this finding is in accord with previous findings (p. 18) and constitutes a more direct proof.

On the basis of the results of the nitrogen experiment, it is assumed that lipase activity was slight or absent in short reaction times.

Since the relative ratios of palmitic : stearic : oleic

acids were constant throughout all the experiments, within the limits of 1 standard deviation, it is concluded that there was no saturase or desaturase activity affecting the original acids present.

The iodine value determinations show that the apparent losses of EFA were real, and the trend of the weight recoveries of lipids (Tables 7 and 8) indicate that FFA losses had occurred. Since there was no great overall loss of FFA in the nitrogen experiment, it is concluded that the overall losses of FFA in mixing experiments in air were not due to re-esterification.

From these considerations it appears that the observed losses of FFA must be attributed to enzymic lipid oxidations. The most probable enzyme systems are lipoxidase and β-oxidation. Although it is recognised that these terms should be broader and should include other possibilities, they are used for convenience in this discussion.

Lipoxidase is specific for EFA, that is those acids containing methylene-interrupted double bonds. β-oxidation is usually more specific for a range of fatty acid chain lengths, being unaffected by the unsaturation which may be present, but there is no information on β-oxidation specificity in wheat. It would be expected that both enzyme systems would act concurrently, and perhaps have a mutually competitive effect since they would both use EFA, and indirectly require oxygen.

(ii) Calculation of oxygen uptake, and activity of fatty acid-oxidising

(ii) Calculation of oxygen uptake, and activity of fatty acid-oxidising

Since the ratios of palmitic : stearic : oleic acids were

virtually constant, although losses of all acids had occurred, it is assumed that β-oxidation used these acids pro rata, and by extension EFA also. It is thus possible to calculate the loss by β-oxidation of these acids from the loss of palmitic acid. In the case of EFA, the ratios relative to palmitic dropped after mixing, due to lipoxidase activity, and to calculate the losses due to β-oxidation the average of the ratios to palmitic before and after mixing is used.

Thus, for flour mixed in air, the loss of palmitic acid was 2.28 - 1.55 = 0.73%, and the average relative ratio of linoleic acid to palmitic acid was (3.47 + 2.50) ÷ 2 = 2.985. The loss of linoleic acid due to β-oxidation was thus 0.73 x 2.985 = 2.18%. The total loss of linoleic acid was 7.91 - 3.88 = 4.03%, and by difference the loss due to lipoxidase was 1.85%.

Since linoleic acid has been taken as representing all EFA, the total lipoxidase activity is calculated to have used 1.85% EFA. The total loss of FFA was 12.0 - 6.5 = 5.5%, so that, by difference, the total β-oxidation activity used 3.65% FFA. This and similar calculations for the other experiments are summarised in Table 10.

It is now possible to calculate the approximate oxygen requirements of these lipid oxidations. On the basis of 1 mole oxygen/mole EFA (144), lipoxidase activity would require 0.26 ml. oxygen/30 g. flour. On the assumption that each acid contained

8 oxidisable C_2 units, and that the system would require 1 mole oxygen/C_2 unit, β-oxidation would require 4.07 ml. oxygen. The total oxygen requirement of these lipid oxidations would thus be 4.33 ml. oxygen. This is close to the observed values at 10 minutes reaction time, and is evidence that the assumed lipid oxidations are a reasonable explanation of the experimentally determined oxygen uptakes of flour-water mixtures (40, 144). It is also interesting to note that there was very little FFA oxidation after 10 minutes reaction time, and that oxygen uptake and oxidation of SH are also largely completed in the same time.

The relative activity of lipoxidase oxidation to β-oxidation based on the FFA oxidised was 1 : 1.2, and based on the oxygen consumed 1 : 15.6.

In the mixing experiment on which the above calculations are based, 1.45% of the EFA was found to be conjugated (Table 9), whereas 23.4% of the EFA was accounted for by lipoxidase activity. Conjugated linoleic acid derivatives can therefore represent only a transient state in the lipoxidase-initiated attack of linoleic acid in flour-water sponges mixed aerobically.

Since it has been shown that all the EFA are not oxidised in even 60 minutes reaction time, the conclusion arrived at in the first part of the discussion (p. 51) is substantiated, as regards high grade flours of relatively low FFA content. The behaviour of a range of lower grade flours remains to be studied, and with the

Table 10

The Calculated Lipoxidase and β-oxidation Activities During Mixing Experiments, Expressed as a Percentage of the Total Lipid

Mixing Experiment	Reaction Time, Minutes	EFA Used by Lipox.	EFA Used by β-oxidn.	FFA Used by β-oxidn.
Air	10	1.85	2.18	3.65
Nitrogen	10	0.62	0.86	1.38
Oxygen	10	1.81	1.26	2.19
Sulphite	10	1.38	1.19	1.82
NEMI	10	1.18	1.34	2.02
NDGA	10	3.79	0.29	0.75
Air	60	1.59	2.64	4.21
Bromate	60	1.35	2.82	4.55

aid of more elaborate and extensive experiments more general conclusions may be arrived at.

(iii) Oxygen requirements of known oxidation reactions in flour.

It is known that the carotenoids of flour are partially bleached in a coupled oxidation involving EFA and lipoxidase, or some similar enzyme (71). It is also known that some oxidation of SH occurs (149), and that it is largely due to secondary oxidation by lipoxidase (145). It follows from these statements that the calculated oxygen requirements should fall within the calculated value for lipoxidase activity, and since there are no other major oxidations which are known, it might be expected that the discrepancy would not be large. The carotenoids of flour are mainly xanthophyll (21, 22, 168), and are present at 0.25 - 4.0 ppm. of flour weight (22, 168). The carotenoids of the flour were extracted with water-saturated n-butanol, and found spectroscopically to be equivalent to 1 ppm. of the flour weight. Only about 30% of the carotenoids are bleached in a normal dough mixed in air (71, 111).

If the carotenoids, calculated as xanthophyll, were bleached by lipoxidase using 1 mole oxygen/mole carotenoid, the oxygen required would be 0.000394 ml./30 g. flour. Taking 10 times this level as a reasonable maximum for the actual bleaching, the uptake of 30 g. flour would be 0.00394 ml.

Flour contains ca. 1.5 μEq. SH/g. (4, 5, 31, 149), and during aerobic dough mixing ca. 30% of the SH is oxidised in 10

minutes, after which little further oxidation occurs (145, 149). Assuming the equation

$$2\ RSH + EFA + O_2 \xrightarrow{\text{lipoxidase}} 2\ RSSR + \text{etc.}$$

the loss of 15 μEq. SH in 30 g. flour during 10 minutes mixing and reaction would require 0.168 ml. oxygen, which is equivalent to 1.21% EFA.

These calculations show that the loss of EFA due to lipoxidase activity is adequate to promote known secondary oxidations, and that it is unlikely that other major secondary oxidations occur at the same time. Since SH is the main substrate for iodate and bromate oxidations, it would be expected that they would act competitively with lipoxidase, and there is evidence of this happening with bromate (33, 36, 42).

From the above discussion it seems probable that the oxygen uptake of flour during mixing is largely due to enzymic lipid oxidations, which are caused by enzyme systems of the lipoxidase and β-oxidation types. Lipoxidase is known to cause secondary oxidation of flour SH, and is thus connected with the oxidative improvement of dough quality (145). In the following discussion, the results of experiments, in which additives which affect dough quality were present, are discussed with particular reference to the probable lipoxidase activity of the sponges. The effect of one antioxidant is also discussed.

It has been shown above that the experimental analysis

of FFA from flour and flour-water sponges mixed in air show differences which can be related to the known oxygen uptake of flour during mixing, if it is assumed that two concurrent FFA oxidations occur - namely, lipoxidase oxidation of FFA, and β-oxidation of FFA. The calculated level of lipoxidase activity corresponds with that required for known secondary oxidations of carotenoids and SH. In the light of these findings, the later experiments, where flour was mixed under varying oxygen tensions or in the presence of certain additives, can now be considered. Since lipoxidase activity, SH oxidation or reduction, and dough strength are all inter-related, and since all the experimental conditions are known to affect at least one of these factors, there should be some alteration in the calculated lipoxidase activity, compared with normal aerobic mixing, in every case.

(iv) The effect of oxygen tension.

Reduced oxygen tension was obtained in the case of the nitrogen experiments, since it was known that all the oxygen could not be purged, and there was evidence of slight oxidation of FFA in the results. The previously discussed experiments mixed in air represented normal oxygen tension, and the experiments mixed in oxygen represented an atmosphere of at least 90% oxygen.

From Table 10 it can be seen that lipoxidase activity changed with increasing oxygen tension, using 0.62 - 1.85 - 1.81% EFA in each case. Taking into account the magnitude of 1 standard

deviation of the weight per cent of FFA recovered, it can be said that there was an increase in lipoxidase activity when there was a change from low to normal oxygen tension, but there was no significant change on further increasing the oxygen tension. Since oxygen can oxidise SH directly under special circumstances (defatted flours mixed under impure nitrogen, air or oxygen), oxygen might compete with lipoxidase for SH in flour mixed under oxygen, and slightly suppress lipoxidase activity, in which case the slight drop in calculated lipoxidase activity from 1.85 to 1.81% FFA consumed could be real, although there is no statistically significant difference.

From Table 10 it can also be seen that β-oxidation was affected by increasing oxygen tension, and 1.38 - 3.65 - 2.19% FFA was oxidised in the three experiments. This rather unexpected behaviour is undoubtedly real, since there are parallel changes in both the palmitic acid and oleic acid recoveries, the former being significantly different. The stearic acid figures cannot be given any weight because of the difficulty of measuring the stearic acid chromatogram peaks accurately. There is no apparent explanation of this inverse effect of higher oxygen tensions on β-oxidation, although a possible explanation has been put forward above in the case of lipoxidase.

(v) The effect of sulphite.

Sulphite, or more usually bisulphite, is added to flour

before mixing in order to greatly weaken the dough formed. It is believed that this effect is due to a reduction of intermolecular SS in gluten, which weakens the structural rigidity, and increases the ease of disulphide-sulphydryl (SS - SH) interchange reactions by forming new SH (78, 109, 126, 145). The reaction is:

$$RSSR + HSO_3^- = RSH + RSSO_3^- \quad (109)$$

At the pH of sponges (5.5 - 6.5) sulphite exists as bisulphite ions, and acts according to the above equation.

The amount of sulphite added was capable of reducing SS to produce 45 μEq. SH and 45 μEq. thiosulphonic acid in 30 g. flour, according to the above equation. Thus the total SH content of 30 g. flour was 90 μEq., and the lipoxidase activity (Table 10), which used 1.38% EFA, was capable of oxidising only 17.3 μEq. SH. There was therefore a large overall increase in the SH content of the flour, which was adequate to cause drastic weakening of the sponge or dough. This conclusion was supported by the fact that the sponge was very thin and runny.

The thiosulphonic acid groups formed from the sulphite might be thought of as blocked SH groups, but the SS - SH interchange system would be unaffected since there was still a large excess of SH present.

From the above considerations it might have been expected that there would have been more lipoxidase activity, due to there

being more oxidisable SH present. This could have led to decreased β-oxidation in a limited oxygen supply. Table 10 shows a proportionate increase in lipoxidase activity, compared with β-oxidation, but both oxidations were at a considerably lower level than in the sponge mixed without additives in air. This is a surprising result, since if oxygen was directly absorbed by all the sulphite to form sulphate, thereby reducing the enzymically available oxygen tension to a level below normal, only 0.504 ml. oxygen would have been removed, whereas the drop in total calculated oxygen uptake was 1.86 ml.

(vi) The effect of N-ethyl maleimide.

N-ethyl maleimide (NEMI) combines irreversibly with SH, and is commonly used to inhibit SH-dependent enzyme reactions. In the sponges considered here the reactions inhibited include β-oxidation (blocking of the SH of Coenzyme A), and secondary oxidation of SH by lipoxidase. Table 10 shows that there was ca. a 50% decrease in β-oxidation, and ca. a 30% decrease in lipoxidase activity, compared with the experiments without additives mixed in air. It is known that NEMI has no effect on the coupled bleaching of carotenoids (111), and the calculated lipoxidase activity was quite adequate in these experiments for normal carotenoid bleaching (cf. section (c) above). If it is assumed that all available gluten-SH were blocked, there is no evidence to suggest the mechanism of the still considerable calculated lipoxidase activity and the coupled oxidations which took

place. Coupled oxidations or destruction of linoleic acid hydroperoxide must have occurred extensively, since there was little diene conjugation present in the lipids (Table 9). β-oxidation was not totally inhibited by NEM, and unless some other FFA oxidation independent of SH occurred, this finding must be attributed to the very incompletely understood conditions pertaining in flour-water sponges.

(vii) The effect of nordihydroguaiaretic acid.

Nordihydroguaiaretic acid (NDGA) was added in an attempt to partially inhibit lipoxidase activity, since it has been shown to prevent carotenoid bleaching, presumably by preventing coupled oxidation by lipoxidase. Table 10 shows, however, that there was about double the lipoxidase activity and 1/6th of the β-oxidation which occurred in the control experiments mixed without additives in air. NDGA therefore had a pro-oxidant effect on lipoxidase in these circumstances. It has recently been shown that in larger amounts (which was the case in the experiments) NDGA accelerates the rate of decomposition of linoleate hydroperoxide (22, 132). The results are therefore consistent with the effects of large doses of NDGA on hydroperoxides, if the accelerated breakdown of linoleic acid hydroperoxide in flour could lead to accelerated formation of the hydroperoxide by lipoxidase by disturbing the reaction equilibrium.

Table 10 also shows that β-oxidation was greatly depressed, and since the increased uptake due to lipoxidase was of the order

of 0.26 ml. oxygen/30 g. flour, this would not sufficiently reduce the oxygen tension in the sponge and have this effect. A possible explanation is that the very heavy dose of NDGA had an inhibitory effect on β-oxidation, since it is known to have this effect on a range of enzymes (106a). Hence the lipoxidase activity might be unaffected, or represent very great activity depressed to the observed level.

The oxygen uptake of the sponge was calculated to be 1.36 ml./30 g. flour, which, as the sole criterion, would suggest that the NDGA had had an antioxidant effect. Since it is necessary to invoke NDGA-catalysed decomposition of hydroperoxides to explain the results, it is feasible that this would prevent secondary oxidation of carotenoids at the same time.

(viii) The effect of bromate.

In doughs bromate oxidises SH more or less specifically (31, 33), and the rate at which this occurs is rather slow. In a dough containing 0.90 mg. bromate/30 g. flour (corresponding to 30 ppm. bromate of flour weight), 0.12 mg. bromate is decomposed in 60 minutes (32, 33), which is equivalent to the oxidation of 4.33 μEq. SH. It is assumed that the rate of bromate decomposition is similar in sponges, in which case the bromate oxidation is assumed to have been as above. The lipoxidase activity (Table 10) was equivalent to 1.35% EFA, which would have oxidised 16.3 μEq. SH,

so that the total SH oxidised would have been 20.6 μEq. This represents an increase over the 15 μEq. of SH which is normally oxidised (145, 149), and would have an improving effect. However, the values for lipoxidase at 10 and 60 minutes reaction time differ by nearly the same amount as the values at 60 minutes with and without bromate, so that no weight can be given to these figures. The only conclusion which can be drawn is that there is no evidence from these experiments to suggest that bromate action is involved with FFA (33, 36, 42), other than by competition with lipoxidase oxidation of SH - which is the conclusion arrived at in the literature review (pp. 20, 21).

There are also differences in the amounts of β-oxidation in the experiments at 10 and 60 minutes, and at 60 minutes with bromate added. The recoveries of palmitic acid on which these are based are not significantly different, and although the trends may be real, no conclusions have been drawn.

(e) Suggestions for Future Investigations

From the literature review and work in this thesis it is apparent that there is a relationship between oxygen uptake, FFA oxidations, secondary oxidations of carotenoids and SH, and oxidative improvement of dough strength. The study of any one of these subjects is a major undertaking, and each has already occupied cereal chemists for many years without being fully understood.

Nevertheless the information which could be gained from a simultaneous study of all these aspects of the chemistry of a piece of dough would be so valuable that the effort would be well worth while. While these are the most important reactions and aspects of the problem of dough improvement, future work may uncover other important reactions and lead to new discoveries concerning dough structure and rheology.

Very little is known about the enzymes which oxidise FFA and EFA in flour, and this will be a difficult but rewarding field for investigation. Most studies are confined to high grade flours, but an extension to include all grades and types of flour is desirable, and this may eventually lead to an understanding of why different flours and wheats have such different dough properties. The technological and economic significance of this has already been mentioned.

The field is not a new one, but the approach to one small aspect, which has been the purpose of this thesis, has given a new insight into the problem, and there are several obvious lines of investigation to follow which must surely yield valuable results.

(f) Conclusions

1. The literature concerning the uptake of atmospheric oxygen by flour-water sponges or doughs was reviewed, and it was deduced that, contrary to what has been assumed by other authors, the uptake of oxygen cannot be solely attributed to lipoxidase-

catalysed oxidation of linoleic and linolenic acids. This was proved experimentally by showing that there is not enough of these acids present to permit the observed oxygen uptakes.

2. It was shown that during aerobic mixing of flour-water sponges changes occurred in the composition of the free fatty acids, but no changes were observed in the esterified fatty acids during reaction times up to 60 minutes.

3. The changes which occurred in the free fatty acids were shown to be due to enzymic oxidation of both essential and other fatty acids.

4. The free fatty acids and esterified fatty acids contain very similar proportions of palmitic, stearic, oleic, linoleic and linolenic acids, and there is nothing to explain the marked effect of free fatty acids as opposed to esterified fatty acids in doughs mixed aerobically, apart from their being unesterified.

5. From an analysis of the recoveries of free fatty acids in mixing experiments it was deduced that two types of free fatty acid oxidation occurred simultaneously, and that these were most probably lipoxidase oxidation of essential fatty acids and β-oxidation of all free fatty acids, or reactions of a similar nature.

6. The known oxygen uptake of flour can be satisfactorily accounted for by the oxygen requirements of these types of enzymic oxidation at the levels found in the experiments.

7. The level of lipoxidase activity which was deduced from the experimental results is in agreement with that required for known lipoxidase-dependent oxidations of carotenoid pigments and sulphydryl groups.

8. Experiments were conducted with the addition of substances which affect the rheological properties of dough, or lipoxidase activity, and the results were discussed in terms of the probable lipoxidase and β-oxidation activity. Most of the results were readily explained, but some points remained inexplicable.

9. Flour lipids were analysed, and it was shown that gadoleic acid is present in significant amounts only in the esterified acids. Storage at 0°- 4° is not sufficient to prevent slow lipase hydrolysis of esterified fatty acids esters. Flour lipase(s) are either non-specific, or the hydrolysed esters are of nearly random fatty acid composition. Lipid constants were calculated from the results, and found to agree with published values. They were:

Iodine value	118
Unsaponifiable matter	7.8%
Mean mol. wt. of fatty acids	275
Saponification value	180
Phosphorus content	less than 0.02%

10. Suggestions for the further development of the study of free fatty acid oxidations during mixing processes have been made.

PART IV

DEVELOPMENT OF METHODS OF ANALYSIS

DEVELOPMENT OF METHODS OF ANALYSIS

To simplify the reading of the actual experimental work and the methods of analysis used therein, all the development work necessary to arrive at these methods has been combined to form Part IV of the thesis.

(a) Solvent Extraction of Flour Lipids

Two problems had to be solved, namely how to consistently extract all the lipids from both flour and freeze-dried dough, and how to remove water, sugars, proteins, polar lipids and other extraneous material by a simple and rapid method. In the first instance it was useless to examine non-polar solvents since they cannot extract all lipids, especially from freeze-dried doughs (129). In a comparison of defatting by percolation with ethanol-diethyl ether 2 : 1 (v/v), and by methanol, followed by methanol-chloroform 1 : 1 (v/v) the yields were 1.044% and 1.053% of crude lipid respectively. Since methanol and chloroform form an azeotrope of lower boiling point than either component they are easy to remove, and since residual methanol is of a lower boiling point than residual ethanol, the ethanol-diethyl ether system had disadvantages and was not further considered.

In the next step methanol-chloroform percolation was compared with extraction using water-saturated n-butanol by the method of Mecham and Mohammed (113), in which an aliquot of butanol

was taken from a centrifuged mixture of flour and butanol. In both cases the crude lipid was re-extracted with chloroform, dried with anhydrous sodium sulphate and filtered through a No.44 Whatman filter paper, or a pledget of cotton wool. The butanol lipids were invariably cloudy, and both systems gave irregular yields and nitrogen : phosphorus (N : P) ratios on analysis of the lipids.

	Methanol-chloroform	Water-satd. n-butanol
Crude lipid yield	1.02 - 1.36%	1.22 - 1.24%
N : P ratio	2.11 - 3.32	1.39 - 2.26

These results are obviously unsatisfactory, and since they were thought to be due to phospholipid, galactolipid and protein variations it was decided to proceed with silicic acid chromatography using the method of Hirsch and Ahrens (77) as applied to wheat lipids (49).

The FFA and Estd.FA components of wheat lipids are eluted from silicic acid by varying amounts of ether in light petroleum up to 100% ether, and polar lipids are eluted by varying amounts of methanol in ether (49). The chromatography was therefore limited to a single elution with ether. At the same time a comparison was made by adding silicic acid to an ethereal solution of crude lipids and then removing it by filtration - this is analogous with the same process using chloroform as solvent (137), but when tried, chloroform did not retain all the desired lipids in solution as shown by low yields. The results of these comparisons were:

Solvent Extraction and Silicic Acid Method	Yield, % of Flour Wt.	% Phosphorus
Methanol-chloroform, chromatography	0.603 - 0.673	0.0068 - 0.0162
Methanol-chloroform, filtered	0.578 - 0.588	0.0092 - 0.0093
Butanol aliquot, chromatography	0.603 - 0.645	0.0159 - 0.0189

The results from the phosphorus analyses are satisfactory and there are only slight variations of yield which do not matter in a comparative study. As butanol is a difficult solvent to remove methanol-chloroform was preferred, and since the procedure of adding silicic acid to an ethereal solution of crude lipid is both simple and rapid it was also adopted. The silicic acid effectively dries the lipids in solution if the bulk of the water removed from the flour by the methanol is substantially distilled off with the solvents.

Previous attempts to remove polar lipids by acetone precipitation or by dialysis (15) were unsatisfactory and are not reported.

Comparison of the infra-red spectra of crude lipids and silicic acid-filtered lipids showed that the silicic acid had completely removed all absorption due to -CO.NH-, -C-N-, -P=O, and that there was no evidence of trans unsaturation. This evidence

showed that the lipids were free from phospholipids and polypeptides, and that changes in fatty acid structure had probably not occurred.

(b) Weighing of Aliquots

Initially "solvent-free" lipids were weighed for phosphorus analyses, but erratic results were eventually traced to the hygroscopic nature of phospholipids and to the difficulty of removing last traces of lipid solvents from bulk lipids. When aliquots of lipid solution in ether were used excellent replication was obtained, and the weighing of small aliquots for calculation purposes proved satisfactory. The procedure for weighing given on pp. 30, 31 was adopted without development troubles.

(c) Phosphorus Determination

In the analysis of "phospholipid-free" lipids the material analysed for phosphorus was almost entirely hydrophobic and caused difficulties during digestion to destroy organic matter by wet oxidation. The use of perchloric acid for wet oxidation of organic matter, as in the method of Allen (1), proved unsuitable because of losses due to spurting and the colour of chlorine compounds which were confused with the charring of organic matter. Digestion with sulphuric acid and hydrogen peroxide to destroy organic matter according to the method of Harvey (69) was satisfactory with casein and similar materials, but with lipids a lot of material steam-distilled up the walls of the digestion tube and again caused

difficulties in completing digestion. The method of Beveridge and Johnson (18) uses a relatively large amount of sulphuric acid and this dissolves triglycerides and gives an easy digestion. This method was therefore adopted, and the only further trouble was caused by insufficient shaking when adding the hydrogen peroxide. Microkjeldahl flasks were found to be better than test tubes for digestions.

There are many methods for the formation of heteropoly blue from the orthophosphoric acid-molybdate complex, but the method of Fogg and Wilkinson (51) appealed because it gives a very stable blue of high optical density. Although the method of Beveridge and Johnson (18) destroys excess hydrogen peroxide which would interfere with the formation of the heteropoly blue, sodium sulphite as used by Fogg and Wilkinson was retained as an extra safeguard - it has no interfering effect (51). No further troubles were experienced, and the final method is given on pp. 31-33. Tests showed that the optical density developed from a standard amount of phosphorus was unaffected by a twofold increase of any reagent, and the method is thus fairly proof against errors of measurement of reagents.

(d) Recovery of Free Fatty Acids

Since it was decided to use silicic acid to remove polar lipids, the best way to isolate FFA appeared to be by selective elution from a chromatographic column. Hirsch and Ahrens (77)

have done so, but others have failed to elute FFA as a pure class, probably due to less elaborate precautions during the preparation of the column (28, 166). In preliminary trials, titration of column eluates of wheat lipids chromatographed according to Fisher (49) showed that the FFA were spread over a range which included triglycerides. Attention was then given to obtaining FFA from the lipids which had been treated by the silicic acid filtering technique which had been adopted.

Ion exchange resins were abandoned after trials in which some of the following faults were observed:

1. Ethyl esters can be formed when eluting FFA with ethanolic hydrochloric acid (140).

2. Recovered acids are partially decomposed, and recovery is incomplete (28).

3. FFA methylated on the resin are not eluted in the same proportions as in their original analysis. Recoveries with $C_{17:0}$ taken as 100%, being $C_{12:0}$ = 41% to $C_{18:2}$ = 172% (81)

4. Ethanolic hydrochloric acid dissolves resin material which can be precipitated from the acids at a later stage.

The long-established method of alkali washing to remove FFA (110) was tried and adopted. The possibility that poor recovery of FFA or oxidative changes might occur was not realised, even when

no precautions were taken to exclude oxygen. The method is given in detail on pp. 34 and 35. The possibility of preferential extraction of some acids (or soaps) from one phase into another was also checked, and found not to occur. Similar results were obtained when extraction of methyl esters after methylation was examined.

(e) Preparation of Methyl Esters

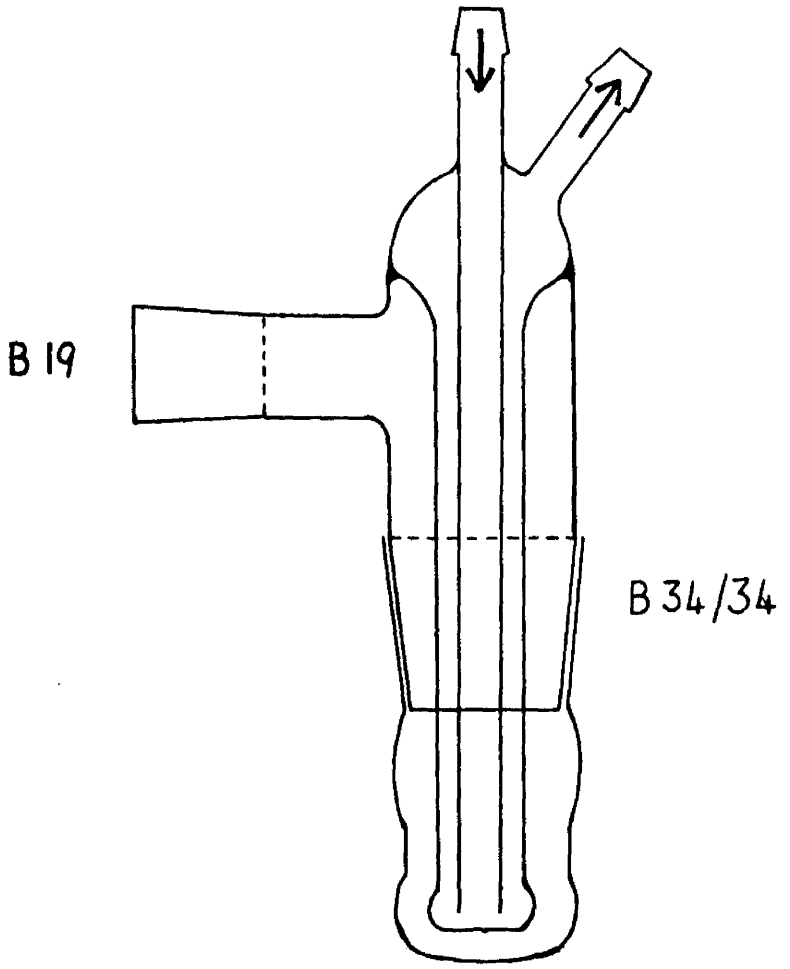
Although FFA can be analysed directly by GLC (117), the use of their methyl esters permits of easier purification by vacuum distillation, and lower temperatures for GLC with less risk of thermal decomposition and much longer column life. Trans-esterification as a means of forming methyl esters (19, 101, 107, 163) was only examined in the case of the boron fluoride-methanol reagent (43), but with a refluxing time of 1 hour only 92% yield was obtained and the GLC analysis was not the same as that from the adopted procedure. This method was not examined further at the time, and methylation of FFA was preferred. Since it had already been shown that diazomethane forms artefacts which interfere with GLC of fatty acid methyl esters (127) the boron fluoride-methanol reagent (117) was used as described on pp. 36 and 37. No difficulties were encountered with this reagent and it was stable for at least several months (117). As with all acid-catalysed methylation procedures it is necessary to use anhydrous methanol, and the special grade for Karl Fisher moisture determination was found to be most convenient for preparing

the reagent.

(f) Vacuum Distillation of Methyl Esters

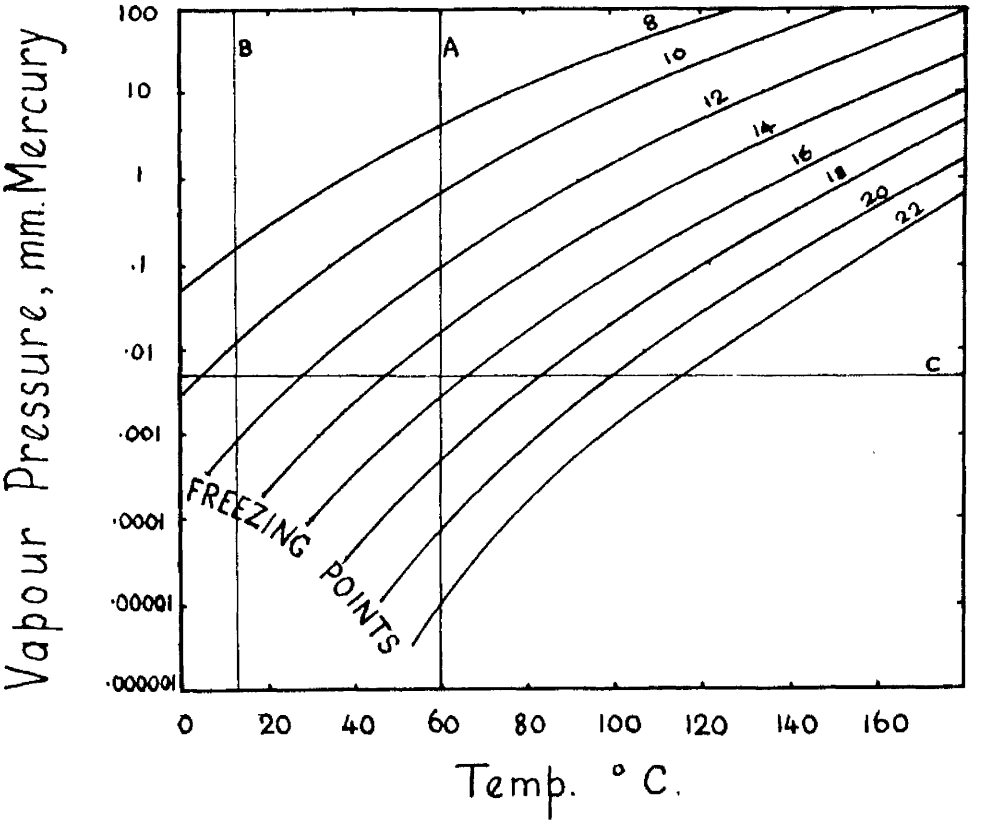
Conditions for micro-scale vacuum distillation of methyl esters (128) have been given for an American type of apparatus (151) which was not described in detail. Accordingly a simple pot still was designed as shown in Fig. 3, and optimal working conditions arrived at by experiment. Fig. 4 shows the vapour pressure/temperature relationships of the C_{10} to C_{20} saturated fatty acid methyl esters (91). Those esters with double bonds and hydroxyl groups for which data are given have vapour pressure properties very close to the corresponding saturated ester (91, 107). The working conditions described (151), and those used in this work, were generally $60^{\circ} \pm 2^{\circ}$ at pressures of 0.05 to 0.005 mm. Hg. Under those conditions esters of C_{14} and lower acids boil, and with C_{10} esters there is considerable danger of the esters not being retained on the condenser at 10 to 15° (163). The C_{16} and C_{18} esters are however the ones of chief interest in this work, and they do not boil under these conditions of distillation. They can therefore only be obtained by a slower process governed by the rate at which the condenser removes esters from the vapour state, and the rate at which the heated pot supplies esters to the vapour state. Higher vacuum could not be obtained with the pump in use, which was an Edwards' Speedivac Model 18C 50 B, and in any case more severe conditions would have increased the

Figure 3



Vacuum Distillation
Pot Still - ½ Scale

Figure 4



A: Pot Temp. B: Cold Finger Temp. C: Working Press.

likelihood of sterols and perhaps other materials distilling.

The mean free path of air is given approximately by

$$\text{mean free path length (cm.)} = \frac{5 \times 10^{-3}}{\text{Pressure (mm.Hg.)}}$$

At 0.05 mm. pressure the mean free path is 0.1 cm. and at 0.005 mm. pressure the mean free path is 1.0 cm. The gap between the condenser and the pot is ca. 0.5 cm. so that, during distillation, conditions approach molecular distillation in which the gap between the condenser and pot is less than the mean free path of the molecules in the vapour state.

Loads of esters of ca. 5 mg. (from FFA) appeared to distil completely in less than 10 minutes, and loads of 40 to 60 mg. (from Estd.FA) in 30 minutes. In practice at least 30 minutes distillation at 60 - 65° and full vacuum (0.005 mm.) were used. There was no evidence of oxidation of the unsaturated esters at any time, and all esters were colourless.

(g) Gas Chromatography and Analysis of Fatty Acid Methyl Esters.

The Pye argon gas chromatograph which was used for analysis of fatty acid methyl esters was made available by Mr. J. Wight of the Food Science Department in which the work in this thesis was done, and no development work was required.

The resolution obtained on the column appeared to be as good as any published, or seen elsewhere, for a column of only 4 ft.

length. Fig. 5 shows two typical chromatograms, 165A from FFA, and 175C from Bstd.FA. The base-line to each peak has been drawn in, together with the starting line from which all measurements of retention times were made. Fig. 6 shows a calibration plot of retention times (measured as chart distance, the chart travelling at 1"/10 minutes) against the measured standard deviations of a range of chromatogram peaks. The standard deviation was taken as the breadth of the peak at 0.885 x peak height, or as $\frac{1}{2}$ the breadth at 0.607 x peak height, or as 1/3 the breadth at 0.332 x peak height. Although a straight line was drawn, there was a sinusoidal scatter which was believed to be a feature of the recorder, since the standard deviation was found to vary with the peak size (Fig. 7) independently of the load of esters or detector voltage. At certain retention times the peaks were always either small or large, because of the fairly constant gas flow rate used and the constant column temperature, hence the sinusoidal calibration pattern.

It is sometimes necessary to apply corrections to obtain the true areas of overlapping peaks, but this was not necessary in the case of stearic and oleic acids, which were the only ones to overlap slightly in the chromatograms.

The method of calculating peak areas (11, 146) and ester composition used here is better than most other methods (including triangulation of the peaks, planimetry, and cutting out and weighing

Fig. 5

165A

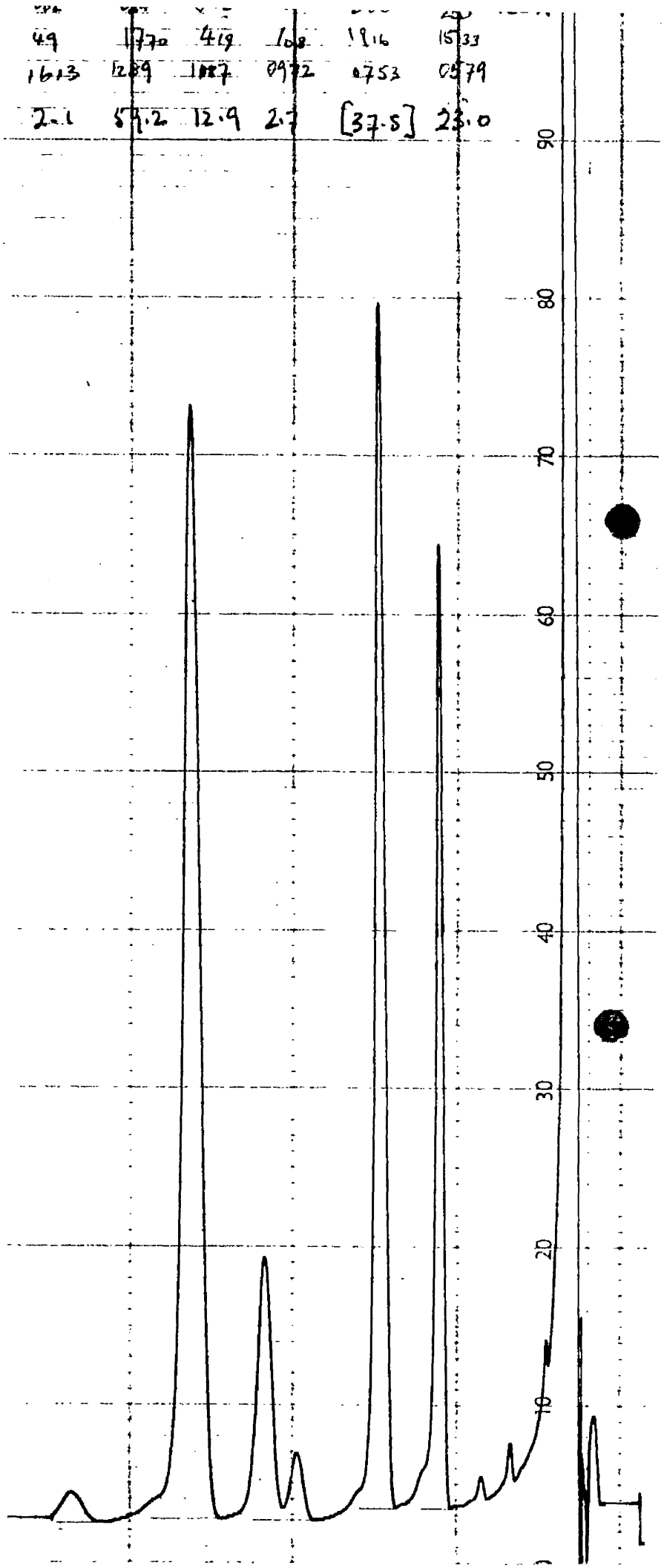

49 1770 419 108 1916 1533
1613 1289 1087 0972 0753 0579
2.1 59.2 12.9 2.7 [37.5] 23.0
90
80
70
60
50
40
30
20
10

Fig. 5
1750

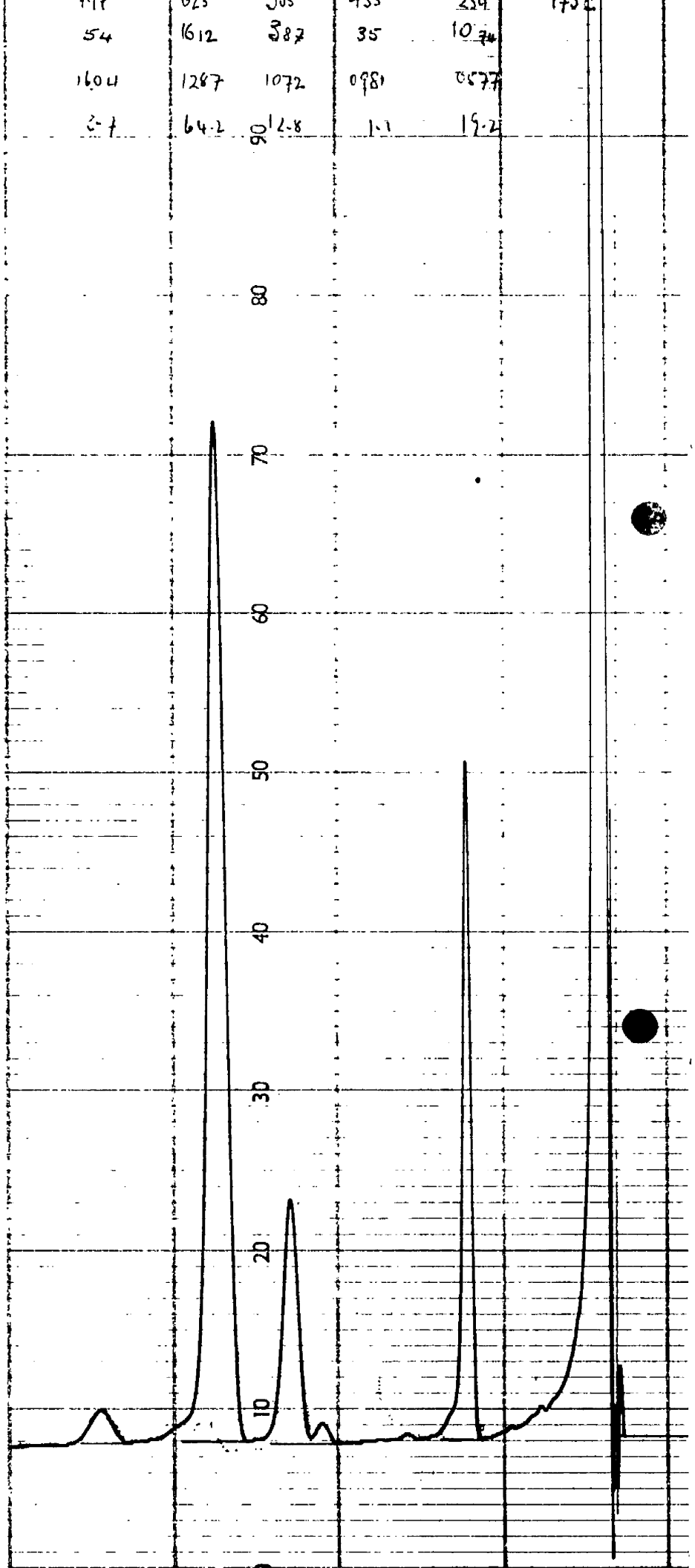
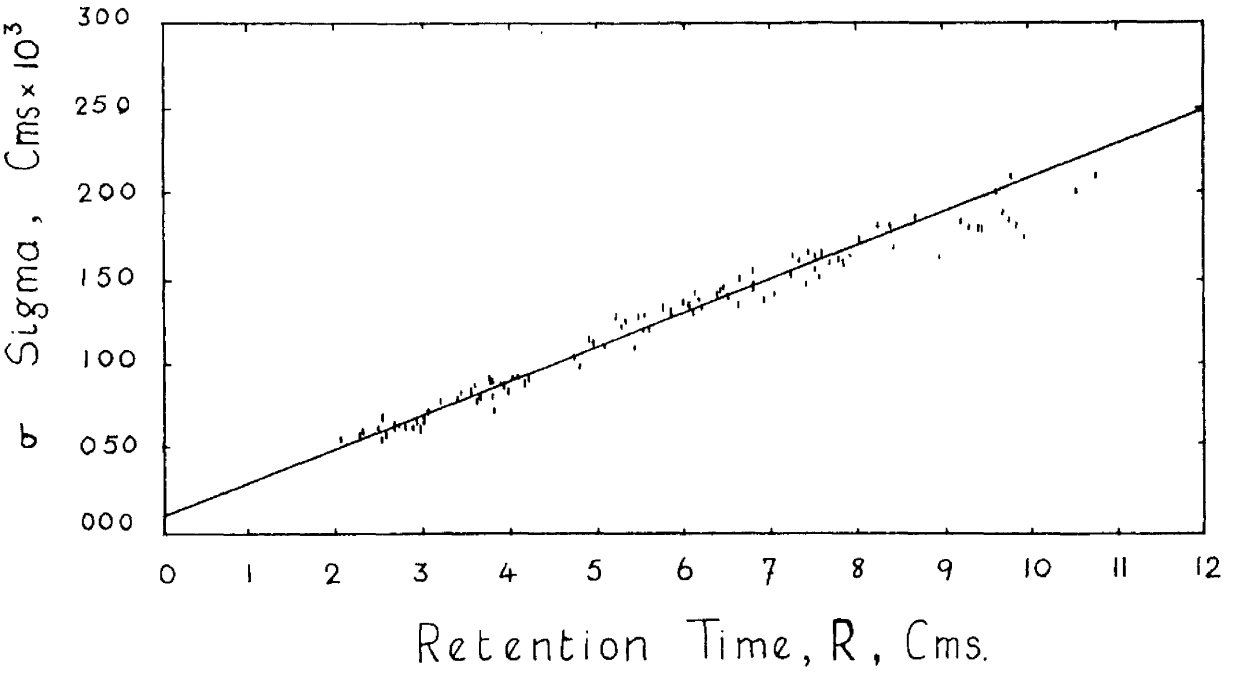
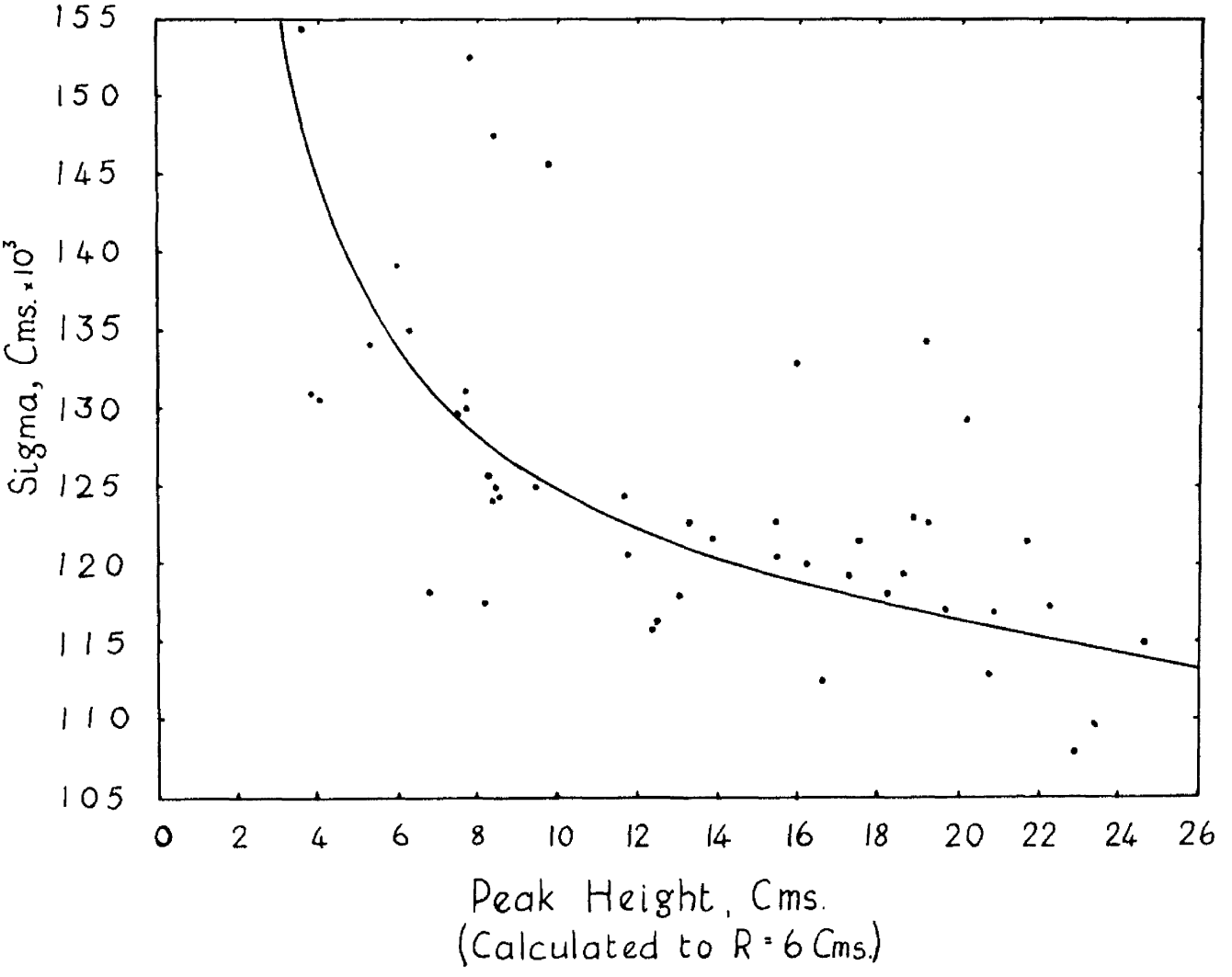

54
1612
387
35
1074
1604
1267
1072
0981
0577
64.2
12.8
1.1
19.2
90
80
70
60
50
40
30
20
10

Figure 6



$\sigma = 0.0198\,R + 0.0100$

Figure 7


155
150
145
140
135
130
125
120
115
110
105
Sigma, Cms. x 10³
0
2
4
6
8
10
12
14
16
18
20
22
24
26
Peak Height, Cms.
(Calculated to R = 6 Cms.)

of the peaks), having a variation of less than 2%, and being free from significant subjective error.

The mole percent composition of the esters (and hence of the original acids) was obtained by summation of the areas of the peaks, dividing each area by the total area, and expressing the result as a percentage. The area of a peak is given by

$$\text{area} = \sqrt{2\pi} \times \text{standard deviation} \times \text{height}$$

In practice the standard deviation was calculated from the retention time and the equation relating retention time to standard deviation (shown in Fig. 6), and the common factor $\sqrt{2\pi}$ was omitted from the calculations. The percentage FFA and recovery of FFA from the crude lipid were calculated using the equations given on p. 39.

Worked examples for chromatograms 165A and 175C are given below.

Chromatogram 165A

Acid	R (cms.)	h (cms.)	σ	σh	mole %
16:0	2.35	15.33	0.0579	0.8890	23.0
17:0	3.30	19.16	0.0753	1.4420	(37.5)
18:0	4.50	1.08	0.0972	0.1049	2.7
18:1	5.02	4.19	0.1187	0.4975	12.9
18:2	6.24	17.70	0.1289	2.2800	59.2
18:3	8.02	0.49	0.1613	0.0791	2.1
Total excluding 17:0				3.8505	99.9

10 ml. aliquot wt. = 16.92 mg.

$$\text{FFA} = \frac{1.5 \times 104}{3.5 \times 16.92 \times 37.5} = 6.75\% \text{ of lipid}$$

Chromatogram 175C

Acid	R (cms.)	h (cms.)	σ	σ h	mole %
16:0	2.34	10.74	0.0577	0.6200	19.2
17:0	3.23	0.03	0.0739	0.00222	(0.068)
18:0	4.55	0.35	0.0981	0.0343	1.1
18:1	5.05	3.87	0.1072	0.4155	12.8
18:2	6.23	16.12	0.1287	2.0740	64.1
18:3	7.97	0.54	0.1604	0.0866	2.7
Total excluding 17:0				3.2304	99.9

$$\text{FFA recovery} = 100 - \frac{0.068 \times 100}{37.5} = 99.82\%$$

PART V

SUPPLEMENTARY WORK

SUPPLEMENTARY WORK

The experimental work given below is not part of the main thesis, but since it is relevant to the work and discussion of the main thesis it has been included. Work concerning rheological dough testing is described in some detail, and published work is mentioned in abbreviated form since reprints of the papers have been bound in at the end.

(a) Dough Testing Using the Chopin Alveograph

(i) Introduction

The Chopin alveograph is one of several empirically designed dough testing instruments for comparing the baking qualities of doughs. It works on the principle of inflating a disc of dough, containing a fixed ratio of solids : water, to a bubble at a constant rate, and recording the pressure on a kymograph to give a tracing called an alveogram. Fig. 8 (top) shows a somewhat idealised alveogram. This procedure was thought to represent the conditions prevailing in a fermenting dough which was expanding due to production of carbon dioxide by yeast, but this is not correct (79). A more fundamental interpretation of the alveogram can be made if the alveograph is considered as a stress-strain recording instrument. The tracing of pressure (height of the alveogram) can be recalculated as the resistance, or pressure at unit bubble wall thickness, and it is then found to become a curve without an inflection near the

Fig. 8

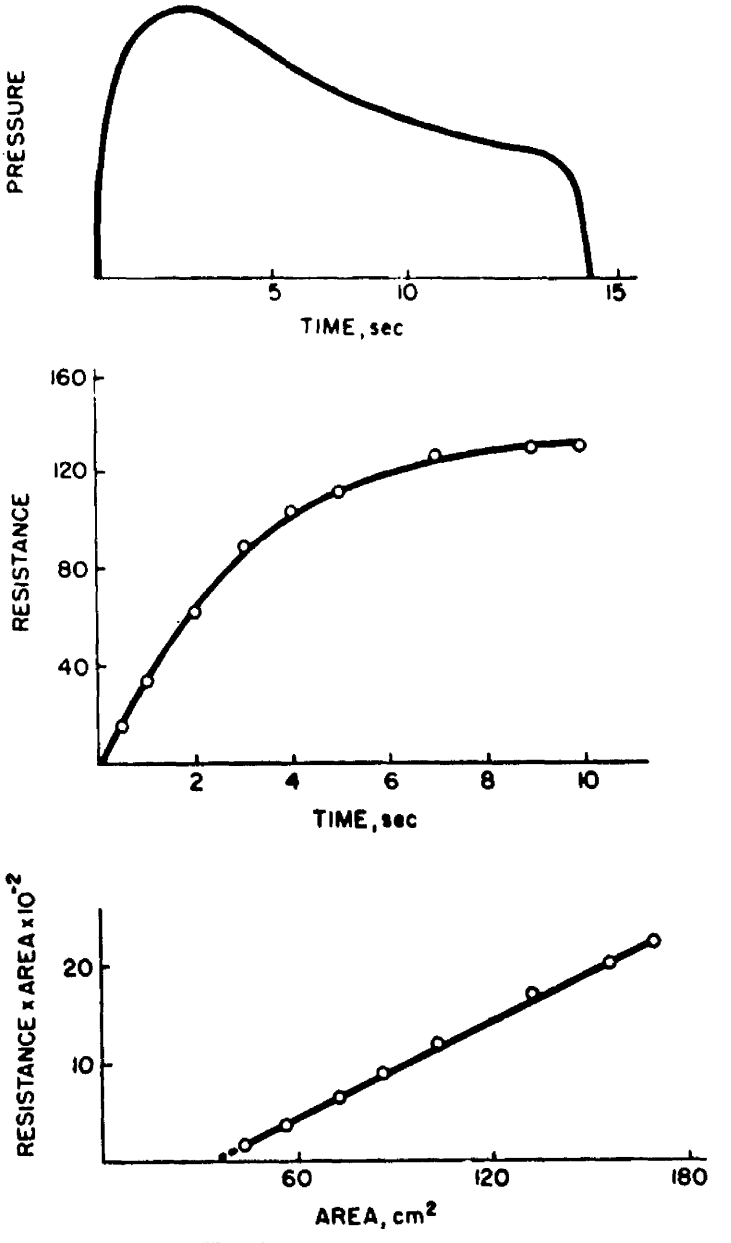


Fig. 2. The alveogram and its transformations.

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beginning (Fig. 8, centre). Since the bubble is blown from a fixed volume of dough it is equally valid to multiply the pressure by the bubble surface area (1/thickness = area/volume). A further transformation of the alveogram can be made by plotting the resistance x area against the area during the bubble blowing procedure, and this gives a straight line relationship (Fig. 8, bottom). Normally it is enough to measure the resistance of a dough as the height of the alveogram at fixed bubble size, and, depending on the dough strength, this was measured as the height at 2.5 or 3.3 cm. from the beginning of the alveogram in the work reported later.

In the normal alveograph procedure 250 g. flour are made up to a dough and extruded as a sheet from the mixer. Discs are cut from this sheet, and after resting are inflated to bubbles as described above. There is a modification of this procedure in which pieces of dough are manually moulded to form discs (16), and this gives a much better representation of dough quality, especially when improvers are present. Since the mixing process is extravagant and clumsy, a small scale process using 40 g. flour was developed, together with minor changes in the moulding procedure, and this is described in the work reported below. Using this procedure the alveograph is more sensitive than most instruments of its kind, and is particularly suited for detecting small changes in dough quality.

This alveograph procedure was developed as a means of

measuring flour and dough changes caused by defatting and other experimental procedures applied to one or two flours. Some of the experiments demonstrated the behaviour of the flour used in Parts II and III under conditions relevant to the main discussion of the thesis, and they are described later. Other work of a similar nature was complete in itself, and was published as a paper entitled "The Effect of Atmospheric Sulphur Dioxide on Wheat Flour". A reprint of this paper is attached at the end of this section of the thesis.

(ii) Materials and methods

Two flours were used; flour A was an Australian flour which was weaker than the flour used in Parts II and III, and flour B was the same type of flour as used in those parts of the thesis.

All reagents were of analytical quality, and distilled water was used for the dough-making, salt solution and other solutions.

A Simon Minorpin pin mixer was used for mixing doughs, and since the power of the motor was rather limited the greatest amount of flour which was mixed was 40 g.

The doughs were made to contain 40 g. flour (14% moisture) and 22 ml. of 2½% (w/v) salt solution, or the same proportions of solids : water where the flour moisture was different. By keeping the workroom and materials at 24° it was found that a dough temperature of 27° was obtained consistently, the temperature rise being due to

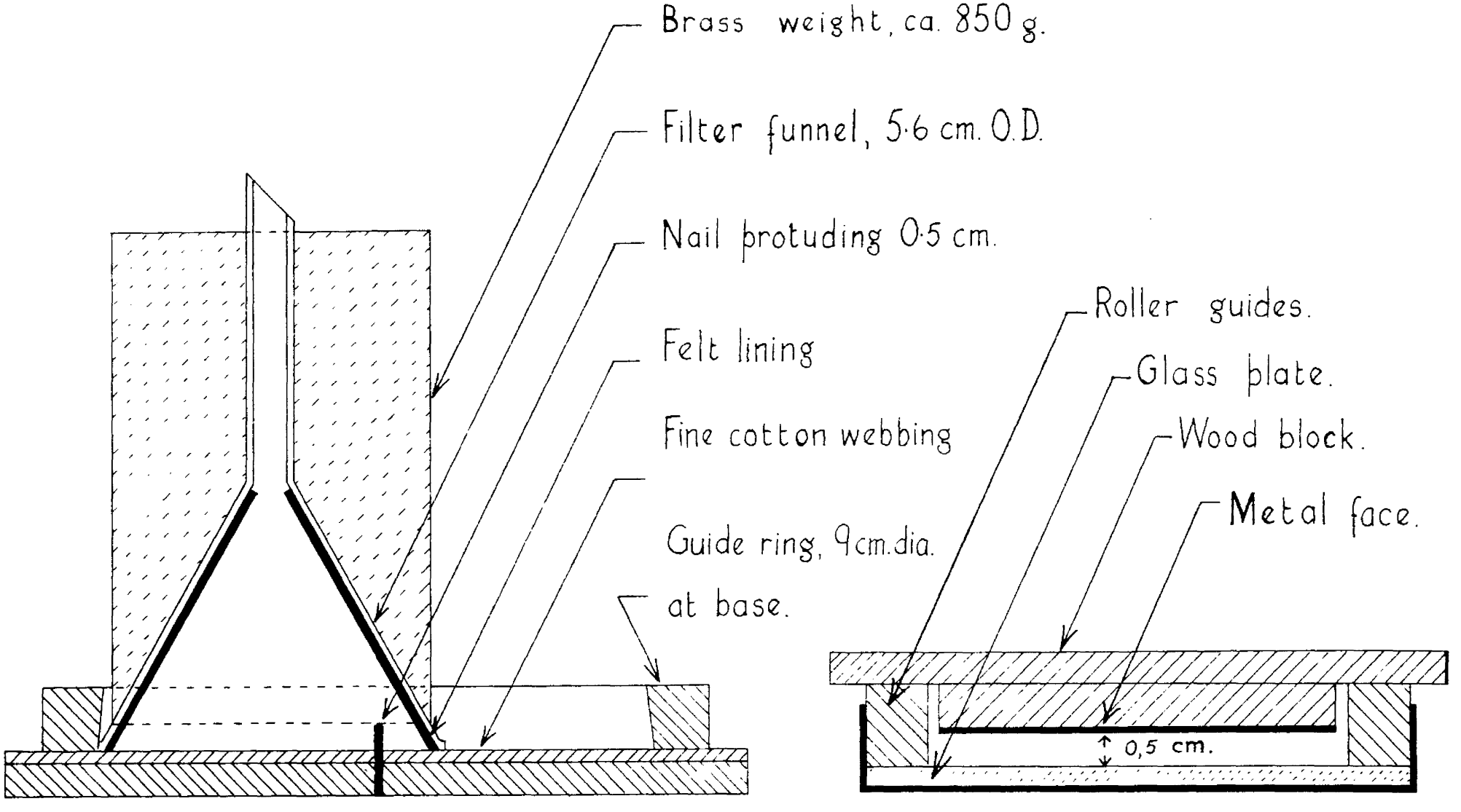
the head of hydration of the flour and to the heat developed by mixing. The alveograph was thermostatically controlled to 27°.

The procedure for making the discs of dough for testing was as follows.

The salt solution was placed in the mixing bowl, followed by the flour, and these were mixed to a dough for exactly 3 minutes. Additives were introduced as a solution in lieu of some of the salt solution. Where an atmosphere of nitrogen or oxygen was required the flour was put into the bowl first, the bowl covered with a close-fitting hood with gas or water inlets, and the flour dry-mixed under the appropriate gas. The salt solution was flushed out with the gas, and after both had been flushed for 20 minutes the salt solution was added and the dough mixed for 3 minutes as before, maintaining a positive gas pressure under the hood.

The dough was then removed, and lightly rolled into a cylindrical shape. Three 20 g. pieces were cut from the dough, and placed under covers to prevent drying. Each piece was then moulded by placing under the funnel of the moulding apparatus (Fig. 9, left) and rotating the funnel within the guide ring 40 times in exactly 15 seconds. The ball of dough was then pressed to a disc in the pressing apparatus (Fig. 9, right), taking 5 seconds to press down, and holding for 5 seconds. The dough was prevented from sticking by oiling the faces of the pressing apparatus with paraffin oil. After pressing, the dough was rested in the alveograph resting

Fig. 9


Brass weight, ca. 850 g.
Filter funnel, 5·6 cm. O.D.
Nail protuding 0·5 cm.
Felt lining
Fine cotton webbing
Guide ring, 9cm.dia.
at base.
Roller guides.
Glass plate.
Wood block.
Metal face.
0,5 cm.

cabinet for 20 minutes and then tested by the normal alveograph procedure.

The alveograms obtained from this procedure by an experienced operator are exactly reproducible, unlike those from the standard procedure in which the average of five curves is used. It is hoped to develop a mechanical moulding procedure, and by so eliminating the major source of variation make the method equally effective in relatively unskilled hands.

Defatted flours were prepared by stirring flour and light petroleum (B.Pt. 40°- 60°) together in a beaker, allowing to settle, decanting the solvent, and repeating till the solvent was no longer coloured by carotenoid pigments. The flour was then assumed to be fully extracted (within the capabilities of the solvent), and was air dried and sieved for use.

(iii) Experimental and results

There are varying reports of the effect of defatting on the mixing properties of doughs (p. 14), and experiments were therefore carried out with flour and defatted flour mixed under atmospheres of air and nitrogen. Flour fat was also returned to defatted flour to determine the effect of the solvent on the flour. Since oxygen has an improving effect on flour it was decided to examine the effect of oxygen on dry flour. Flour was stored overnight in air and in oxygen in desiccators, then mixed in air, and the results compared

with the original flour mixed under oxygen. The results of these experiments are summarised in Table 11.

Arising from the work on the effect of sulphur dioxide on flour, experiments were done with the two flours to determine the effect of various reducing agents on dough quality. The results are shown graphically in Fig. 10.

Several other experiments arising from the results shown in Fig. 10 were also done, and these are described in Table 12.

(iv) Discussion

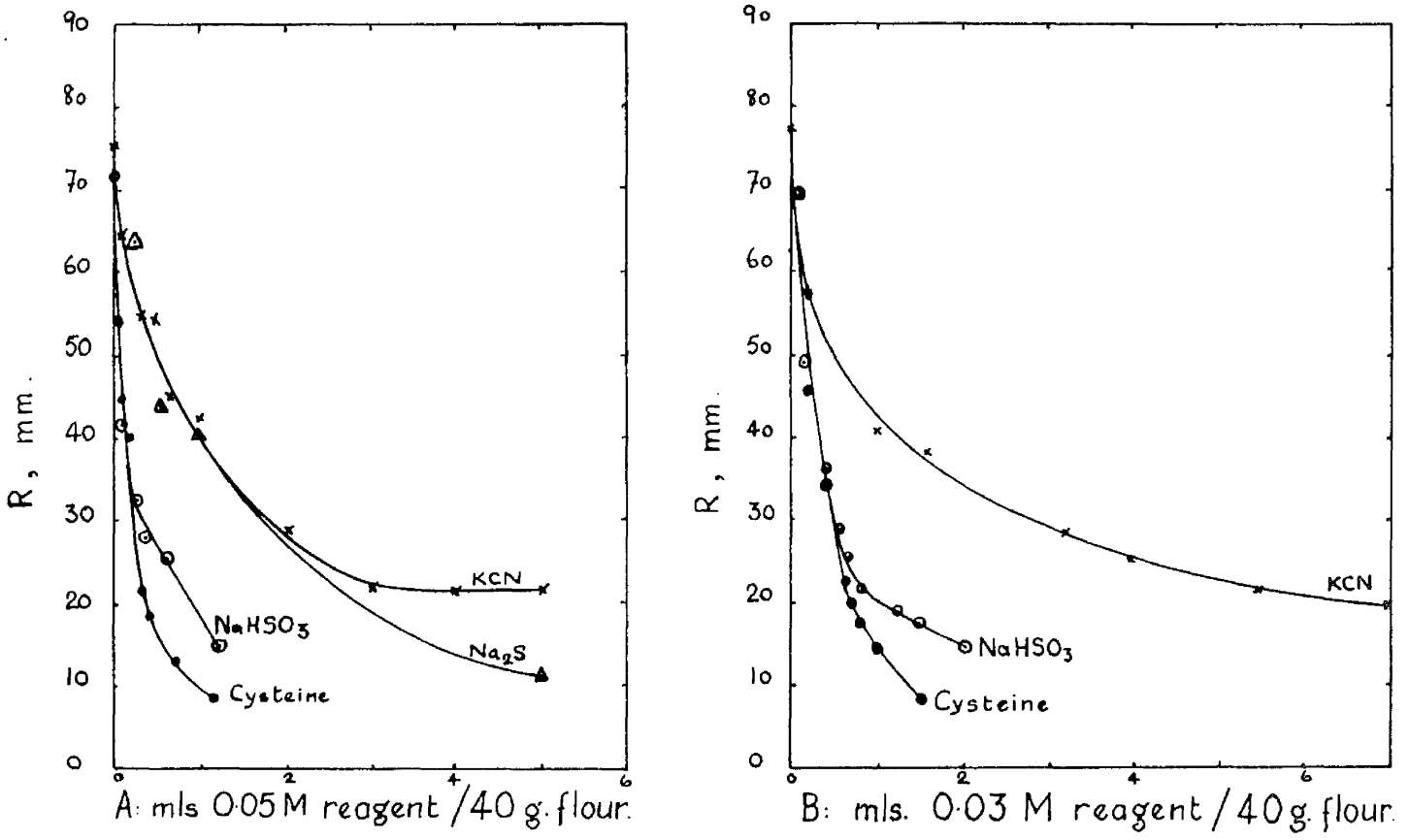
The effect of oxygen tension on doughs is shown in Table 11 (nos. 1, 2 and 8). These results are in accord with all the observations on the "Oxygen Effect" (p. 13), and were found with several other flours which were similarly tested.

The effect of defatting flour has been reported to have an improving effect in some cases, and in other cases to have a detrimental effect on dough quality (p. 14). The results in Table 11 (nos. 1-5) show that the defatted flour was more oxygen sensitive, and when mixed under nitrogen residual traces of oxygen still had an improving effect. When the extracted fat was returned to the flour before the start of mixing the dough was found to be normal, from which it is concluded that the solvent had no irreversible effect on the flour. This agrees with other authors' findings (145). There is no evidence in these results to implicate EFA in the improving

Table 11

	Alveograph Experiments with Flour B	
No.	Experiment	Resistance, mm., measured at 3.3 cm.
1.	Flour mixed in air (control)	80
2.	Flour mixed in nitrogen	66
3.	Flour defatted, mixed in air	94.5
4.	Flour defatted, mixed in nitrogen	69.5
5.	Flour defatted, fat re-added, mixed in air	79
6.	Flour stored in air, mixed in air	80
7.	Flour stored in oxygen, mixed in air	80
8.	Flour stored in air, mixed in oxygen	over 110

Fig. 10


R, mm.
90
80
70
60
50
40
30
20
10
0
0
2
4
6
KCN
Na_2S
$NaHSO_3$
Cysteine
A: mls 0·05 M reagent /40 g. flour.
R, mm.
B: mls. 0·03 M reagent / 40 g. flour.

action of oxygen, and this is probably due to the sensitivity of SH in the defatted flour to oxygen (145).

The improving effect of oxygen apparently occurs only in wetted flour (Table 11, nos. 6-8), which is consistent with an enzymic mechanism such as secondary oxidation by the lipoxidase system.

Fig. 10 shows the effect of various amounts of substances which weaken doughs, and it can be seen that the qualitative effects are the same in the two flours, although the quantitative effects differ, as would be expected since one flour was classed as strong and the other as soft. The probable reactions of cyanide and bisulphite in dough at pH 5 - 6 are

$$HCN + RSSR = RSH + RSCN$$

$$HSO_3^- + RSSR = RSH + RSSO_3^-$$

These reactions are similar, and it might be expected that the rheological effects would be similar, but this was clearly not the case. A similar trend has been found when the SH of gluten dispersions was measured in the presence of increasing amounts of bisulphite (109). The effect of sulphide is obscure, and since there was copious evolution of hydrogen sulphide during the mixing, quantitative conclusions cannot be drawn. Cysteine probably acts by supplying mobile SH for the SH/SS interchange reaction. The fact that the cysteine curves are identical to the bisulphite curves in the initial

stages probably means that both exert the maximum weakening effect on the same readily accessible SS of gluten at these levels, but react differently at the later stage where the curves diverge. The The points where the cyanide curves end represent the stages at which further additions of cyanide had no more weakening effect. The points where the other curves end represent the stages at which the doughs became unworkably soft and sticky. Since the latter do not represent complete weakening it is not possible to usefully correlate them with the SS content of the flour. It was shown (p. 62) that the end of the bisulphite curve corresponded to a doubling of the flour SH content, but there would still be 6 - 8 moles SS/mole SH in the dough (4, 5, 31, 149).

Further experiments were done with addition of these reagents (Table 12). It was found that sulphite treated dough was still oxygen sensitive (nos. 11-13), and this is attributed to the ability of the flour lipoxidase system to counteract the effect of sulphite in the presence of oxygen (145). The weakening effect of cyanide is also slightly reversible by increasing the oxygen tension (nos. 16, 17) or by adding hydrogen peroxide (nos. 6-9). Iodate, which is a powerful and rapid improver, could not reverse the effect of cyanide (nos. 1, 3-5).

Since cysteine is supposed to act by facilitating SH/SS interchange reactions, it is not surprising that reduced oxygen tension did not further weaken dough treated with a large dose of

Table 12

Alveograph Experiments with Flour A and Additives

No.	Experiment	Resistance, mm., measured at 3.3 cm.
1.	Flour mixed in air	68
2.	Flour mixed in oxygen	104
3.	Flour mixed in oxygen, + 5 ml. 0.05 M KCN	17
4.	Flour mixed in air, + 50 ppm. KIO_3	over 110
5.	Flour mixed in air, + 50 ppm. KIO_3, + 5ml. 0.05 M KCN	below 15
		Resistance, mm., measured at 2.5 cm.
6.	Flour mixed in air	64
7.	Flour mixed in air, + 1 ml. "1 volume" H_2O_2	70.5
8.	Flour mixed in air, + 1 ml. "1 volume" H_2O_2, + 5 ml. 0.05 M KCN	17
9.	Flour mixed in air, + 5 ml. "1 volume" H_2O_2, + 5 ml. 0.05 M KCN	28
10	Flour mixed in air, + 1 ml. 2×10^{-5}M cytochrome C.	63.5
11.	Flour mixed in air, + 2 ml. 0.03M sulphite	15
12.	Flour mixed in oxygen, + 2 ml. 0.03M sulphite	28
13.	Flour mixed in nitrogen, + 1 ml. 0.03M sulphite	4
14.	Flour mixed in air, + 1 ml. 0.03M cysteine	10
15.	Flour mixed in nitrogen, + 1 ml. 0.03M cysteine	10
16.	Flour mixed in air, + 0.3 ml. 0.05M KCN	57
17.	Flour mixed in oxygen, + 0.3 ml. 0.05M KCN	61.5

cysteine (nos. 14 and 15).

Cytochrome C was added in one experiment (no. 10) to determine whether a haematin could promote oxidative improvement by a mechanism similar to lipoxidase, but it was found to have no effect. Since the methanol treated flour showed no FFA losses in mixing (p. 54) it seems probable that haematins and their degradation products are not the prime cause of FFA losses or of oxygen uptake.

These experiments show that the mechanisms by which doughs can be weakened are complex and that the weakening is not directly related to the total SS which has been reduced. There is a wide field for investigation here which it is hoped will be developed in the future. The results however serve to illustrate that the flour used behaved normally under the experimental conditions used in Part III of this thesis, and therefore add weight to the calculations and assumptions involved in the Discussion in Part IV.

(b) Methylation of Long Chain Fatty Acids with Diazomethane.

When the work reported in Part IV was being done, some colleagues in another laboratory were having difficulty with their GLC analyses of fatty acid methyl esters from blood plasma. The difficulty appeared in the form of peaks in the chromatograms, which only appeared in some cases, and represented acids which had never been reported in blood lipids before. The problem was referred to this author, and in due course the peaks were traced to artefacts

formed from the diazomethane which had been used to methylate the acids. The investigation formed the subject of a paper entitled "By-products Formed During the Methylation of Long Chain Fatty Acids with Diazomethane", and a reprint of this paper is attached at the end of this section of the thesis.

(c) Thioctic Acid in Wheat Flour

Mention has been made of the presence and possible role of thioctic acid in wheat flour (p. 8). When it was learned that thioctic acid had been identified in wheat flour, it was decided that it would be of interest to isolate it. Taking the long term view this could open up ways for the further study of a potentially very active sulphur-containing co-enzyme in the oxidation mechanisms of flour-water doughs.

This author initiated work which led to the identification of thioctic acid in whole wheat flour, using novel techniques for this substance. This work has recently been published in a paper entitled "Thioctic Acid in Wheat Flour", and a reprint of the paper is attached at the end of this section of the thesis.

In the paper it is stated that further work is in hand to prepare thioctic acid extracts which are much less contaminated by impurities. This work is at present being carried out, and so far has shown that the contamination is largely due to EFA which have been autoxidised during the acid digestion stage, giving rise to

conjugated triene and tetraene acids. The ultraviolet absorption of the conjugated tetraene acids has an absorption maximum sufficiently close to that of thioctic acid to cause the abnormal absorption spectrum found for thioctic acid extracts. Present work is being concentrated on the removal of these conjugated acids.

(d) Summary

Experiments in which flours were rheologically tested to demonstrate the oxygen effect on normal and defatted flours have been described. Other experiments, in which the effect of reducing agents on doughs was examined, have been described and discussed in relation to the discussion in Part III of this thesis.

Work which was carried out supplementary to the main work of this thesis has been introduced, and three papers describing the work are attached on the pages following this one.

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